Mechanisms of action of the chemopreventive agent indole-3-carbinol in breast cell lines.

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by

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ABSTRACT

Mechanisms of action of the chemopreventive agent indole-3-carbinol (I3C) in breast cell lines.

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The chemopreventive agent indole-3-carbinol (I3C) is found in cruciferous vegetables such as broccoli, cabbage and Brussels sprouts. Clinical trials have shown I3C to favourably alter urinary 2-OH:16α estrone ratios, which may prove beneficial for decreased risk of ER+ve breast cancer. However, molecular mechanisms for potential chemopreventive effects in ER-ve breast cancer have yet to be elucidated.

I3C inhibited proliferation of the immortalised HBL 100, and tumour-derived MDA MB468, T47D and MCF7 breast cell lines. Approximate IC_{50}s were 122μM, 33μM, 73μM and 67μM respectively, with MDA MB468 cells exhibiting four-fold greater inhibition than HBL 100 cells.

I3C caused significant induction of apoptosis in the MDA MB468 cells only, possibly explaining this difference in sensitivity. This was not due to immediate effects upon members of the Bcl-2 family, although downregulation of the anti-apoptotic Bcl-2 protein may have contributed to apoptosis at later time points.

I3C caused a dose-dependent decrease in Akt phosphorylation and activity in the MDA MB468 cells, which possessed constitutive Akt activity caused by loss of PTEN expression. I3C was shown to similarly inhibit Akt phosphorylation and induce apoptosis in the low-PTEN, phospho-Akt-overexpressing LNCaP prostate cell line, but not in the high PTEN/low phospho-Akt expressing DU145 prostate cells or the HBL 100 line.

I3C inhibited PI3K activity in the MDA MB468 cells (50μM) and HBL 100 cells (500μM) when added directly into an *in vitro* kinase assay. Induction of apoptosis in the MDA MB468 and LNCaP cells by the PI3K inhibitor LY294002 supported the hypothesis that inhibition of PI3K signalling may contribute to apoptosis. However, induction of apoptosis by I3C also occurred via an Akt-independent pathway that may involve NF-κB, as I3C, but not LY294002, decreased NF-κB DNA-binding (as shown by EMSA) in the MDA MB468 cells.

This investigation into the molecular mechanisms of action of I3C, indicates a potential role for its use in chemoprevention against phospho-Akt-overexpressing, PTEN-negative, highly aggressive breast tumours, independent of estrogen receptor α status.

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I wish to dedicate this thesis to my family, and thank them for all that they have done.

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ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein-1
APAF-1	Apoptosis protease activator factor-1
ASK-1	Apoptosis stimulating kinase-1
Asp	Aspartate
ATP	Adenosine 5'-triphosphate
Bcl-2	B-cell lymphoma/leukaemia-2
BH	Bcl-2 homology
BIR	Baculovirus IAP repeat
BRCA1/2	Breast cancer 1/2
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CARET	beta-carotene retinol efficacy trial
CDK	Cyclin dependent kinase
Chk1	Checkpoint homologue 1
CIN	Cervical intraepithelial neoplasia
CK2	Casein kinase 2
CKI	CDK inhibitor
COX	Cyclooxygenase
CTet	Cyclic tetramer
CTr	Cyclic trimer
CYP	Cytochrome P450
Cys	Cysteine
DIM	3,3'-di-indolylmethane
DISC	Death inducing signalling complex
DMBA	7,12-Dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
DYRK1A	Dual specificity tyrosine phosphorylation regulated kinase 1A
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGF	Epidermal growth factor
EGTA	Ethyleneglycoltetraacetic acid
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERBB2	v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2
ERK	Extracellular signal regulated kinase
FACS	Fluorescence activated cell sorting
FADD	Fas associated death domain
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
G1	Gap 1 phase of the cell cycle
G2	Gap 2 phase of the cell cycle
GSK-3	Glycogen synthase kinase-3
HA	Haemaglutinin
HEPES	2[-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]
HRP	Horse radish peroxidase
IκB	Inhibitor of kappa B
I3C	Indole-3-carbinol
IAP	Inhibitor of apoptosis
IC_{50}	Concentration for 50% inhibition
ICZ	Indolo[3,2-b]carbazol
IKK	IκB Kinase
IP	Immunoprecipitation
JNK	c-jun N-terminal kinase
KDa	Kilodalton
LTet	Linear tetramer

LTr	Linear trimer
Lck	Lymphocyte-specific protein tyrosine kinase
M	Mitosis
MAPKAP-K1	MAP kinase activating protein kinase 1
MBq	Mega bequerel
MEK	MAPK/ERK Kinase
mg	Milligramme
MKK	MAPK kinase (MEK)
MMP	Matrix metalloproteinase
MSK1	Mitogen- and stress- activated protein kinase-1
NF-κB	Nuclear factor kappa B
NIK	NF-κB inducing kinase
NSAIDS	Non steroidal anti-inflammatory drugs
OH	Hydroxy
PARP	Poly (ADP ribose) polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PDK	3-Phosphoinositide-dependent kinase
PGE2	Prostaglandin E2
p-gp	p-glycoprotein
PH	Pleckstrin homology
PhIP	2-amino-1-methyl-6-phenylimidazopyridine
PHK	Phosphorylase kinase
PI	Propidium Iodide
PI3K	Phosphatidylinositol-3-kinase
PIP	Phosphatidylinositol phosphate
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid]
PKA	Protein kinase A
PKC	Protein kinase C
PRAK	Mitogen-activated protein kinase-activated protein kinase
PTEN	Phosphatase and tensin homologue deleted from chromosome 10
RANK	Receptor activator of NF-κB

Rb	Retinoblastoma
ROCK-II	Rho-associated, coiled-coil containing protein kinase II
RPMI	Roswell Park Memorial Institute
S	DNA synthesis phase of the cell cycle
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SGK	Serum/glucocorticoid regulated kinase
SH2	Src homology domain 2
SH3	Src 3 homology domain
SHIP	SH2-containing Inositol polyphosphate-5-phosphatase
SLE	Systemic lupus erythematosus
SSC	Disodium citrate
SSPE	Disodium phosphate EDTA
TAD-1	Transactivation domain-1
TAE	Tris acetate EDTA
TBST	Tris buffered saline-tween
TCF	T cell factor
TE	Tris/EDTA
TEMED	Tetramethyl ethylene diamine
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNFR associated death domain
UV	Ultra violet
VEGF	Vascular endothelial growth factor
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
XRE	Xenobiotic response element

CHAPTER 1

INTRODUCTION

1.1 CANCER CHEMOPREVENTION

It has been estimated that approximately two thirds of all cancers may be preventable through lifestyle modifications (Oliveria *et al.*, 1997), with epidemiological studies showing a correlation between the ingestion of certain food groups and the incidence of cancer (Goodman, 1993, Fung *et al.*, 2003). The idea that 'prevention is better than cure' is not a novel one, but the truth in this old adage is particularly relevant with respect to cancer and the current treatments available. Once a cancer is established, the existing therapies whether they be surgical, radiochemical or drug based are generally of an unpleasant, invasive nature and in many cases have little guarantee of success. The concept of cancer prevention therefore offers an attractive proposition within anti-cancer research.

Chemoprevention can be defined as the use of natural or synthetic chemicals to reverse the onset, suppress the development or prevent progression of the carcinogenic process (Sporn *et al.*, 1976, Wattenberg, 1993, Gescher *et al.*, 1998).

The process of tumourigenesis has been proposed by Hanahan and Weinberg (2000) to require six essential alterations to normal cell physiology: the ability to provide their own growth signals, insensitivity to growth inhibition, evasion of apoptosis, immortalisation, sustained angiogenesis, and tissue invasion and metastasis.

The development of an invasive cancer is a complex and ongoing process consisting of many individual steps, but can be broadly categorised into three main areas:
Initiation is the primary step in the onset of the carcinogenic process occurring via many different mechanisms (eg: chemical interaction, irradiation, spontaneous mutation) but ultimately resulting in DNA damage that may confer growth advantages over the normal cellular population. Agents that are able to target this primary stage are known as blocking agents, and can prevent occurrence of the initiation step by preventing carcinogenic agents reaching a critical site, or by the scavenging of free radicals. Other effects include the ability of the agent to modify phase I/II drug metabolising enzymes leading to increased conjugation and elimination of carcinogens, altering rates of DNA repair or limiting rates of adduct formation (Wattenberg, 1997, Manson *et al.,* 2000). Prevention of this initial step

may prove difficult in many respects as it begins very early on in life. However, it is unlikely that a single initiating event will result in carcinogenesis, rather it is an accumulation of further promoting signals and damage that will progress the carcinogenic process.

Promotion can occur many years after the initiation step and is an ongoing process, resulting in the clonal expansion of initiated cells, giving rise to a cellular population primed for tumourigenesis.

Progression may be seen as an extension to the promotional clonal expansion procedure, and will ultimately result in tumour formation. These two latter phases may be targeted by suppressing agents, which exert their effects by slowing or preventing clonal expansion of initiated cells via a host of mechanisms including: scavenging of reactive oxygen species, modulation of signal transduction pathways, suppression of growth factor or hormone activity, inhibition of arachidonic acid metabolism, changes to oncogene function or inhibition of polyamine synthesis, ultimately leading to inhibition of proliferation and/or induction of apoptosis (reviewed in Gescher *et al.,* 1998). In theory, it is therefore possible to target all 3 stages of the carcinogenic process through chemoprevention over the lifetime of an individual as shown in *figure 1.1.*

Early primary prevention may include the avoidance or removal of certain triggers to slow or prevent the initiation and promotion processes, and can often be simple measures such as lifestyle changes, prior to more invasive secondary prevention measures such as hormone therapy. However, once a tumour reaches a palpable mass (approximately 1g - the point at which it is most readily detectable), it is often at an advanced stage where treatment is difficult, yet essential for patient survival. Tertiary prevention measures usually involve radical surgery together with aggressive chemotherapy. This highlights the importance of readily accessible and easily detectable biomarkers during the initiation and promotion phases, in order to target intervention strategies prior to invasion and metastasis. The principle of chemoprevention allows a non-aggressive intervention regime, targeting all phases of tumour growth.

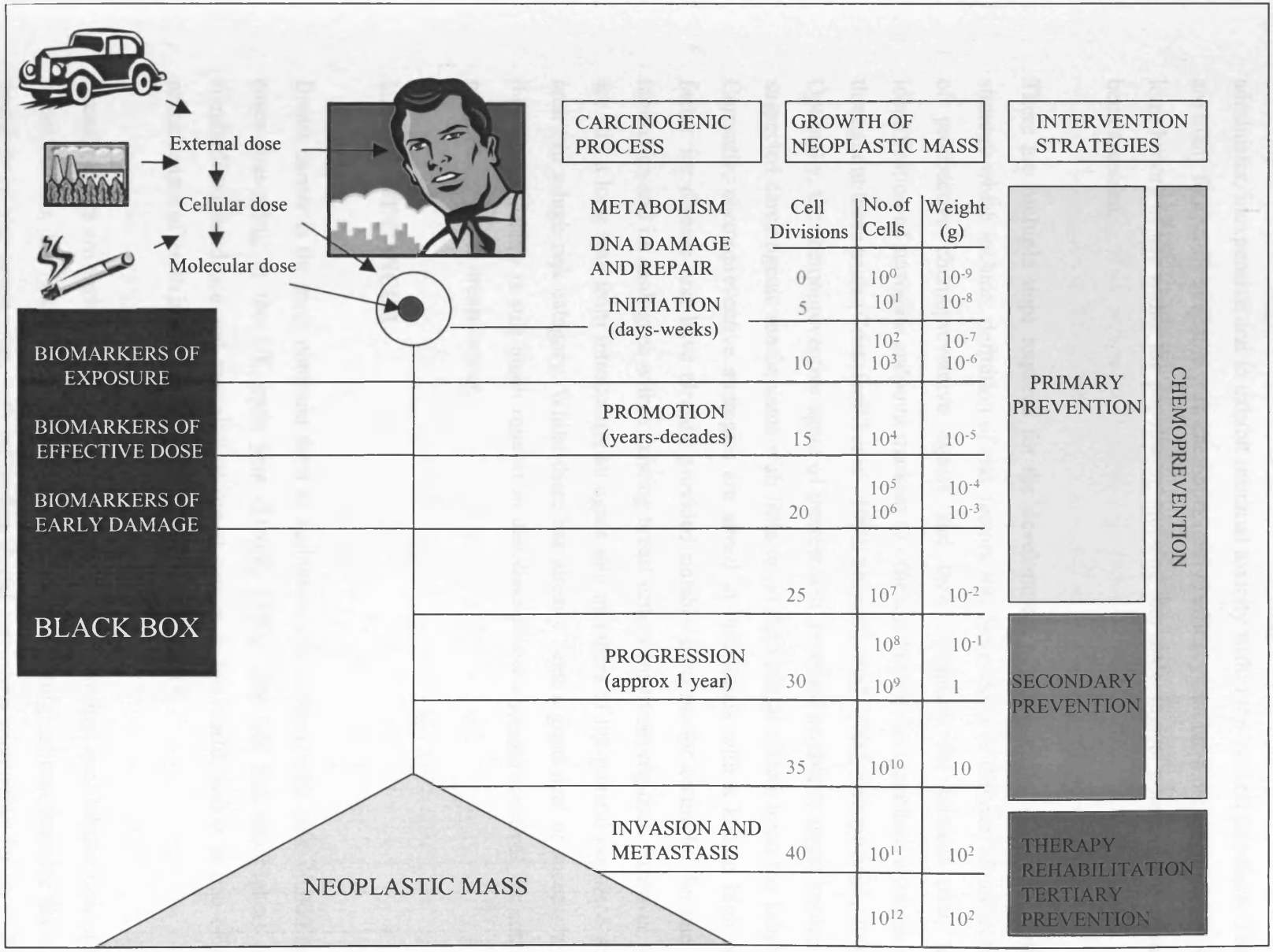


Figure 1.1 Intervention strategies against cancer, related to tumour mass and the carcinogenic process. *Adapted from De Flora et al., Mutation Research (2001)*

For an agent to have chemopreventive potential, it is required to be highly effective, easy to administer, inexpensive and to exhibit minimal toxicity within the general populace. There are many foodstuffs available with chemopreventive efficacy that have the advantage of a long history of use within the population, meaning that toxicological potential has already been assessed.

There are multiple steps required for the development of a successful chemopreventive strategy which include: definition of risk factors and biomarkers of disease, identification of prospective chemopreventive agents and their suitability for clinical trial, and identification of surrogate endpoint markers of efficacy that can be quantitatively assessed throughout these trials (Cristofanilli *et al.,* 1998, Shureiqi *et al.,* 2000, Sharma *et al.*, 2001). Optimally, the chemopreventive agent of interest will therefore be able to target known or suspected carcinogenic mechanisms with little or no detrimental effects upon the subject. Currently, chemopreventive strategies are aimed at individuals with a known high risk factor for disease and have already provided notable success, for example, the use of tamoxifen and its analogues within existing breast cancer treatment regimes. However, we are still a long way from introducing an agent into members of the general populace who aren't in a high risk category. Whilst there has already been a good deal of success with these agents, there is still much interest in developing new chemopreventives for such a noxious disease as breast cancer.

1.2 BREAST CANCER

Breast cancer is the most common form of malignancy in women, with over 40,000 new cases presenting in the UK each year (Dixon, 1997). The UK has the highest age standardised incidence and mortality of breast cancer in the world, and it is one of the prime causes of death in women between the ages of 40 and 55.

Breast cancers are derived from epithelial cells within the terminal duct lobular unit of the breast (Dixon, 1997) and are classified as invasive (infiltrating) or non invasive (in situ) ductal or lobular carcinomas, with infiltrating ductal carcinoma accounting for up to 75% of breast cancers (Harris *et al.,* 1992). Once breast cancer has been identified, there are many factors that determine the prognosis for the disease, influencing selection of

appropriate treatment for patients. General prognostic factors include tumour size and localisation, occurrence of metastasis and histological grade. At a molecular level, however, there has been significant progress in the determination of prognostic biomarkers related to the carcinogenic process such as estrogen receptor (ER) status, oncogene expression (erbB2, myc, Ras), cell cycle regulators (Rb, cyclin D1, cyclin E), angiogenic factors (VEGF, FGF), adhesion molecules (E-cadherin) and mutation of various tumour suppressor genes (p53, Rb and BRCA1/2) (Landberg and Roos, 1997, Stearns *et al.,* 1998).

ER status of breast tumours provides one of the most important markers with regard to current treatment regimes, with the use of selective estrogen receptor modulators (SERMs) such as Tamoxifen in ER+ve (estrogen receptor positive) breast cancers, and in individuals at high risk. Estrogen-induced proliferation is mediated mainly through ERα, with increased levels of the receptor observed in women with breast cancer. Estrogen signalling through the estrogen receptor can result in stimulation of Ras-dependent signalling pathways via recruitment of adaptor proteins such as Grb2 (Santen *et al.*, 2002), ultimately resulting in aberrant signalling through the MAPK (mitogen activated protein kinase), PI3K (phosphatidylinositol-3-kinase) and NF-κB (nuclear factor kappa B) cascades. Targeting of anti-estrogens to the estrogen receptor, can therefore remove the proliferative stimulus of estrogens (reviewed in Hilakivi-Clarke *et al.,* 2002). However, ER-ve (estrogen receptor negative) tumours give rise to highly aggressive breast cancers resistant to hormone therapy, which are typically found in younger women. These tumours often possess grossly deregulated cell cycle and signalling properties and have a highly invasive, metastatic phenotype.

Her2/neu/erbB2 over expression is commonly observed in breast cancers. ErbB2 is a cellular transmembrane glycoprotein of the EGFR family, overexpression of which is generally associated with poor prognosis. It is thought that erbB2 overexpression may confer resistance to hormone-based therapy in ER+ve tumours and may also be involved in doxorubicin-based chemotherapy resistance (reviewed in Stearns *et al.*, 1998). Signalling through erbB2 again results in deregulation of cell signalling pathways, leading to uncontrolled cell cycling, differentiation and proliferation. Signalling by erbB2 through Ras is thought to result in deregulation of the G1/S checkpoint possibly through over expression and activation of cyclins D1 and E (Ryo *et al.*, 2002).

Only approximately 10% of all breast cancers are due to inherited mutations, generally within the breast cancer susceptibility genes *BRCA1* and *2* (Polyak, 2001). However, there are many factors that can increase the risk for breast cancer, with relative risk being indicated by 'the incidence among persons possessing a characteristic in question divided by the incidence among otherwise similar persons without the characteristic' (Harris *et al.,* 1992). Known risk factors for breast cancer include: gender, a family history of breast cancer, age at menarche, age at birth of first child, age at menopause, benign breast disease, exposure to ionising radiation, obesity, high birth weight, height, ethnic background, oral contraceptive use, hormone replacement therapy and alcohol intake (Dixon, 1997, Sanderson *et al.*, 2002). The most definitive risk for breast cancer is that of a family history, with a 20% chance of a woman under 40 developing breast cancer if they have a first degree relative diagnosed with the disease under 40 years of age (Easton, 2002).

Geographical location may also be considered a risk factor for breast cancer, with incidence varying widely between countries, and also between urban and rural areas (Laden *et al.,* 1998). Lower breast cancer incidences primarily in Asian countries compared to the USA and UK, have been widely reported (Ziegler *et al.*, 1993). However, migrants from Japan to the USA have shown a rapid increase in breast cancer risk, becoming comparable to that of the host nation within a generation, particularly if migration is prior to 35 years of age (Nagata and Shimizu, 1996). This change can probably be attributed to alterations to lifestyle and environment.

It is thought that one of the major changes to lifestyle that could account for increased cancer risk, is that of dietary alteration. Western diets often consist of a high caloric intake due to over-consumption of saturated fats, red meat and processed foods, and are often lacking in fresh fruit and vegetable intake when compared to many Asiatic countries. An increased cancer risk from such a diet may not be entirely due to caloric intake, high fat and lack of fibre, but may be in part due to a dietary deficiency of the valuable vitamin and phytochemical components of plant-derived material that may provide a chemopreventive/protective effect. There are many studies suggesting the benefits of a healthier diet, resulting in the Department of Health recommendation that we consume five portions of fruit and vegetables per day. The European prospective investigation into cancer and nutrition (EPIC) is an ongoing study investigating food and alcohol

consumption patterns within nine European countries, with a follow up for cancer incidence and death. This is one of the largest studies underway, attempting to find true cause and effects between diet and lifestyle, and disease incidence and death.

Treatment of breast cancer has undergone radical changes in the past few years with the advent of chemoprevention via the cytostatic anti-estrogen Tamoxifen, that is administered both as a therapeutic and preventive agent. Tamoxifen greatly decreases breast cancer incidence in high-risk patients (Fisher *et al.,* 1998, Osbourne, 1999), whilst adjuvant tamoxifen therapy following primary surgery for breast cancer has also been associated with up to a 30% decrease in risk of death (Fisher *et al.,* 1989). Whilst the benefits of tamoxifen therapy outweigh the detrimental effects, which include a doubling of endometrial cancer risk, and a significant increase in thrombosis in women over 50 (Hortobagyi. 1998), there is much interest in developing lower toxicity second generation estrogen antagonists which also act as anti-estrogens in endometrial tissue, such as Raloxifene (Valgus, 1999).

The success of tamoxifen as a breast cancer therapeutic agent in the treatment of estrogen receptor positive tumours, and as a chemopreventive agent for women in high risk groups, has also led to a wealth of natural and synthetic agents being further investigated for chemopreventive potential. There is particular interest in agents that may be more effective against the aggressive, estrogen receptor negative tumours which are difficult to treat and represent approximately 30% of breast cancers (Biswas *et al.,* 2000). Such agents include retinoids, phytochemicals, monoterpenes, selenium and non steroidal antiinflammatory drugs (NSAIDs) (Carolin *et al.,* 2000). Current emerging therapies for breast cancer include anti-Her2/neu antibodies which give a non-chemotherapeutic based treatment alternative (Herceptin) (Decensi and Costa, 2000), Capecitabine which is converted to 5-fluorouracil once absorbed (so decreasing side effects) (Valgus, 1999) and agents that may be used in combination therapy with tamoxifen such as the natural retinoid fenretinide (Decensi and Costa, 2000). Future directives may be able to use specific targeting of aberrant signalling molecules via administration of antisense oligonucleotides against erbB2 (Vereb *et al.*, 2002), Bcl-xL (Mercatante *et al.,* 2002) or Bcl-2 (Gutierrez-Puente *et al.*, 2002).

Of particular interest in this thesis is the glucobrassicin-derived indole, ***indole-3-carbinol.***

1.3 INDOLE-3-CARBINOL (I3C)

1.3.1 Chemistry

Members of the *Cruciferae* family include broccoli, Brussels sprouts, cabbage and kale, all of which are rich in glucobrassicins (otherwise known as glucosinolates). Glucobrassicins have a basic chemical structure consisting of a thioglucose group linked via a sulphonated oxime group to an R group, where R may be aryl, alkyl or indolyl (Das *et al.,* 2000. Refer to *figure 1.2*).

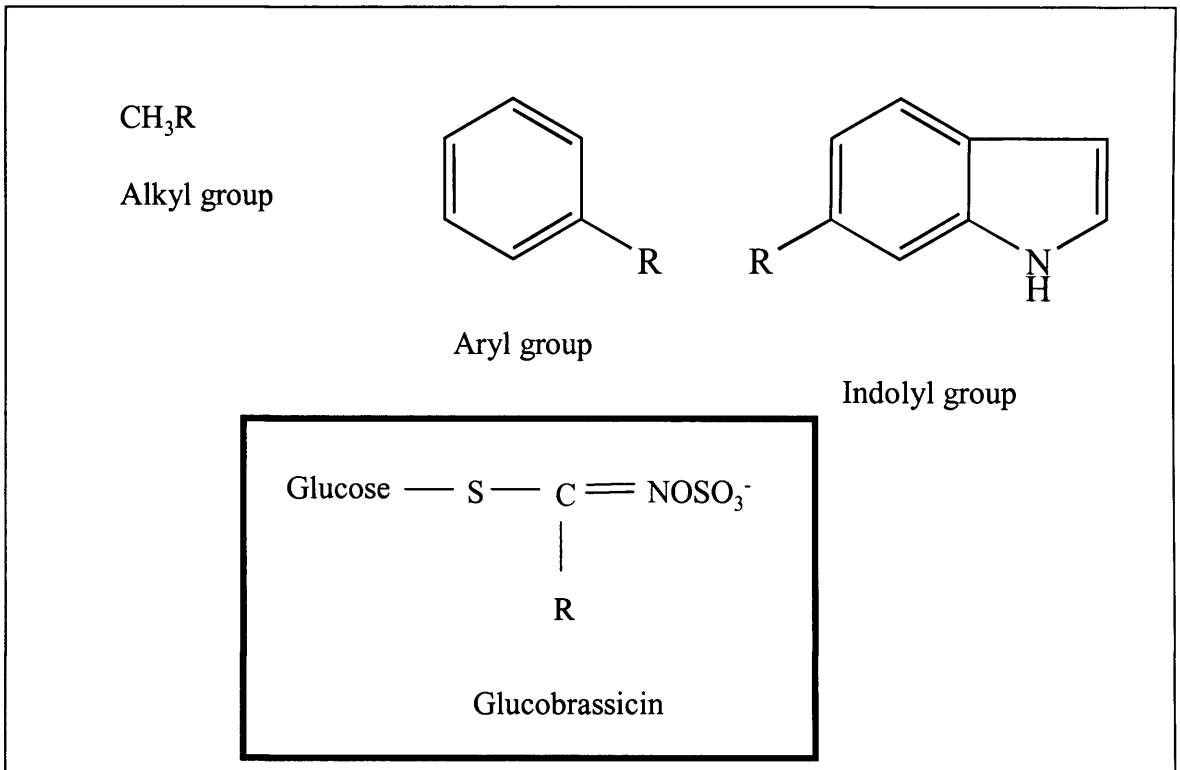


Figure 1.2 Glucobrassicin chemical structure

Glucobrassicins in plants, are formed by amino acid precursors, with indole glucobrassicins being derived from D,L-tryptophan. I3C is formed upon glucobrassicin hydrolysis by the enzyme myrosinase, which is released when the plants undergo mechanical damage, such as via maceration (van Poppel., 1999).

Indole-3-carbinol is the major indole derivative from this process. At neutral pH, glucobrassicin forms an unstable isothiocyanate that degrades to I3C and a thiocyanate ion (Verhoeven *et al.,* 1997). I3C is then able to dimerise at acid pH to form the variety of condensation products shown in *figure 1.3*, including 3,3'-di-indolylmethane (DIM), indolo[3,2-b]carbazol (ICZ), 3-(methoxymethyl) indol and cyclic (CTr) and linear trimer (LTr) products (Shertzer and Senft, 2000).

Brussels sprouts have been reported to contain the highest glucobrassicin levels of approximately 0.5 – 3.2mmol/kg, although almost half of the brassicin content is lost upon boiling (Broadbent and Broadbent, 1998), and it has been estimated that the average UK resident consumes approximately 6.4mg glucobrassicins/day (Sones *et al.,* 1996).

Little is currently understood about the fate of I3C in cells, despite a great deal of evidence for its chemopreventive potential using cellular models. It has been suggested that in MCF7 breast cells, glutathione *S*-transferase is responsible for conversion of I3C primarily to a 3-cysteine I3C conjugate. MCF7 cells metabolised I3C very slowly (with an I3C half life in medium of approximately 40 hours). Very low intracellular I3C levels (nM) suggested that it was not readily taken up by the cell, which may consequently prove to be a major activity-limiting factor (Staub *et al.,* 2002).

1.3.2 I3C and chemoprevention

The chemopreventive potential of the parent I3C and its acid condensation products has been the subject of much discussion over the past 20 years. Following case studies of diet and cancer incidence, evidence was found to suggest a decreased cancer frequency in human populations following high consumption of cruciferous vegetables (reviewed in Verhoeven *et al.,* 1996, Van Poppel *et al.,* 2000, Murillo and Mehta, 2001). Whilst some mechanisms of action for crucifer derivatives have become better defined, there are still many to be further elucidated.

The mechanisms by which I3C exerts its effects have been partially expounded, and can be categorised into blocking or suppressing actions. I3C exhibits blocking activity, as it is able to induce upregulation of certain detoxification enzymes that are important xenobiotic regulators. Feeding studies with I3C have resulted in significant induction of mRNA expression and activity over time, of rat cytochrome P450s 1A1 (Nho and Jeffery, 2001),

1A2, 3A and 2B1, glutathione-*S*-transferase and quinone reductase. Suppressing actions included inhibition of total tyrosine kinase, flavin-containing monooxygenase and ornithine decarboxylase activities (Manson *et al.,* 1997, 1998, Katchamart *et al.*, 2000). I3C is able to induce xenobiotic response element-containing genes such as CYP1A1 and 1B1 in female rat neonates when fed to pregnant animals, so may also have the potential to alter xenobiotic metabolism in the fetus in response to transplacental xenobiotic exposure (Larsen-Su and Williams, 2001).

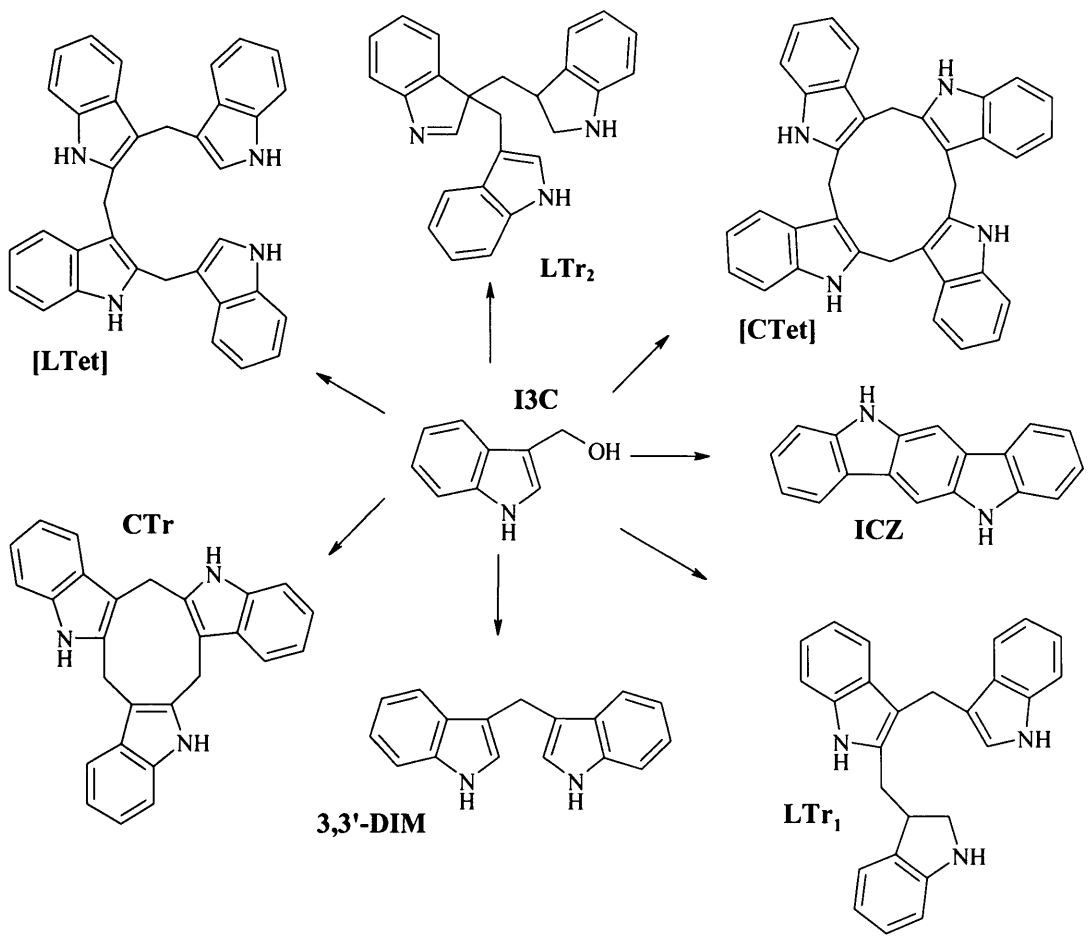


Figure 1.3 Major acid condensation products of I3C *(Grose and Bjeldanes, 1992). CTet=Cyclic Tetramer, LTet=Linear Tetramer, ICZ=Indolocarbazole, LTr_2=Linear Trimer 2, CTr=cyclic trimer, DIM=Diindolylmethane, LTr_1=Linear Trimer 1*

Several genes of drug metabolising enzymes are modulated by the aryl hydrocarbon receptor (AhR), including those encoding CYP1A1, 1A2, UDP-glucuronosyltransferase and glutathione *S*-transferase, and may provide a target through which I3C and its derivatives can elicit their effects (Vang *et al.,* 1999). The AhR is translocated to the nucleus on ligand binding, whereupon it binds to the aryl hydrocarbon receptor nuclear translocase (ARNT). This complex is then able to bind to XRE (xenobiotic response element) gene promoter regions (Ciolino *et al.*, 1998). I3C is only able to bind weakly to the AhR and so it is unlikely that this is the main CYP inducer, whereas DIM and ICZ in MCF7 cells have been shown to bind with greater affinity, resulting in rapid translocation of the AhR to the nucleus and induction of XRE containing genes such as CYP1A1 (Chen *et al.,* 1998).

I3C has been observed to inhibit many *in vivo* chemical models of carcinogenesis, but it has also been proposed to be an inducing agent in some studies. *Table 1.1* shows studies undertaken with I3C acting as a promoting or inhibitory agent.

Examples of studies that show I3C to inhibit the carcinogenic process include: dimethylbenzanthracene (DMBA)-initiated skin (Srivastava and Shukla, 1998) and mammary tumourigenesis (Bradlow *et al.,* 1991) and nitroquinolone 1-oxide- induced rat tongue carcinogenesis (Tanaka *et al.*, 1992). It is also able to prevent DNA adduct formation resulting from exposure to environmental carcinogens such as cigarette smoke (Arif *et al.,* 2000) and dietary carcinogens such as 2-amino-1-methyl-6-phenylimidazopyridine (PhIP) (He *et al.*, 1997), and can inhibit cyclophosphamide-induced chromosomal aberrations (Agrawal and Kumar, 1999). I3C and its acid condensation products may be useful agents for use in conjunction with classical chemotherapeutic agents, as they have demonstrated an ability to reverse multi-drug resistance via inhibition of p-glycoprotein activity (Christensen and LeBlanc, 1996), and an *in vitro* synergistic effect with tamoxifen in MCF7 cells (Cover *et al.*, 1999).

As with many foodstuffs, there is potential for adverse effects from the microconstituents present if consumed in high enough quantities. It has long been known that excessive glucobrassicin intake in animals (1-2 kg in large animals) can have toxic effects, causing

I3C dosing regimen	Action	Site	Animal	Reference
Initiation	Inhibits	Forestomach	Rat	Wattenberg 1978
	Inhibits	Forestomach	Rat	Wattenberg 1990
	Inhibits	Breast	Rat	Wattenberg 1990
	Inhibits	Breast	Rat	Stoewsand 1988
	Inhibits	Breast	Rat	Bresnick 1990
	Inhibits	Breast	Mouse	Bradlow 1991
	Inhibits	Breast	Rat	He 1997
	Inhibits	Breast	Rat	Chen 1996
	Inhibits	Bone marrow	Mouse	Agrawal 1999
	Inhibits	Bone	Mouse	Malloy 1997
	Inhibits	Colon	Rat	Wargovich 1996
	Inhibits	Tongue	Rat	Tanaka 1992
	Inhibits	Lung	Rat	Arif 2000
	Inhibits	Liver	Trout	Nixon 1984
	Inhibits	Liver	Trout	Dashwood 1989
	Inhibits	Liver	Rat	Tanaka 1990
	Inhibits	Liver	Rat	Kim 1984
	Inhibits	Liver	Rat	Ito 1988
	Inhibits	Liver	Rat	Ito 1989
	Inhibits	Liver	Rat	Manson 1998
	Inhibits	Colon	Rat	Guo 1995
	Inhibits	Colon	Rat	Xu 1996
	Inhibits	Skin	Mouse	Srivastava 1998
Post Initiation	Inhibits	Colon	Rat	Guo 1995
	Enhances	Colon	Rat	Pence 1986
	Enhances	Colon	Mouse	Temple 1987
	Enhances	Thyroid	Rat	Kim 1997
	Enhances	Pancreas	Hamster	Birt 1987
	Enhances	Liver	Trout	Bailey 1987
	Enhances	Liver	Trout	Dashwood 1991
	Enhances	Liver	Rat	Kim 1997
	Enhances	Liver	Rat	Ito 1988
	Enhances	Liver	Rat	Ito 1989
	Inhibits	Liver	Rat	Manson 1998
	Inhibits	Liver	Mouse	Oganesian 1997
	Inhibits	Liver	Rat	Kassie 2003
	Inhibits	Breast	Rat	Stoewsand 1988
	Inhibits	Skin	Mouse	Srivastava 1998

Table 1.1 Summary of in vivo studies using I3C. *Studies utilised tumour size/burden and biomarkers such as the presence of GST-P-positive liver foci as indicators of efficacy. Initiation relates to I3C exposure during, or before and during carcinogen treatment. Post-initiation relates to I3C exposure after, or during and after carcinogen treatment. Adapted from Dashwood, 1998.*

thyroid gland hypertrophy and inhibiting iodine uptake by the thyroid, although there is little evidence of such effects occurring in man (Heaney and Fenwick, 1995). Xu *et al.,* (1997) found that I3C may provide both anticarcinogenic or tumour promoting effects dependent upon dose, exhibiting similar properties to other chemopreventive agents such as genistein (Zava and Duwe, 1997). I3C appears able, at very low levels (similar to amounts ingested in a normal diet), to facilitate DNA adduct formation by certain carcinogens (reviewed by Dashwood, 1997). Studies in trout (Oganesian *et al.*, 1999) have also shown I3C to be a tumour promoter, although isoforms of phase I/II metabolising enzymes do differ between species, which may account for some detrimental effects of I3C. The upregulation of certain P450s may also result in a detrimental effect, in that they may metabolise certain carcinogens to a more toxic form. Promotion by I3C at lower dietary levels, may in part be explained by estrogenic activity of I3C and its acid condensation derivatives, which act to promote chemically-induced hepatocarcinogenesis (Oganesian *et al.*, 1999), but may contribute to the anticarcinogenic effect in breast cancer (Riby *et al.*, 2000a). It may also be that I3C is more likely to have a potentially detrimental effect at a post initiation stage as opposed to administration prior to initiation (Dashwood, 1998), although this was not observed in the study by Manson *et al.,* (1998). A detrimental effect of I3C has been reported in its ability to alter immune function via suppression of natural killer cell cytotoxicity (Exon and South, 2000, 2001), resulting in a decrease in immune response effectiveness against aberrant cells or potential cancer-causing pathogens. The choice of endpoint markers in some studies may however, not truly reflect the chemopreventive effects of I3C, as increased GST-P positive foci were observed at early time points in rat livers by Manson *et al.,* when I3C was given post initiation, yet this did not correlate with tumour burden at the end of the study.

I3C may offer chemopreventive potential for all 3 stages of the carcinogenic process as it is able to act both as a pre-initiation blocking agent, and as a post-initiation suppressing agent in liver (Manson *et al.*, 1998), skin (Srivastava and Shukla, 1998) and breast carcinogensis (Stoewsand *et al.*, 1988, reviewed by Shertzer and Senft, 2000). Despite the potential for a protective effect in many forms of cancer, I3C and its derivatives have been most widely investigated with respect to their potential role in breast cancer prevention.

1.3.3 I3C and breast cancer

Estrogen is a critical factor in initiation and progression of mammary gland carcinogenesis, and modulators of enzymes involved in this pathway may be able to alter the carcinogenic process in estrogen sensitive tissues. Estrogen undergoes an initial oxidation of its 17β-hydroxy group to form estrone, which is then available for metabolism via hydroxylation at the C-2 or 16α position. Metabolites hydroxylated at the 16α position have been closely correlated with mammary tumour incidence due to their genotoxicity to breast cells (Bradlow *et al.,* 1985, 1994). Estrogen metabolism is partially regulated by constitutive P450s such as CYP1A1, 1A2, 2B1 and 3A, which increase metabolism of estrogen via the 2-OH non-genotoxic pathway. The increase in P450s associated with I3C and DIM-stimulated activation of the AhR, correlates well with the extent of 2-hydroxylation and down-regulation of the nuclear estrogen receptor (Ritter *et al.*, 2001), leading to a decrease in transcription of ERE (estrogen response element)-containing genes. Activation of the AhR by I3C or its metabolites, may also result in cross talk between the AhR and estrogen receptor, as the AhR transactivation domains (TAD) are able to inhibit 17β-estradiol-induced activation of the ER (Reen *et al.*, 2002). In agreement with animal model data, I3C and its derivatives are also able to induce estrogen-2-hydroxylase through increased AhR activity (via direct binding) in a variety of breast cell lines, resulting in an increased 2OH:16αOH ratio (Jellinck *et al.*, 1993, Niwa *et al.,* 1994, Tiwari *et al.*, 1994, Telang *et al.,* 1997, Chen *et al.*, 1998, Vang *et al.*, 1999).

This provides one of the mechanisms by which I3C treatment can ultimately result in a decreased breast cancer risk, as shown via inhibition of DMBA and nitrosomethylurea-induced mammary tumours in Sprague-Dawley rats (Stoewsand *et al.,* 1988, Chen *et al.*, 1998). It is also thought that I3C and its condensation products can elicit anti-estrogenic activity due to direct estrogen receptor binding (Liu *et al.,* 1994), resulting in a dose-dependent inhibition of estrogen-dependent transcriptional activity of ERα (Meng *et al.,* 2000c).

It was initially proposed that estrogen receptor activity was required for I3C and its derivatives to have an effect upon breast cell proliferation. It has now been demonstrated that not only can LTr_1 inhibit estrogen receptor function and activate the AhR, but it is also able to inhibit both the estrogen-dependent proliferation of MCF-7 cells, and the estrogen-

independent proliferation of MDA MB231 cells (Chang *et al.,* 1999). DIM and ICZ are also able to affect MCF-7 cell proliferation via mechanisms independent of ER activity (Liu *et al.,* 1994, Riby *et al.*, 2000b).

1.3.4 I3C in clinical trials

There is great advantage to using naturally derived chemopreventive agents that have a prolonged history of use within the human population. Most agents, however, are administered at much higher doses than would normally be ingested, so it is important to firstly determine any toxic effects within a small, healthy population. Most trials with I3C to date have used the increase in the ratio of urinary 2:16α OH estrogen metabolites as an end point biomarker suggestive of chemopreventive potential. Human trials using I3C are listed in *table 1.2.*

Study	Reference
Long term responses of women to indole-3-carbinol or a high fibre diet.	Bradlow *et al.* 1994
Dose ranging study of indole-3-carbinol for breast cancer prevention.	Wong *et al.* 1997
Increased estrogen 2-hydroxylation in obese women using oral indole-3-carbinol.	Michnovicz. 1998
Preliminary results of the use of indole-3-carbinol for recurrent respiratory papillomatosis.	Rosen *et al.* 1998
Role of estrodiol metabolism and CYP1A1 polymorphisms in breast cancer risk.	Taioli *et al.* 1999
In vitro and *in vivo* inhibition of human flavin-containing monooxygenase form 3 (FMO3) in the presence of dietary indoles.	Cashman *et al.* 1999
Placebo-controlled trial of indole-3-carbinol in the treatment of CIN.	Bell *et al.* 2000
Effects of anabolic precursors on serum testosterone concentrations and adaptations to resistance training in young men.	Brown *et al.* 2000
Indole-3-carbinol in women with SLE; an effect on estrogen metabolism and disease activity.	McAlindon *et al.* 2001

Table 1.2 Human clinical trials using I3C.

The first phase I trial for I3C recruited healthy subjects at high breast cancer risk. Subjects received 400mg/day I3C orally over a period of 3 months. The treatment was well tolerated by all and a significant increase in 2-OH estrone to estriol ratio was observed (Bradlow *et al.*, 1994).

Further dose ranging studies have since been carried out in women from similarly high risk groups, with the minimum effective dose reported to be 300mg/day, and only 5% of women exhibiting any toxic effects (Wong *et al.*, 1997). High levels of 16α estrogen metabolites are often present in obese women, and these levels have subsequently been shown to decrease following a regime of 400mg/day I3C, to levels comparable to those seen in non-obese women (Michnovicz, 1998).

This effect of dietary I3C upon estrogen metabolism has implications for many other hormone-dependent cancers such as cervical cancer. Here, intervention can occur at the cervical intraepithelial neoplasia (CIN) pre-invasive stage. In a placebo controlled trial of I3C in the treatment of CIN, no patients in the placebo group had complete CIN regression, whereas 50% showed complete regression at 200mg I3C/day and 44% at 400mg/day (Bell *et al.,* 2000). Trials such as this are encouraging, as they suggest that I3C may prove to be a useful therapy agent, particularly when administered prior to the fully invasive stage of tumourigenesis.

That I3C is able to exert a protective effect in women at high breast cancer risk is certainly encouraging in its role as a chemopreventive agent. However all trials to date have focussed upon its ability to affect estrogen metabolism. Cell studies have shown that I3C is also able to inhibit proliferation of breast cancer cells via an estrogen-independent mechanism, which could be a valuable finding with regards to its potential in inhibition of highly aggressive, estrogen-insensitive breast tumour growth. The use of breast cell lines in determining the mechanisms of action of I3C at a molecular level, will provide important information with regards to these effects upon cellular proliferation. Interaction of many cellular signalling pathways ultimately provide the stimulus for cells to proliferate or die, and it is the effect of I3C upon these pathways that is of major concern within this thesis.

1.4 THE CELL CYCLE

Progression of cells through the cell cycle determines the rate at which they are able to proliferate. The cell cycle consists of four main stages all of which are heavily regulated to ensure the correct order of events for each individual step. These stages consist of the G1 (gap1), S (synthesis), G2 (gap2) (which make up the interphase, during which time the cell is undergoing continuous growth) and the M (mitosis) phase. During the S phase, the cell is able to double its DNA content. Restriction points exist towards the end of the G1 and G2/M phases, crossing of which, allows progression to the next stage of the cell cycle. Once the restriction point in G1 has been passed, cells are able to progress from G1 to S phase, switching from a growth factor-dependent to -independent state (Nguyen and Jameson, 1998).

Progression past the G1 restriction point is overcome by phosphorylation of the retinoblastoma protein (Rb). Important regulators of the cell cycle include the transcription factor E2F, Rb and Rb associated proteins. Rb is a nuclear phosphoprotein that acts as a tumour suppressor gene, as its deregulation is associated with uncontrolled cell division leading to tumourigenesis. Rb strictly controls G1 progression, and is affected by direct binding of cyclin dependent kinases, which are under the regulation of cyclin proteins. Loss of Rb function has been shown to be associated with numerous malignancies, including retinoblastoma, osteosarcomas, lung cancer and bladder cancer (Hickman *et al.,* 2002).

In its resting state, Rb exists in a hypophosphorylated form. Growth factor induced activation of the cyclin-dependent kinases (cdks 4 and 6) in G1 occurs via their association with primarily cyclin D proteins, which persist throughout most of the cell cycle, or as long as growth factor availability is sustained (Nguyen and Jameson, 1998). This results in partial phosphorylation and inactivation of Rb, leading to release of E2F family members. Progression through the restriction point in G1 then requires the presence of cyclin E. On release of E2F, cyclin E begins to accumulate, complexes with Cdk2 and results in the phosphorylation and degradation of the cell cycle inhibitor p27. This cyclin E complex is also responsible for further phosphorylation of Rb and full activation of the E2F family (Jones and Kazlauskas, 2001). E2F then stimulates transcription of many S-phase target genes involved in proliferation such as c-myc, APAF-1, pRb, E2F-1, cyclin A and cyclin E (Krauss, 2001). G1 cell cycle arrest may occur if cells cannot progress through the

checkpoint at the end of G1. This may transpire due to DNA damage, growth factor withdrawal or the presence of agents that may enhance activity of cell cycle inhibitory proteins. Cells then progress readily from S phase to G2/M, with the integrity of the S phase monitored at a further checkpoint before entry into the M phase. Continuation through the S and G2/M phases relies on the expression of cyclin B proteins. Cdk1 (cdc2) interacts with cyclin B to form the 'mitosis promoting factor', which determines entry into mitosis, therefore driving progression of G2 to M phase (Castedo *et al.,* 2002) – a process that can be negatively regulated by p53 and p21.

Important regulators of progression through the cell cycle are the Cdks, which are themselves regulated by phosphorylation events in conjunction with the binding of cyclins. Cyclin family members become activated during specific phases of the cell cycle, and are mostly localised in the nucleus, with the exceptions of cyclins B1 and B2 that are eventually translocated there upon activation. *Figure 1.4* shows some of the regulatory components within each phase of the cell cycle.

Cdk inhibitors negatively regulate Cdk activity (and so slow progression through the cell cycle) and can be divided into two groups. The CIP/KIP family includes $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$, with the INK family compromising of $p15^{ink4b}$, $p16^{ink4a}$, $p18^{ink4c}$ and $p19^{ink4d}$ (Harper and Adams, 2001, Ortega *et al.*, 2002). The major cyclins, associated Cdks and their inhibitors are shown in table *1.3*.

p53 is one of the most frequently mutated genes in human cancer (Scherr, 1996), and functions as an essential cell cycle checkpoint regulator responsible for ensuring that in response to genotoxic damage, cells arrest in G1 prior to the onset of replication and that repair genes are activated. It exists in the cell in a constantly repressed state, until damaging signals occur (including DNA damage, constitutive activation of growth factors or hypoxia), whereupon it can mediate many antiproliferative responses. Genes and proteins regulated by p53 (reviewed in Stewart *et al.,* 2001, Guimaraes and Hainaut, 2002) include p21, cyclin A, Bax, Bcl-2, Cox-2 and Mdm2, which are involved in cell cycle, apoptosis and stress activated protein kinase regulation. Loss of p53 therefore allows duplication of damaged chromosomes to continue unchecked, and so gives rise to cells that are more likely to contain mutations and become tumourigenic, and acts as an important regulator of both proliferation and cell death.

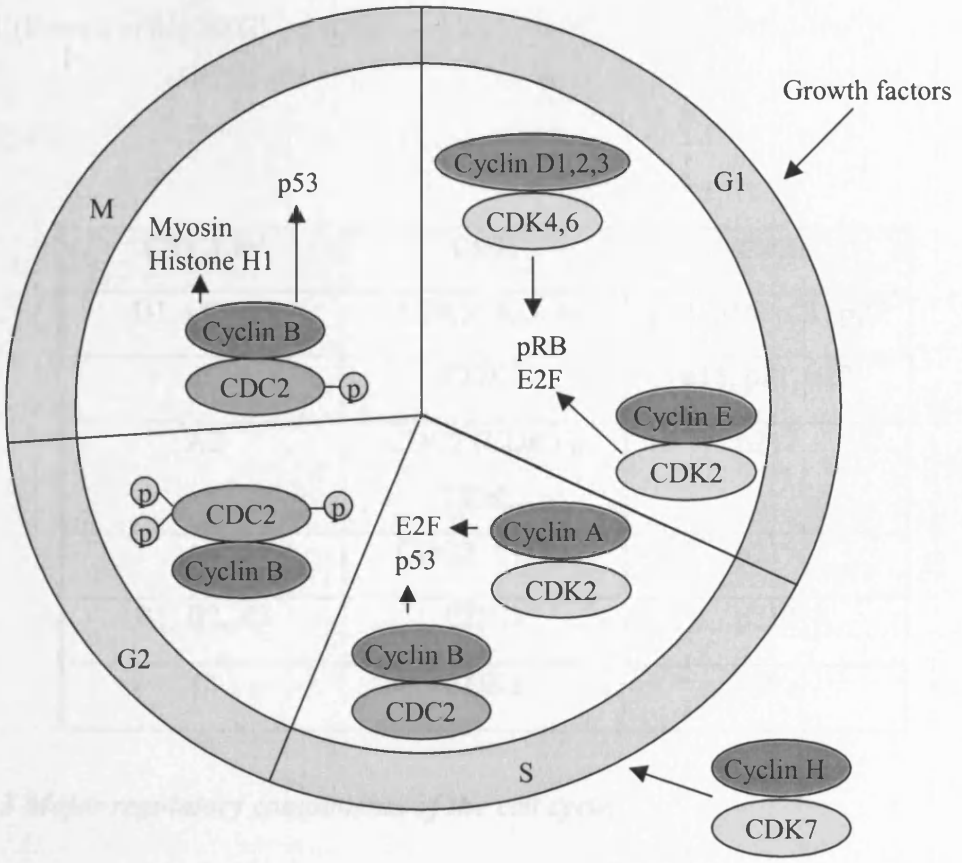


Figure 1.4 Activation of phase specific CDKs during the cell cycle. *Adapted from Biochemistry of Signal Transduction and Regulation. Ed. Gerhaud Krauss.*

The loss of normal cell cycle control has been implicated in mammary tumourigenesis via uncontrolled proliferation, with many breast cancers exhibiting aberrant expression or amplification of cyclins D1 (Hulit *et al.,* 2002) or E (Loden *et al.,* 1999). It has been proposed that cyclin D1 overexpression offers a valuable prognostic marker for breast cancer and de-regulated cell cycling, which may arise from aberrant regulation of numerous signalling pathways associated with the carcinogenic process (Sweeney *et al.*, 1997, Ingram and Geradts, 1998). Cyclin E has recently been proposed as a potential prognostic factor in breast cancer, with abnormal expression patterns observed in tumours from breast, prostate and cervical carcinomas (Erlandsson *et al.,* 2003). p53 mutations have also proven to be a common event in mammary tumourigenesis, with inactivation of p53 conferring a proliferative advantage to breast tumour cells (Landberg and Roos, 1997). Other cell cycle regulators such as $p16^{ink4a}$ have been observed to exhibit decreased

expression patterns in breast tumours (Silva *et al.,* 2003), as has the downstream effector of p53, p21 (Powell *et al.*, 2002).

CYCLIN	CDK	CKI
D1, D2, D3	CDK2, 4, 5, 6	p15, p16, p21, p27
E	CDK2	p15, p21, p27
A2	CDC2 (CDK1), CDK2	p21?
A1	CDC2, CDK1	p21?
B1, B2, B3	CDC2	p21?
C	CDK8	?

Table 1.3 Major regulatory components of the cell cycle.

1.5 APOPTOSIS

Apoptosis is a mechanism by which superfluous cells can be eliminated in a targeted manner, and it plays a central role in tissue homeostasis, developmental and differentiation processes, maintenance of the immune system and elimination of damaged cells (Greenwood *et al.,* 2002). The apoptotic process can be initiated in response to a variety of extracellular factors including growth factor withdrawal (Zhou, X.M. *et al.*, 2000), death receptor activation and cellular stress (Bratton *et al.,* 2000). The apoptotic process is very distinct from the necrotic process, and involves a number of characteristic changes in cell morphology such as chromatin condensation, degradation of DNA, cell shrinkage, nuclear fragmentation, membrane blebbing and formation of apoptotic bodies (Igney and Krammer, 2002). Necrosis on the other hand, exhibits none of the distinct fragmentation processing seen in apoptosis, but involves release of the cell contents directly into the surrounding stroma, thus resulting in induction of an inflammatory response. *Figure 1.5* shows the interaction of various signalling pathways that can ultimately lead to the execution of the apoptotic cascade. Initiation of apoptosis can be channelled via two major

pathways: one that is mediated by cell surface death receptors (extrinsic) and one that is mediated by the mitochondria (intrinsic), both of which are maintained by a series of signalling cascades that ultimately result in the activation of a family of proteases known as caspases (Bratton *et al.*, 2000, Wajant, 2002).

Caspases are the main effector components of the apoptotic cascade, and are initially formed as inactive pro-enzymes of 30-50kDa, which are then activated upon proteolytic processing (Zhivotovsky et *al.,* 1999). Caspases are specifically named for their properties, in that they use a Cys residue as a nucleophile, and cleave the substrate after an Asp residue (Slee *et al.*, 1999). There are currently 14 known caspases, which can be divided into *initiator caspases* (such as caspases 8 and 9) that are able to activate the *effector caspases* (such as caspases 3, 6 and 7) responsible for the execution of apoptosis (Chen *et al.,* 2002). The caspases can be further split into those directly associated with cell death (2, 3, 6, 7, 8, 9, 10) and those associated with cytokine processing (1, 4, 5, 11), although comparatively little is known about caspases 12, 13 and 14 (Slee et al., 1999).

Activation of the effector caspases can occur in a direct manner i.e., activation of a pro-caspase by an upstream initiator caspase that is already activated, or indirectly, by the processing of a pro-caspase effector into a mature caspase, through its close proximity to other molecules such as apoptosis protease activating factor-1 (APAF1) or cytochrome c which then initiates an apoptotic response due to this proximity (Zhivotovsky et *al.,* 1999). Caspases that are activated indirectly (caspases 1, 2, 4, 5 and 9) contain a caspase recruitment domain (CARD) that allows aggregates to form in the presence of cofactors (Nunez *et al.*, 1998).

The Bcl-2 family can be broadly split into pro-apoptotic and anti-apoptotic molecules. All possess homology in 4 regions called the Bcl-2 homology (BH) domains, which are responsible for mediating protein interactions in order for them to assume their function. Anti-apoptotic members of the Bcl-2 family include Bcl-2, Bcl-xL, BclW and Mcl-1, and are frequently found to be overexpressed in oncogenesis. Pro-apoptotic Bcl-2 family members include Bad, Bax, Bcl-xS and Bak, and are able to interact with other Bcl-2 family members by virtue of their BH domains. This allows dimerisation of Bcl-2 family molecules that results in modulation of their function to provide either pro- or anti-

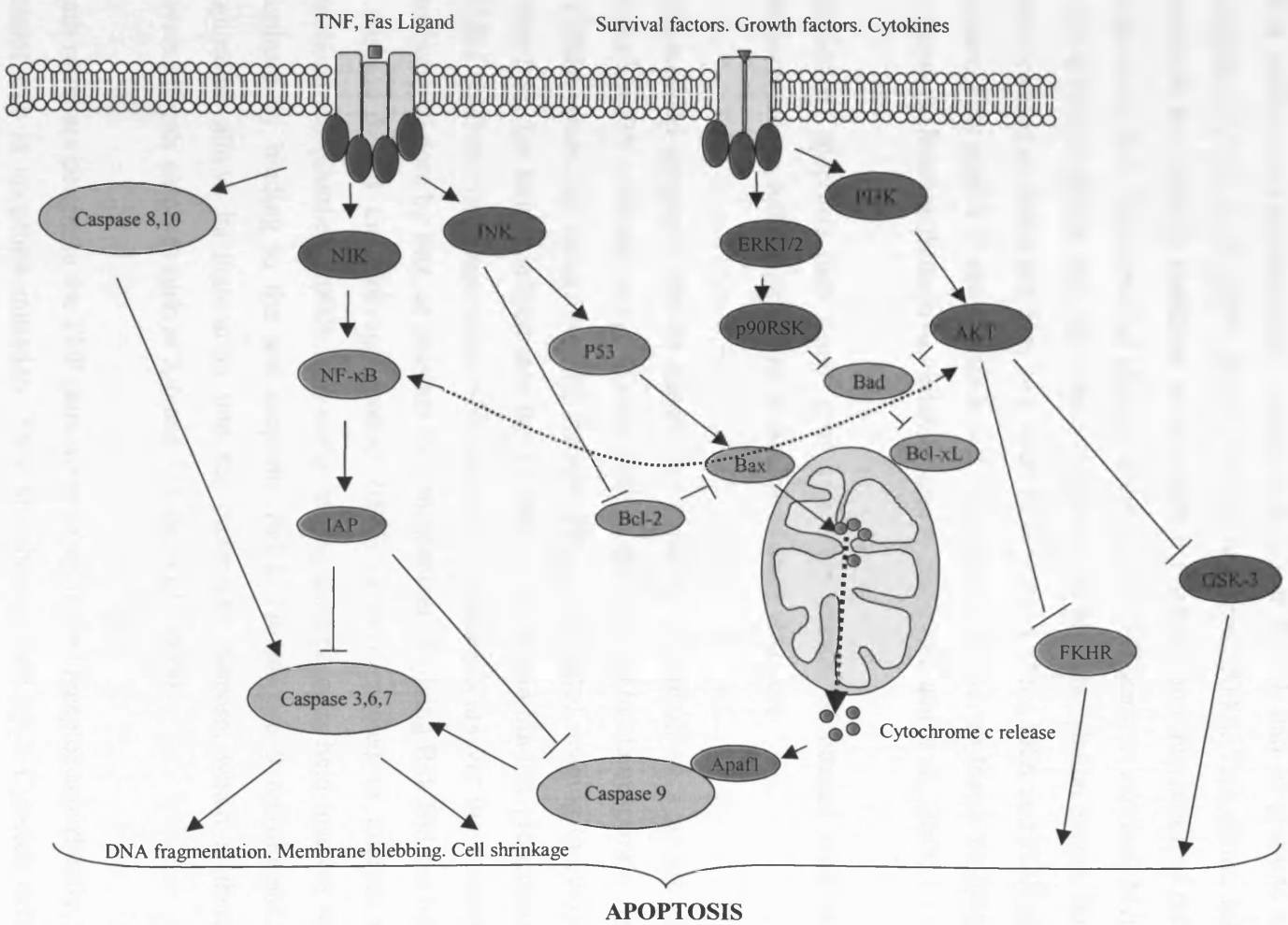


Figure 1.5. Interaction of signalling cascades that feed into the apoptotic process.

apoptotic signals. For example, the BH1 and BH2 domains are required for Bcl-2 and Bcl-xL to interact with Bax and suppress apoptosis, whereas the BH3 domain of Bak, Bax and Bad is sufficient to promote their binding to Bcl-2 or Bcl-xL and so promote apoptosis (reviewed in Cross *et al.*, 2000, Antonsson and Martinou, 2000). This allows interaction between all Bcl-2 family members, so it is often the balance and interaction of pro- versus anti-apoptotic Bcl-2 proteins that ultimately determines cell death or survival. Members of the Bcl-2 family such as Bad, may also by regulated by phosphorylation events. Bad can be phosphorylated on serine residues as a result of activation of the PKA and PI3K signalling cascades. This results in sequestration and inactivation of Bad, so that it no longer has a pro-apoptotic function through its ability to bind to Bcl-2 (Harada *et al.*, 1999).

Induction of apoptosis may occur upon two main stimuli of interest with respect to chemoprevention: cellular stress and activation of death receptors.

Stress-induced apoptosis can be caused by a variety of stimuli such as growth factor deprivation, DNA damage or cytotoxicity such as that induced by staurosporine. As a result of cellular stress, the integrity of the mitochondria is disrupted, leading to cytochrome c release from the mitochondrion into the cytosol, where it can bind to pro-caspase 9 and APAF-1 to form the apoptosome. Cytochrome c release occurs via the formation of a mitochondrial pore by Bax, or possibly by a mechanism involving Bid. Bid can be cleaved by caspase 8 and the cleavage product (tBid) is then enabled to interact with the mitochondrion (Gottlieb, 2000). Bax under resting conditions, is held inactive within the cytoplasm by binding to the anti-apoptotic Bcl-2. Pro-caspase 9 recruitment into the apoptosome allows its maturation into the proteolytic caspase, which is then able to activate effector caspases such as 3, 6 and 7 (Cain *et al.,* 1999).

Death receptors belong to the TNF (tumour necrosis factor) receptor superfamily, and play a major role in apoptosis initiation. They are characterised by a Cys-rich extracellular domain and a similar, intracellularly located 'death domain', as found in the Fas receptor and the TNF receptor. These receptors are activated by ligands such as CD95L, TRAIL, TNF or FasL. These ligands bind to their respective receptor, causing recruitment of the adaptor molecule FADD (TRADD for TNF) via binding of its death domain (DD) to the receptor (Ashkenazi and Dixit, 1998). Pro-caspases are then recruited to the FADD death

domain by a similar region within their 'pro' domain, resulting in formation of the DISC (death-inducing signalling complex) complex, which allows caspase maturation and subsequent activation of effector caspases (Couzinet *et al.*, 2002, Igney and Krammer, 2002). Once the effector caspases have been activated, the cells are committed to undergoing the apoptotic process. Cleavage of the nuclear enzyme poly (ADP-ribose) polymerase (PARP), which acts as a substrate for caspase 3, is often used as confirmation of caspase-mediated apoptosis.

Apoptosis in breast cancer

Normal development of breast tissue is reliant upon a balance between cell proliferation, and apoptosis, and there is increasing evidence that de-regulated apoptotic mechanisms may play an equal role alongside uncontrolled proliferation in the progression of breast tumourigenesis.

In normal breast tissue, there are dramatic changes to the Bcl-2 family proteins during puberty and pregnancy, which helps dictate the rapid tissue restructuring required both during and after these stages of development. During the menstrual cycle, there is also a cycling between proliferation and apoptosis mainly occurring in the lobular unit of the terminal duct within the breast (Anderson, 1999, Kumar *et al.,* 2000). In breast cancers, apoptosis is surprisingly often increased, but accompanied by high levels of proliferation, with high levels of apoptosis often correlating with poor survival (Lipponen *et al.*, 1994). In humans, the anti-apoptotic Bcl-2 is over expressed in approximately 80% of breast cancers (Krajewski *et al.*, 1999) and tends to correlate with the amount of estrogen and progesterone receptors present (Parton *et al.*, 2001), low p53 levels and a low proliferative rate, which generally indicates a favourable prognosis (Lipponen, 1999, Daidone *et al.,* 1999). The pro-apoptotic protein Bax has also been investigated as a potential prognostic marker, with some studies revealing reduced Bax levels to be associated with poor response to chemotherapy (Krajewski *et al.*, 1999). Other proteins that may have potential as predictive biomarkers include the X-linked inhibitor of apoptosis (XIAP). The presence of the caspase inhibitor XIAP may explain why there is a reduction in apoptosis in a subset of tumours despite the presence of caspase 3, possibly due to decreased processing of the pro-caspase to its active form (Krajewski *et al.*, 1999).

Apoptosis and therapeutic applications

There are many areas within the apoptotic cascade that offer potential targets for the action of chemotherapeutic/chemopreventive agents, not least because of the interaction with so many intracellular signalling cascades. A large number of studies to date have found a variety of chemopreventive agents to induce apoptosis in many models of carcinogenesis. I3C and its acid condensation derivatives have been shown to induce apoptosis in breast and prostate cell lines (Ge *et al.*, 1996, 1999, Chinni *et al.*, 2002, Howells *et al.*, 2002, Frydoonfar *et al.,* 2003), with green tea and derivatives inducing apoptosis in oral carcinogenesis (Li et al., 2002), breast cell lines (Yanaga et al., 2002) and skin carcinogenesis (Conney et al., 2002). Other naturally-derived chemopreventive agents which induce apoptosis include, curcumin in breast cells (Squires *et al.*, 2002), oral carcinogenesis (Li *et al.*, 2002), prostate cells (Deeb *et al.*, 2003) and rat thymocytes (Jaruga *et al.*, 1998), genistein in breast and prostate cells (Dampier *et al.*, 2001, Katdare *et al.*, 2002, reviewed in Sarkar and Li, 2002) and resveratrol in breast cells (Pozo-Guisado *et al.*, 2002). These compounds represent but a few that are currently under investigation, yet provide an insight into how chemopreventives may prove useful as anti-cancer agents.

Apoptosis and cell cycling are closely linked with many other cell signalling pathways that play an equally important role in proliferative control, and of particular interest is the phosphatidylinositol-3-kinase signalling pathway.

1.6 PHOSPHATIDYLINOSITOL-3-KINASE SIGNALLING PATHWAY

1.6.1 Phosphatidylinositol-3-kinase (PI3K)

PI3K is a key mediator of many processes including cell survival, cellular proliferation, cell cycle progression, glucose metabolism and cytoskeletal changes. The role of PI3K in cell survival is particularly relevant to cancer, as this disease state is associated with gross deregulation of survival and proliferative processes.

Signalling via PI3K depends upon its recruitment to the cell membrane upon activation of a variety of cell surface receptors. PI3K is able to initiate signalling cascades via its ability to catalyse the transfer of phosphate from ATP to the hydroxyl group at the D3 position of the inositol ring of membrane-localised phosphoinositides (Datta *et al.*, 1999). This results in the generation of the second messengers phosphatidylinositol 3-phosphate (PI(3)P)

phosphatidylinositol 3,4-bisphosphate (PI(3,4)P) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P) which are critical substrates for many kinases, phosphatases and phospholipases (Martelli *et al.,* 2002).

There are 3 classes of mammalian PI3K, classes I, II and III. Class IA PI3Ks have been the most intensely studied to date, and are heterodimers consisting of a regulatory subunit p85 (α/β) or p55 (α/χ) and a catalytic subunit p110 (α,β,δ), giving a potential of 12 distinct heterodimers. Class IA PI3Ks preferentially phosphorylate PI(4,5)P (although they will phosphorylate phosphatidylinositol and PI(4)P) and are receptor regulated, whilst class IB PI3Ks (PI3Kγ) are activated by G-protein subunits (Walker *et al.,* 1999). Class IB PI3Ks consist of the p110γ catalytic subunit and a p101 regulatory subunit, but are less widely distributed throughout the tissues than the class IA isoforms, being expressed mainly in haematopoietic tissues (Vanhaesebroek and Waterfield, 1999). Interestingly, Benistant *et al.,* (2000) have proposed distinct roles for p110α and p110β PI3Ks within the carcinogenic process, with p110α involved in cell survival and p110β required for *de novo* DNA synthesis in cancer cells.

Class II PI3Ks (PI3KC2$\alpha/\beta/\gamma$) possess alternate regulatory domains to p85 and p101 and preferentially phosphorylate phosphatidylinositol and PI(4)P, but similarly to class I PI3Ks, respond to a diverse range of receptor-mediated signalling processes. Insulin has been shown to activate the class IIα isoform, but unlike class I PI3Ks, it does not result in its association with IRS-1 (insulin receptor substrate 1) or the insulin receptor (Brown *et al.,* 1999). It is likely that the class II PI3Ks also have a role in oncogenesis, whilst class III PI3Ks use only phosphatidylinositol as a substrate, and are not usually considered to be involved in the proliferative and oncogenic processes associated with malignancy (Kraslinikov, 1999).

Not all subunit combinations have been fully investigated to determine whether they are physiologically viable, but there is evidence to suggest that there may be site-specific expression and function of some of these combinations (Beeton *et al.*, 2000, Vanhaesebroeck and Waterfield, 1999).

The class I PI3Ks can be activated by a diverse range of cell surface receptors including G-protein coupled receptors, receptors with intrinsic or associated protein tyrosine kinase activity and by the action of small G proteins such as Ras. Catalytic activity of the PI3K upon tyrosine receptor phosphorylation is thought to occur for two reasons; the close proximity of the recruited p110 subunit to its lipid substrate, and the fact that the interaction between the p85 subunit with the receptor tyrosine kinase (RTK) may relieve an inhibitory effect of p85 on p110 due to a conformational change (Vivanco and Sawyers, 2002). The p85 isoforms possess 2 Src homology 2 domains (SH2) and an N- terminal Src 3 homology domain (SH3) that are responsible for recruitment of the class I PI3Ks to the membrane. This interaction may occur via binding of tyrosine phosphorylated residues of p85 within the cytoplasmic domain of the receptor (Vivanco and Sawyers, 2002) or by binding of the phosphorylated receptor to the 'inter' SH2 sequence which separates the two SH2 domains (Krasilnikov, 1999). The p85α subunit is able to protect p110 from degradation and inhibits its enzymatic activity until receptor-mediated stimulation of p85 occurs (Yu *et al.,* 1998). It may be that the C-terminal region of p85α is responsible for the regulation of sequential PI3K activation by tyrosine kinases and Ras (Jimenez *et al.,* 2002).

The PI3K pathway is heavily involved in the regulation of apoptosis and consequently, proliferation in many cell lines, impinging on a variety of signalling pathways that are similarly crucial in normal growth and developmental processes. There have been several key investigations that have determined an active role for PI3K (particularly class I) in oncogenic transformation. The retroviral oncogene v-p3k which codes for a homologue of p110α has been shown to transform chicken embryo fibroblasts in vitro (Chang *et al.*, 1997) and amplification of the cellular-p3K (c-p3K) human counterpart (PIK3CA) has been reported in a variety of human ovarian cancer cell lines (Shayesteh *et al.,* 1999), although it is likely that p3k oncogenicity requires constitutive lipid kinase activity (Aoki *et al.*, 2000). There is also increasing evidence of PI3K involvement in carcinogenesis in both rodent and cell models (Phillips *et al.,* 1998, Perez-Tenorio, 2002). In many breast cancers, there is an amplification of the erbB family members, which are able to signal via and activate PI3Ks (Ignatoski *et al.,* 2000, Neve *et al.*, 2000, Hermanto *et al.,* 2001), with tumour cells that overexpress erbB2 often showing constitutive Akt activity (Nicholson and Anderson, 2002). Increased expression of the cytoplasmic protein tyrosine kinase Src, is

also seen in a high percentage of breast cancers, and enhances coupling of the EGF receptor to PI3K (Biscardi *et al.*, 2000) to increase signalling via this pathway.

1.6.2 PTEN/MMAC1/TEP1

The tumour suppressor gene PTEN (Phosphatase and Tensin homologue deleted from chromosome 10) located on human chromosome 10q23.3, is one of the most commonly mutated genes in human cancers. Germ line mutations of PTEN are associated with three autosomal dominant diseases in humans (Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome) that are characterised by multiple benign tumour formation, increased susceptibility to malignancy and certain growth defects (Waite and Eng., 2002). PTEN has been shown to be mutated or deleted in glioblastomas (Tada *et al.*, 2001), prostate cancers (McMenamin *et al.*, 1999, Dong *et al.*, 2001) and endometrial cancers (Risinger *et al.,* 1997, Simpkins *et al.,* 1998) to name but a few. It has also been shown that despite a low PTEN mutation rate, there is frequent loss or reduction of PTEN protein in primary breast tumours (Perren *et al.,* 1999, reviewed by Petrocelli and Slingerland, 2001).

PTEN possesses a structural similarity within its amino terminal sequence to that of dual-specificity phosphatases (Di Cristofano, 2002), which have the ability to dephosphorylate phosphotyrosine, phosphoserine and phosphothreonine residues. However, it is thought that the main mechanism of action for PTEN relies upon its lipid phosphatase abilities. PI(3,4,5)P is the major phospholipid substrate for PTEN, and is specifically dephosphorylated at the D3 position of the inositol ring. This results in a regulatory mechanism by which the pro-survival downstream effectors of the PI3K cascade can be controlled, resulting in a tumour suppressor function (Di Cristofano and Pandolfi, 2000).

The tumour suppressor SHIP (SH2-containing inositolpolyphosphate-5-phosphatase) is likewise a lipid phosphatase but is only able to dephosphorylate phosphoinositides at the 5' position. Up until recently, it was proposed that SHIP played only a very small role in regulation of this pathway, but Scheid *et al.,* (2002) proposed that SHIP not only regulates Akt activity, but also its subcellular localisation.

1.6.3 AKT (protein kinase B)

Akt is a major downstream effector protein of the PI3K pathway, and can regulate many proteins within a multitude of signalling pathways involved in cellular growth and proliferation and cell death (*figure 1.6*). Indeed, it has been shown that overexpression of constitutively active Akt is sufficient to rescue cells from PTEN-dependent cell death (Li *et al.*, 1998).

c-Akt is the cellular homologue of the transforming oncogene of the AKT8 retrovirus (Summers *et al.*, 1998). There are three functional Akt isoforms found in mammalian cells that are encoded by different genes, but with at least 85% sequence homology. All three isoforms possess conserved threonine and serine residues that are required for activation of the kinase; Akt1-thr308 and ser473, Akt2- thr309 and ser474, Akt3- thr305 and ser472 (Nicholson and Anderson, 2002). The isoforms are ubiquitously expressed in mammalian tissues, but exhibit different levels of both mRNA and protein expression between cell types. The isoforms consist of Akt1 - which is the predominant form in most tissues, Akt2 - which is highly expressed in insulin responsive tissues, and Akt3 - which has relatively low expression levels except in certain tissues such as the testis, brain and pancreas (Kandel and Hay, 2000). This differential expression between tissues would suggest that the isoforms may have varying function throughout growth and development.

Akt in oncogenesis

There is increasing evidence for Akt to be directly implicated in the carcinogenic process, due to the many functions it has within cell survival and cell cycle progression. The highly oncogenic effects elicited by membrane-targeted (and therefore constitutively active) Akt1, 2 and 3 appear indistinguishable (Mende *et al.*, 2001), and so the downstream targets that are relevant to transformation are probably shared by the three isoforms.

An increase in Akt1 activity is often associated with prostate and breast tumours that present with a poor prognosis, with Akt3 mRNA being expressed at 30-60 fold higher levels in ER-ve breast cancer cell lines when compared to ER+ve lines (Nakatani *et al.*, 1999). Page *et al.*, (2000) showed that MDA MB468 breast cells and LNCaP prostate cells expressed high levels of Akt phosphorylation associated with a lack of PTEN and increased Stat3 expression, giving rise to invasive, migratory phenotypes.

I3C is able to significantly inhibit cell adhesion, invasion and migration in the MCF7 and MDA MB468 cells in an estrogen-dependent (MCF7) and estrogen-independent manner. This is accompanied by an increase in E-cadherin, catenin and BRCA1 expression, so dramatically suppressing the invasive phenotype of these tumour cells (Meng *et al.,* 2000b). Many breast cancers overexpress the HER-2/neu (erbB2) receptor that is associated with poor prognosis and resistance to chemotherapy, and this also has been correlated with elevated phosphorylated Akt levels (Liu *et al.,* 1999, Hermanto *et al.,* 2001). A recent study by Stal *et al.,* (2003) found Akt 1 to be more prevalent in tumour samples than Akt 2, with overexpression of erbB2 significantly correlated with phosphorylated Akt. They also demonstrated a more significant benefit from tamoxifen treatment in patients that did not overexpress Akt1, Akt2 or phosphorylated Akt.

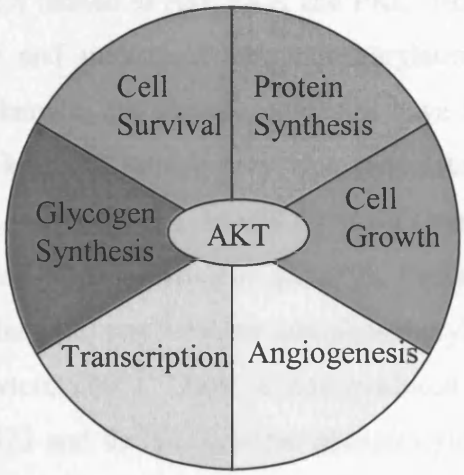


Figure 1.6 Central role of Akt in signalling processes involved in cell growth, proliferation and differentiation. *Adapted from Brazil and Hemmings. 2001.*

1.6.3.1 Regulation of Akt

In its resting state Akt is localised within the cytoplasm, and it is only upon cellular stimulation that it migrates to the plasma membrane and binds via its pleckstrin homology (PH) domain. Recruitment to the inner surface of the plasma membrane occurs upon generation of the phosphoinositide PI(3,4,5)P, which binds with high affinity to the PH

domains within the Akt molecule. Full activation of Akt requires phosphorylation of ser473 (c-terminal hydrophobic motif) and thr308 (activation loop) or equivalent sites on Akt 2 and 3, and occurs via the actions of two kinases (see below) termed phosphoinositide dependent kinases 1 and 2 (PDK1 and 2) (Vanhaesebroek and Alessi, 2000).

It is thought that in order for Akt to be phosphorylated by the PDKs, all 3 kinases are first recruited to the plasma membrane by virtue of the high affinity of their PH domains for PI3K-generated PI(3,4,5)P. This functions to increase the proximity of Akt to the PDKs and induces a conformational change within the Akt molecule, allowing PDK1 to phosphorylate the thr308 site. A large proportion of PDK1, however, remains as a cytosolic fraction and may be important in the phosphorylation of other proteins such as p70S6K (Kandel and Hay, 1999). In its unmodified form, PDK1 is unable to phosphorylate the ser473 site, although its presence may be required for the molecule putatively named PDK2 to phosphorylate ser473. PDK1 encodes a 63kDa protein containing a PH domain and a consensus domain closely related to Akt, PKA and PKC. However, although unlike Akt, it is constitutively active and undergoes autophosphorylation (Alessi *et al.*, 1996). The identity of PDK2 is unknown, but several molecules have been proposed for its activity, including PKC-related kinase-2 (which may also modulate PDK1, enabling it to act as PDK2 (Balendran *et al.*, 1999, 2000)), MAPKAP-K1/2 (Vanhaesebroek and Alessi, 2000) and integrin-linked kinase (Delcommenne *et al.*, 1998, Persad *et al.,* 2001). Alternatively it is possible that the serine site may become autophosphorylated upon phosphorylation of thr308 (Toker and Newton, 2000). There is also evidence to suggest that in addition to phosphorylation of ser473 and thr308, tyrosine phosphorylation of Akt may be important for its biological function (Chen, R.Y. *et al.*, 2001). It has also been suggested that Akt may be activated in a PI3K-independent manner by cyclic AMP elevating agents, formation of a calcium:calmodulin complex, heat shock and through PKA (Filippa *et al.*, 1999). Phosphorylated Akt may then be translocated to the nucleus (Borgatti *et al.*, 2000, Neri *et al.,* 2002), suggesting a further role for Akt perhaps as a transcription factor or an involvement in nuclear protein phosphorylation.

Negative regulation of Akt occurs mainly through the lipid phosphatases PTEN and SHIP which block its activation, although direct dephosphorylation of Akt can occur, particularly through the actions of protein phosphatase 2A. Akt activity is also modulated to some

extent by caspases, which are able to cleave the Akt molecule, resulting in accelerated cell death by turning off survival pathways (Rokudai *et al.*, 2000).

1.6.3.2 Functions of Akt

Akt plays a major role in regulation of glucose metabolism due to its ability to phosphorylate and inactivate glycogen synthase kinase-3 (GSK-3) and to activate phosphofructo-2-kinase to stimulate glycolysis (reviewed in Coffer and Woodgett, 1998). It also has a role in translational regulation and protein synthesis due to its ability to phosphorylate and inactivate certain translational repressors such as 4EBP1. However, within this thesis the role of Akt in the mediation of cell survival processes, and how this may contribute to the oncogenic process will be discussed in more detail. *Figure 1.7* shows the interaction between PI3K, PTEN and Akt, and illustrates some potential downstream targets for Akt.

The role of Akt in the cell survival process.

It is now well established that Akt plays a key role in the cell survival process. In its phosphorylated and active form it has the ability to block apoptosis induced by a wide variety of stimuli, promoting survival in many cell types. That Akt is important in cell survival has been demonstrated in many cellular models using the inhibitors of PI3K, wortmannin and LY294002, and by the transfection of constitutively active or kinase dead Akt constructs into various cell types (Alessi *et al.*, 1996). Overexpression of Akt has been shown to block apoptosis induced by many stimuli such as growth factor withdrawal, UV irradiation, DNA damage and treatment of cells with anti-Fas antibodies (Datta *et al.*, 1999). However, in cells that do not express, or have a mutated form, of the tumour suppressor PTEN, even though there is aberrantly high Akt activity, these cells appear to have an increased susceptibility to induction of apoptosis (Cantley and Neel, 1999).

The definition of the consensus sequence of the major site phosphorylated by Akt (RXRXXS/T-bulky hydrophobic site, Alessi *et al.*, 1996), has suggested several Akt target proteins that are essential components of the apoptotic machinery.

One mechanism by which Akt may block apoptosis is via the phosphorylation of the pro-apoptotic molecule Bad at the serine 136 residue, resulting in its sequestration by the chaperone protein 14-3-3. This sequestration protects Bad from dephosphorylation and also

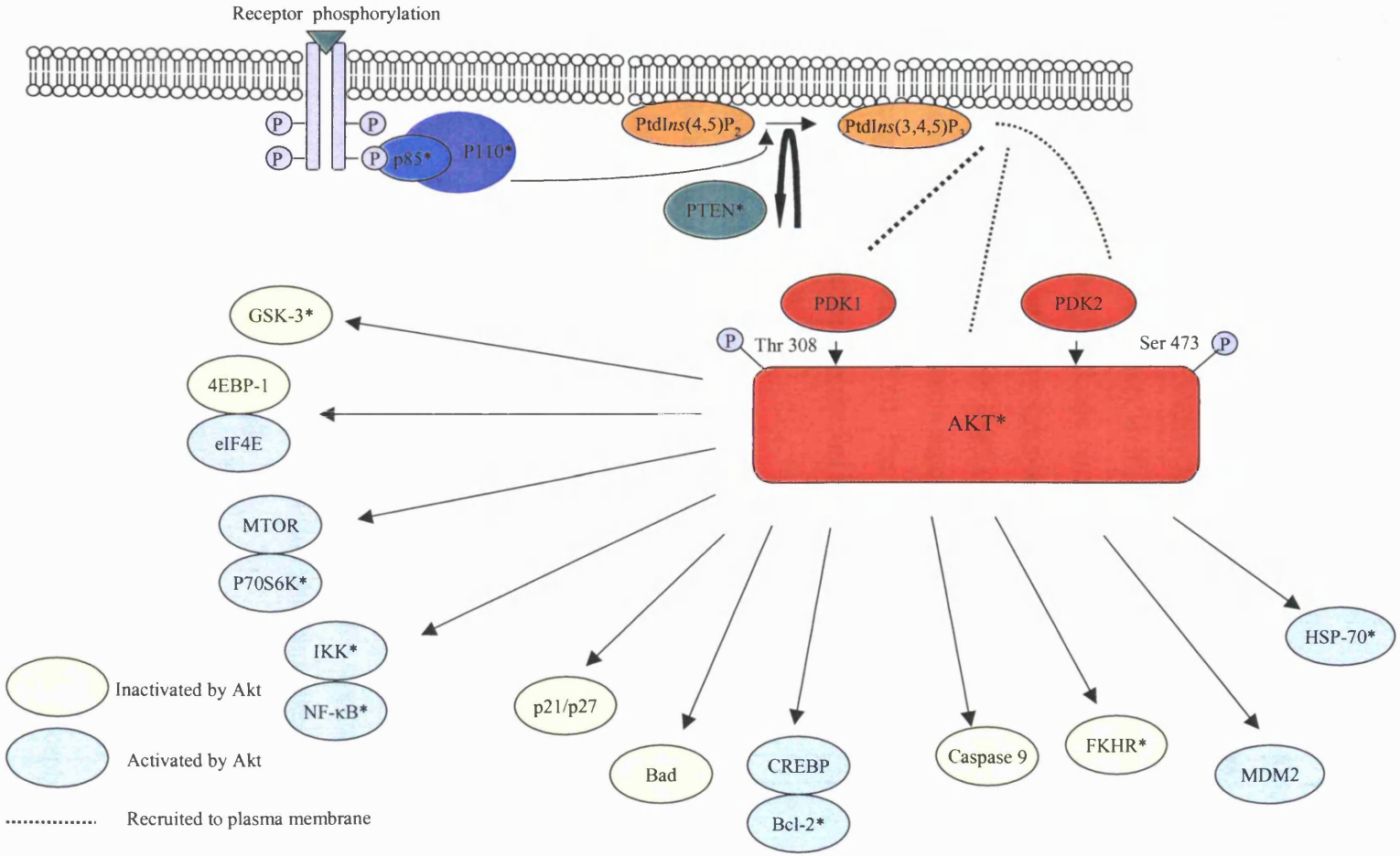


Figure 1.7 Interaction of members of the PI3K signalling pathway and downstream targets. ** represents those molecules investigated within this thesis*

acts to remove Bad from its mitochondrial targets, so inactivating its ability to promote cell death (Scheid and Duronio., 1998).

Similarly, Akt can phosphorylate and inactivate the superfamily of forkhead transcription factors. The forkhead family consists of approximately 90 members, with all members showing high sequence homology within their 'winged helix' domain to the fork head protein of *D. melanogaster*. The most well studied forkheads include AFX, FKHRL1 and FKHR, which once phosphorylated, are removed from the nucleus and transported away from their sites of action into the cytoplasm via sequestration by 14-3-3 (Kops and Burgering, 1999). Phosphorylation of these proteins may be due to the ability of the active Akt molecule to detach from the plasma membrane and translocate to the nucleus (Meier *et al.,* 1997, Borgatti and Martelli, 2000). The forkheads are key pro-apoptotic molecules in that they are transcription factors for the Fas ligand gene promoter.

Akt is also able to maintain the integrity of the mitochondria by preventing cytochrome c release by decreasing pore formation by the Bax molecule, possibly due to an increase in the ratio of anti-apoptotic (such as Bcl-2, Bcl-xL) to pro-apoptotic molecules. CREB is phosphorylated by Akt (Pugazhenthi *et al.,* 1999), resulting in an increase in its transcriptional activity. It has also been proposed however, that Akt may suppress neural apoptosis via a postmitochondrial mechanism, that does not suppress cytochrome c release but occurs prior to activation of caspase 9 (Zhou, H.L. *et al.,* 2000). Human caspase 9 phosphorylation and inactivation by Akt has also been proposed as a mechanism for Akt-induced cell survival (Cardone *et al.,* 1998). However, the true importance of this as an anti-apoptotic mechanism is as yet unclear, as this phosphorylation site is not conserved throughout other species (Lazebnik, 2000).

Akt and cell cycle

Akt is able to modulate intracellular levels of cyclin D1 in several ways. Akt phosphorylates and inactivates GSK-3β. GSK-3β when active, phosphorylates cyclin D1, whereupon the latter is redistributed from the nucleus to the cytoplasm for degradation (Diehl *et al.,* 1998). Inhibition of GSK-3 by Akt, therefore preserves nuclear levels of cyclin D1 by increasing its protein stability. Increased Akt activity is also able to elevate cyclin D1 levels by inducing mRNA translocation and translation (Muise-Helmericks *et*

al., 1998), and can induce other factors that directly enhance cell cycle progression such as c-myc, with myc upregulation being abrogated upon PTEN overexpression (Ghosh *et al.* 1999, Lilja *et al.* 2001). Cyclin D1 regulation by Akt may also occur through NF-κB signalling.

High levels of active Akt appear to phosphorylate and inactivate the cell cycle inhibitors $p21^{WAF}$ and $p27^{KIP}$, with loss of p27 function being associated with a decrease in androgen dependence in prostate cancer cells. Prevention of p21 and p27 entry into the nucleus will ultimately result in cell cycle deregulation (Graff *et al.,* 2000, Rossig *et al.,* 2001, Blain and Massague, 2002). Retention of p21 and p27 in the cytoplasm upon phosphorylation due to 14-3-3 binding and sequestration (El-Deiry, 2001, Fujita *et al.,* 2002), also results in p21 binding to apoptosis-signal-regulating kinase (ASK1) and decreased JNK activity, leading to inhibition of apoptosis. ASK1 is also directly phosphorylated at ser83 by Akt, similarly resulting in changes to JNK and p38-mediated transcriptional events (Kim, A.H. *et al.*, 2001). Enhanced signalling through Akt therefore results in the modulation of signalling molecules in favour of proliferation, by inhibiting apoptosis and preventing inhibition of the cell cycle.

Akt and EGFR signalling

It can be seen that Akt plays an important role in pro-survival signalling, which may be further enhanced due to 'cross talk' with the Raf-MEK-ERK pathway. Whilst Ras can interact with and stimulate PI3K activity, Akt is able to directly phosphorylate and inhibit Raf-1. Raf-1 is a downstream effector of Ras that is able to phosphorylate and activate the ERK pathway (Moelling *et al.,* 2002). Inhibition of Raf-1 via protein 14-3-3 association, leads to a reduction in its signalling activity through the ERK pathway (Zimmerman *et al.*, 1999, Ignatoski *et al.*, 2000). This cross talk between Akt and Raf is thought to be dependent upon ligand type, ligand concentration, the intensity of signalling and upon the duration of the signal (Moelling *et al.*, 2002). In contrast to this, PI3K is able to upregulate the ERK pathway at the level of Raf, and so enhance cell survival (Mograbi *et al.,* 2001). This suggests a complex interaction between PI3K-Raf-Akt that may be responsible for 'fine-tuning' the survival signals through the MAPK pathway.

In human oesophageal cancer cells, all 3 Akt isoforms appear to be regulated through EGFR-mediated stimulation of PI3K in a partially Ras-dependent manner (Okano *et al.*, 2000), although Neve *et al.,* (2002) have proposed that the functions of EGF-stimulated EGFR and PI3K are quite distinct in T47D cells, with each pathway necessary for proliferation, but only occurring upon their cooperation. Biswas *et al.*, (2000) demonstrated a positive link between EGF signalling, PI3K and NF-κB DNA binding, by using LY294002 to block EGF stimulated NF-κB activation.

Akt and GSK-3/Wnt signalling

The Wnts are secreted glycoproteins that mediate many stages of development, including cellular proliferation and differentiation. Overexpression of the Wnt ligand and associated deregulated Wnt signalling has been revealed in a variety of human tumours, and in particular, breast cancer (Kirikoshi *et al.,* 2002, Katoh, 2003). Akt is itself stimulated by Wnt signals, and is thought to play a pivotal role in Wnt signalling due to its ability to phosphorylate and inactivate GSK-3 (Fukumoto *et al.,* 2001). Akt activity on its own, is not sufficient to mimic a Wnt signal, and there is still some uncertainty as to how prolonged Wnt signalling is able to activate Akt (Fukumoto *et al.*, 2001). Binding of Wnt-1 to its frizzled receptor (fzr) results in the phosphorylation and activation of dishevelled (dvl), which is then able to phosphorylate and inactivate GSK-3 (Hollman *et al.,* 2001). (Refer to *figure 1.8).* Recent evidence suggests that ILK may interact with phosphorylated dvl and phosphorylate Akt to enable its participation in the Wnt signalling cascade (Yoganathan *et al.*, 2000).

GSK-3 was originally thought to be purely involved in glucose metabolism, but is now known to act as a key signalling molecule within the cell, particularly with respect to the Wnt signalling pathway. In the absence of a Wnt signal, active GSK-3 is present in a complex consisting of β-catenin, Axin and the protein product of the *APC* gene. Active GSK-3 is able to phosphorylate all components of this complex. Axin phosphorylation results in stabilisation of this protein, acting as a scaffold for the complex, whereas APC phosphorylation facilitates binding of GSK-3 to β-catenin. Once β-catenin has been phosphorylated, a recognition site for ubiquitin ligase is created, leading to β-catenin degradation and decreased availability for its role as a transcription factor (Nakamura *et al.,*

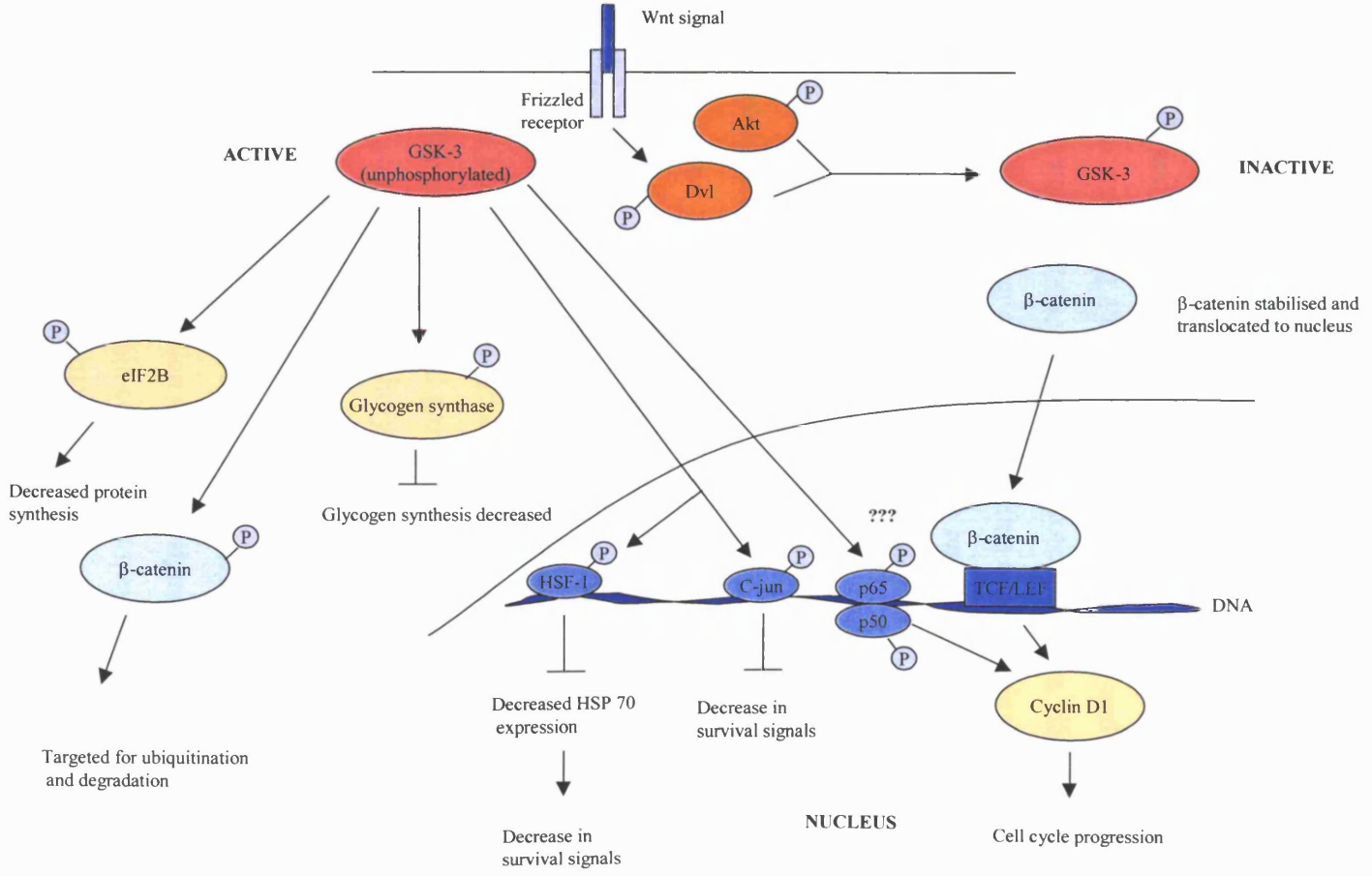


Figure 1.8 GSK-3 signalling in the presence or absence of a Wnt/Akt signal

1998, Frame and Cohen, 2001). Binding of Wnt to its frizzled family receptor results in GSK-3 being inactivated due to its phosphorylation by dishevelled. This then results in dephosphorylation of β-catenin and its transport to the nucleus where it is transcriptionally active, and can upregulate the TCF/LEF transcription factors. Potential targets of this pathway include adhesion molecules (MUC1, E-cadherin), cyclin D1 (which have a LEF binding site within the promoter region), c-jun, c-myc and heat shock factors (D'Amico *et al.,* 2000, Cohen and Frame, 2001). Heat shock factor-1 (HSF-1) is negatively regulated through GSK-3 in a partially PI3K dependent manner (Bijur and Jope, 2000), ultimately resulting in the downregulation of heat shock proteins such as HSP-70 and so decreasing survival signals (Nylandsted *et al.*, 2000*).* Induction of apoptosis was also observed to be a direct consequence of increased GSK-3 activity resulting from PI3K inhibition (Pap and Cooper, 1997), suggesting a further role for constitutive Akt phosphorylation in cell survival.

PI3K and p70S6Kinase (p70S6K)

The 70 KDa serine threonine kinase p70S6K, is a member of the cyclic AMP-dependent/cyclic GMP-dependent/protein kinase C subfamily of protein kinases, and has been seen to be amplified in breast cancers (reviewed by Salh *et al.* 2002). p70S6K phosphorylates the 40 S ribosomal protein S6 which encodes a variety of translation elongation factors and ribosomal proteins (Gonzales-Garcia *et al.*, 2002). It therefore plays a central role in the regulation of cell growth and survival through regulation of these key protein synthesis components. Phosphorylation of p70S6K is thought to occur via a PKC-dependent pathway, and a PKC-independent pathway that is mediated through PI3K signalling. PDK-1 is able to directly phosphorylate and activate p70S6K on the thr212, 229 and 412 sites present in the catalytic domain (Balendran *et al.,* 1999). It is also thought that p70S6K can undergo phosphorylation via MAPK signalling (Iijima *et al.,* 2002). It is also able to directly affect BH3 domain-containing proteins, such as those involved in the apoptotic cascade (Harada *et al.*, 2001).

1.7 NF-κB SIGNALLING

NF-κB (nuclear factor kappa B) is a transcription factor acting as a central regulator of the inflammatory response, which induces the expression of many early response genes

following cellular exposure to a diverse range of signals including cytokines, mitogens, toxic metals, intracellular stresses, viral or bacterial products and UV light (Chen *et al.*, 2001). Five NF-κB proteins exist across two classes of NF-κB and are generally present as dimeric complexes composed of the Rel family of polypeptides including p50 (NF-κB1), p52 (NF-κB2), p65, RelB or c-Rel, with the most commonly active heterodimer consisting of p50:p65. The gene products of NF-κB1 and 2 consist of p105 and p100 which undergo proteolytic processing to form p50 and p52 respectively (Karin and Lin, 2002). The C-terminal regions of p65, RelB and c-Rel contain a transactivation domain that is important for NF-κB mediated gene transactivation, whilst the C termini of p50 and p52 subunits are more important in promoter region binding (Siebenlist *et al.*, 1995). The NF-κB dimer under resting conditions is usually held inactive within the cytoplasm via its binding to the inhibitory protein IκB. There are 3 known IκB isoforms (α, β, ε) of which IκBα is the most common. Phosphorylation of conserved serine residues within the IκB N-terminal domain by IKK, results in rapid targeting of IκB for degradation via the 26S proteasome and the release of NF-κB, whereupon it is able to translocate to the nucleus (*figure 1.9*).

1.7.1 NF-κB and apoptosis

TNF receptor-mediated activation of NF-κB induces expression of anti-apoptotic proteins such as the caspase inhibitors cIAP1, cIAP2 and XIAP (Stehlik *et al.,* 1998), cell cycle regulatory proteins such as cyclin D1, TNF receptor-associated factors TRAF1 and TRAF2 (Guttridge *et al.,* 1999, Hinz *et al.*, 1999) and mitochondrial membrane stabilising proteins such as Bcl-xL and Bcl-2 (Kurland *et al.,* 2001). However, NF-κB can also induce cell death via activating expression of the Fas ligand (Barkett and Gilmore, 1999) depending upon the nature of the stimulus, and may be required in p53-mediated apoptosis (Ryan *et al.,* 2000). The precise mechanisms that determine the switch from an anti- to pro-apoptotic molecule for NF-κB have not yet been clearly defined (Perkins, 2000). There is also evidence to suggest that apoptotic molecules such as receptor interacting protein (RIP) can regulate NF-κB function (Hur *et al.* 2003), as can caspases, following studies of p65 and IκBα cleavage (Barkett *et al.,* 1997).

1.7.2 NF-κB and cell cycle

NF-κB activation has been found to be required in cell cycling of estrogen receptor negative breast cancer cells (Biswas *et al.*, 2000), with identification of two NF-κB binding sites in the human cyclin D1 promoter providing further evidence of its involvement. The involvement of NF-κB in p53 regulation has also been shown in a variety of breast cell lines. Induction of p53 can activate NK-κB, with loss of NF-κB activity resulting in a

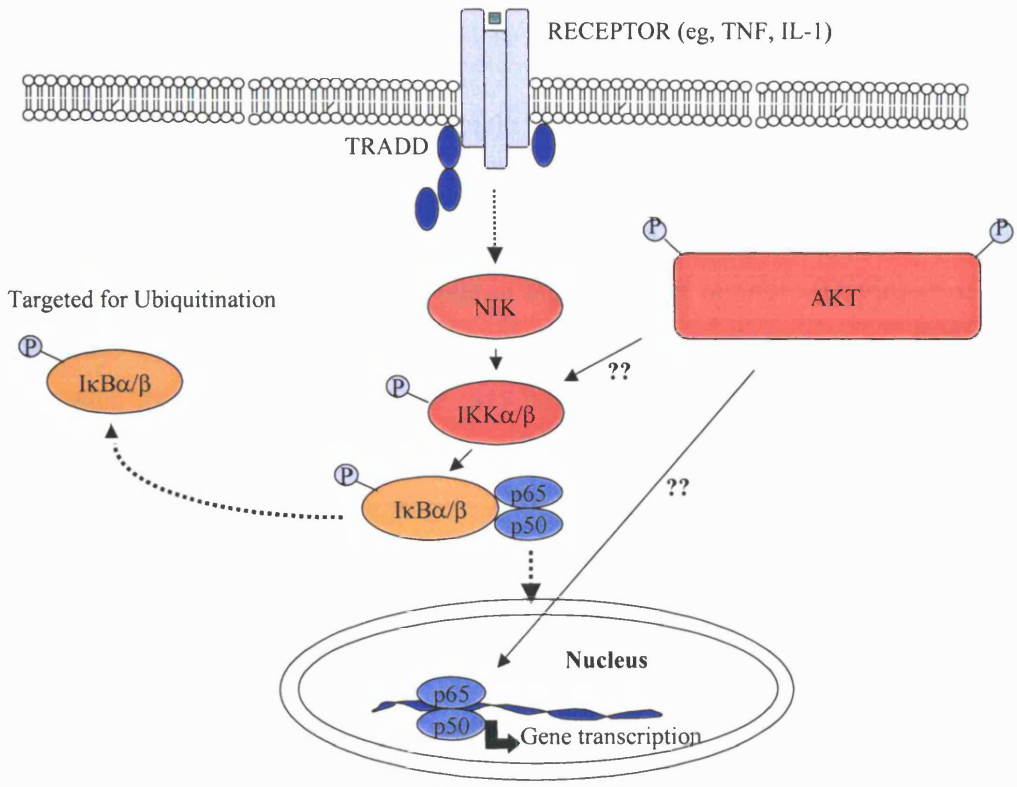


Figure 1.9. Activation of the NF-kB pathway via TNF receptor signalling, and points at which Akt signalling may interact.

decreased ability of p53 to induce apoptosis, indicating that NF-κB plays an essential role in p53-mediated cell death (Ryan *et al.,* 2000).

1.7.3 Role of NF-κB in breast cancer

The exact cause of NF-κB overexpression in breast cancers is not known, but it is possible that oncogenic stimuli common to breast cancers may increase signalling through the NF-κB pathway (Bhat-Nakshatri *et al.*, 2002). NF-κB also plays an important role in normal breast tissue growth and development during pregnancy, and acts to stimulate cell proliferation by increasing transcription of the cyclin D1 gene due to autocrine production of the TNF family member Receptor Activator of NF-κB (RANK). It is possible that during the carcinogenic process, RANK or RANK ligand production may be deregulated, acting to increase cyclin D1 transcription and enhancing the proliferative capacity of the cells (Cao *et al.,* 2001).

Cyclooxygenase 2 (Cox-2), a highly inducible enzyme catalysing prostaglandin synthesis, is upregulated by growth factors, cytokines, hormones and mitogens. Cox-2 possesses two NF-κB binding sites within its promoter region (Surh *et al.*, 2001) and previous studies have reported elevated prostaglandin levels in human breast tumours (Davies *et al.,* 2002, Karuppu *et al.,* 2002) and transformed breast cell lines (Subbaramaiah *et al.,* 1996). It has been suggested that prostaglandin levels may be indicative of metastatic potential and of decreased survival (Tapiero *et al.,* 2002), and that Cox-2 inhibition could provide a potential therapeutic target and surrogate endpoint biomarker in breast carcinogenesis (Parrett *et al.,* 1997, Vainio., 1998).

NF-κB also plays a central role in the transcription of genes encoding the inhibitors of apoptosis (IAPs), and can itself be regulated by the IAPs via a negative feedback mechanism (Levkau *et al.,* 2001). The IAP gene family encodes a set of proteins that are able to directly inhibit caspases 3 and 7 (Deveraux *et al.,* 1997) and are defined by a domain known as a baculovirus IAP repeat (BIR). These domains are themselves able to inhibit caspases 3 and 7, and can inhibit caspase activation induced by cytochrome c (Deveraux *et al.,* 1998). Mammalian IAPs are able to block apoptosis caused by a wide variety of stimuli, although there also appears to be an increasing role for BIR proteins (BIRP) in cell cycle regulation and signal transduction pathways (Levkau *et al.,* 2001). In human melanoma cell lines, the chemopreventive agent curcumin has been reported to inhibit the NF-κB survival pathway and suppress XIAP expression (Bush *et al.*, 2001),

making the overexpression of IAPs in tumour cell lines a potential target for therapeutic strategies.

Akt and NF-kB signalling.

There is increasing evidence that Akt is implicated in survival mechanisms through the involvement of the transcription factor NF-κB. Akt may enhance the degradation of IκB and so increase NF-κB translocation to the nucleus, allowing promotion of transcription of various survival genes. Akt was found to be involved in the induction of NF-κB transcriptional activity by TNF, PDGF and Her2/neu signalling (Ozes *et al.,* 1999, Romashkova *et al.,* 1999, Zhou, B.P. *et al.*, 2000), via a direct phosphorylation of thr23 on IKKα resulting in IκB degradation and increased NF-κB translocation to the nucleus.

Anti-apoptotic and proliferative genes that are under NF-κB transcriptional regulation include Bcl-2, c-myc, cyclin D1 (Hinz *et al.*, 1999), p53, Cox-2 and the inhibitors of apoptosis (IAPs). It has also been recently demonstrated that reintroduction of PTEN into a PTEN negative prostate cell line inhibited Akt activity as expected, but also blocked TNF-induced transcriptional activity of NF-κB and removed its anti-apoptotic function (Mayo *et al.,* 2002). However, alternative mechanisms have been proposed for the action of Akt in NF-kB signalling that are independent of proteosomal IκB degradation, such as via calpain (a cysteine protease that when activated via calcium mobilisation, is able to degrade IκB) (Pianetti *et al.,* 2001), or via a direct interaction between Akt and nuclear NF-kB.

It has been proposed that Akt can stimulate NF-kB activity by upregulating the transactivation potential of the p65 subunit via stimulation of transactivation domain 1 (TAD1), although it is unlikely that Akt is able to directly phosphorylate this region as it does not contain the consensus Akt phosphorylation site (Madrid *et al.*, 2000). The mechanism of IκB-independent induction of p65 transactivation potential has been further elucidated as an indirect mechanism involving Akt induction of the MAPK p38. Stimulation of p65 may occur via p38 cooperation with the CREB-binding protein/p300 (CBP/p300) transcriptional coactivator (Madrid *et al.,* 2001), which functions as a 'bridging' protein between transcription factors and the transcription complex (Snowden and Perkins, 1998). Further interactions between PI3K signalling and NF-κB have been demonstrated by potentiation of TNF-induced apoptosis upon PI3K inhibition (Koul *et al.*,

2001), and by overexpression of PTEN, which inhibited TNF-induced p65 transactivation, and blocked TNF-stimulated expression of NF-κB-dependent genes (Mayo *et al.,* 2002). There is recent evidence to suggest that NF-κB may be implicated upstream of Akt, with overexpression of p65 leading to the phosphorylation of Akt in the absence of any extracellular stimulatory factors (Meng *et al.*, 2002).

The complex nature of proliferative processes within carcinogenesis makes it extremely difficult to target naturally derived chemopreventive agents with great specificity. It is likely that these agents target and interact with a variety of signalling molecules that can result in a host of downstream consequences. Ultimately, it is hoped that the integration of I3C-induced signalling disturbances will result in the balance being tipped in favour of cell death and decreased proliferation within tumourigenic cell lines.

AIMS

The aims of this study were to investigate the potential chemopreventive mechanisms of action of I3C that are not reliant upon alteration of signalling events through ERα.

From historical data, we proposed that I3C would be able to inhibit the growth of both ER+ve (T47D, MCF7) and ER-ve (HBL 100, MDA MB468) breast cell lines. We then aimed to determine the mechanism by which I3C-mediated growth inhibition occurred in these cell lines, employing flow cytometric analysis to establish the induction of cytostatic (cell cycle arrest) or cytotoxic (apoptosis or necrosis) events.

The susceptibility of cell lines to I3C-mediated growth inhibition is likely to be under the influence of signalling pathways involved in proliferation and cell survival. It was hypothesised that I3C may elicit differential effects on proliferation as a consequence of varying expression of key signalling molecules between cell lines.

Survival pathways that are often deregulated in breast cancer include the PI3K and NFκB signalling cascades. Expression of components of these pathways was examined in the HBL 100 and MDA MB468 cells, as was the effects of I3C upon them. Furthermore, these pathways also confer proliferative advantages on cellular populations through events that do not directly interfere with the apoptotic process. Targets of the PI3K and NFκB pathways include molecules involved in cell cycle, angiogenic, proliferative and metastatic processes. The hypothesis that the chemopreventive effect of I3C may be partially mediated through an ability to alter expression of some of these targets was subsequently investigated.

The pleiotropic nature of I3C would suggest that there are many more potential targets through which this agent could engender a chemopreventive effect. It was proposed that use of global-expression microarrays would further contribute to our understanding of the mechanisms of action of this compound.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Company Limited, Poole, Dorset, U.K., and solvents from Fisher Scientific, Loughborough, Leicestershire, U.K.

Acrylamide (30% acrylamide: bis acrylamide)	Anachem
^{32}P Adenosine triphosphate	Amersham
Agarose (high melting point)	Gibco BRL
Akt kinase assay kit	Cell Signalling Technology
Annexin V kit	Bender MedSystems
AP-1 consensus oligonucleotide	Promega
Beta galactosidase reporter assay kit	Promega
BioRad protein assay reagent	Bio-Rad
Cot-1 DNA	Gibco BRL
Cy3-dUTP	Amersham
Cy5-dUTP	Amersham
DH5-α competent cells	Gibco BRL
DNA molecular weight markers	Gibco BRL
dNTP	Pharmacia
ECL detection kit	Amersham
ECL-hyperfilm	Amersham
Epidermal growth factor	Gibco BRL
Endofree MaxiPrep kit	Qiagen
Fetal calf serum	Gibco BRL
Fugene transfection reagent	Boeringer Mannheim
Glutamax	Gibco BRL
Hybond nitrocellulose	Amersham
IκBα protein	Santa Cruz Biotechnology
Luciferase reporter assay kit	Promega
LY294002	Calbiochem
Marvel (dried milk powder)	Premier Brands
Microcon columns	Millipore

Miniprep DNA kit	Promega
Mitotracker	Molecular Probes
NF-κB consensus oligonucleotide	Promega
Optiphase safe scintillation fluid	Beckman Coulter
Platelet-derived growth factor	Gibco BRL
Prostaglandin E2 kit	Cayman Chemicals
^{3}H phosphatidyl-3-inositol	Amersham
Protein molecular weight markers	Bio-Rad
Vectashield	Vector Laboratories
RNAsin	Promega
tRNA	Gibco BRL
RNeasy kit	Qiagen
Superscript II reverse transcriptase	Gibco BRL
T4 polynucleotide kinase	Promega
Trypsin/EDTA	Gibco BRL
Xylene cyanol	BDH
Zeocin	Invitrogen

2.1.2 Antibodies to the following proteins

Phospho- Akt 1,2,3 (serine 473)	New England Biolabs
Phospho- Akt 1,2,3 (threonine 308)	New England Biolabs
Total Akt 1,2,3	New England Biolabs
Total Akt 1	Santa Cruz Biotechnology
Total Akt 2	Santa Cruz Biotechnology
Total Akt 3	Upstate Biotechnology
Phospho-Bad	New England Biolabs
Total Bad	Santa Cruz Biotechnology
Bax	Pharmingen
Bcl-2	Dako
Bcl-xL	Transduction Laboratories
Cox-2	Santa Cruz Biotechnology
Cyclin D1	Santa Cruz Biotechnology
Cytochrome c	Molecular Probes
Total 4EBP-1	Santa Cruz Biotechnology

Total eIF4E	Santa Cruz Biotechnology
EGFR	Santa Cruz Biotechnology
ERK 1	Santa Cruz Biotechnology
Phospho-ERK 1/2	Santa Cruz Biotechnology
Phospho- FKHR	New England Biolabs
Phospho- FKHRL1	Upstate Biotechnology
Phospho- GSK-3α/β	New England Biolabs
Total GSK-3β	Transduction Laboratories
HA-tagged antibody	Cell Signalling Technology
HSP-70	Bioquote Limited
IKKα	Santa Cruz Biotechnology
PARP	Gift from Dr. M. MacFarlane
PTEN	New England Biolabs
p85α subunit of PI3Kinase	Upstate Biotechnology
p110β subunit of PI3Kinase	Santa Cruz Biotechnology
NF-κB p65	Gift from Dr. N. Rice
NF-κB p50	Gift from Dr. N. Rice
NF-κB RelB	Gift from Dr. N. Rice
Phospho- p70S6K (ser 411)	Santa Cruz Biotechnology
Phospho- p70S6K (thr 389)	New England Biolabs
β-Tubulin	Oncogene Research Products
Phospho-Tyrosine	Santa Cruz Biotechnology
XIAP	Gift from Dr. M. Butterworth
Anti-goat HRP linked secondary antibody	Santa Cruz Biotechnology

2.1.3 Plasmids

pTK-Luc	Gift from Dr. P. Bauerle
pCMVβ	Promega
pNF-κB	Gift from Dr. P. Bauerle
pHA-Akt1	Gift from Dr. D. Alessi
pHA-Akt1-kinase dead	Gift from Dr. D. Alessi
pvgRXR	Invitrogen

2.1.4 Suppliers addresses

Amersham Pharmacia Biotech, Buckinghamshire, U.K.

Anachem, Bedfordshire, U.K.

ATCC, Manassas, V.A., USA.

Bender Medsystems, Vienna, Austria

BDH, Darmstadt, Germany

Bio-Rad, Hertfordshire, U.K.

Bioquote Limited, York, U.K.

Boeringer Mannheim, Basel, Switzerland

Calbiochem, Darmstadt, Germany

Cayman Chemicals, Michigan, USA

Cell Signalling Technology, Hertfordshire, U.K.

Dako, Glostrup, Denmark

Gibco-BRL (Invitrogen Life Technologies), Paisley, U.K.

Millipore, Massachusettes, USA

Molecular Probes, Leiden, Holland

New England Biolabs, Hertfordshire, U.K.

Oncogene research products, California, USA

Pharmingen, San Diego, California

Premier Brands, Wirral U.K.

Promega, Mannheim, Germany

Qiagen, West Sussex, U.K.

Santa Cruz Biotechnology, California, USA

Transduction laboratories, Kentucky, USA

Upstate Biotechnology, Milton Keynes, U.K.

Vector Laboratories, California, USA

2.1.5 With thanks to:

Dr. D. Alessi, MRC Protein Phosphorylation Unit, University of Dundee, Scotland.

Dr. R. Barber, Dept. Genetics, Leicester University, Leicester, LE1 7RH.

Dr. P. Bauerle, Institute for Immunology, Munchen, Germany.

Dr. M. Butterworth, MRC, Hodgkin Building, Lancaster Road, Leicester, LE1 9HN.

Prof. Sir P. Cohen, MRC Signalling Unit, University of Dundee, Scotland.

Dr. T. Gant, MRC, Hodgkin Building, Lancaster Road, Leicester, LE1 9HN.

Mrs. R. Jukes-Jones, MRC Hodgkin Building, Lancaster Road, Leicester, LE1 9HN.

Dr. M. MacFarlane, MRC, Hodgkin Building, Lancaster Road, Leicester, LE1 9HN.

Dr. K. Ridd, MRC Hodgkin Building, Lancaster Road, Leicester, LE1 9HN.

Dr. L. Stephens, Babraham Institute, Babraham, Cambridge.

Prof. R. Walker, Breast Cancer Research Unit, Glenfield General Hospital, Leicester.

2.2 BUFFERS

A113 buffer for mitochondrial and cytosolic protein preparation

220mM mannitol, 68mM sucrose, 50mM PIPES-KOH (pH 7.4), 50mM KCl, 5mM EGTA, 2mM $MgCl_2$, 1mM DTT and 1x protease cocktail inhibitor added immediately prior to use. The stock was made up to 200ml in distilled water and stored at 4°C.

Akt kinase assay buffer (10x stock)

The Akt kinase assay buffer was diluted to a 1x working concentration prior to use.
The 10x stock consisted of 250mM Tris (pH 7.5), 50mM β-glycerolphosphate, 20mM DTT, 1mM Na_3VO_4, 100mM $MgCl_2$ made up to 20ml in distilled water and stored at -20°C.

Annexin buffer

10mM HEPES (pH 7.4), 150mM NaCl, 5mM $MgCl_2$, 1.8mM $CaCl_2$. The stock was made up to 500ml in distilled water and stored at room temperature.

Cell lysis buffer (10x stock)

The lysis buffer was diluted to a 1x working concentration prior to use, with the addition of 1x protease inhibitor cocktail. The 10x stock consisted of 200mM Tris (pH 7.5), 1.5M NaCl, 10mM EDTA, 10mM EGTA, 10% triton X-100, 25mM sodium pyrophosphate, 10 mM β-glycerolphosphate, 10mM sodium orthovanadate made up to 500ml in distilled water. The 10x stock was stored at 4°C.

Denhardt's Reagent (50x)

1% w/v Ficoll 400, 1% w/v polyvinylpyrrolidone and 1% w/v bovine serum albumin made up in 100ml distilled water, filtered and stored at -20°C.

DNA loading buffer

0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, made up to an appropriate volume in distilled water and stored at room temperature.

EMSA binding buffer

8% ficoll, 40mM HEPES (pH 7.5) made up to 10ml in distilled water and stored at 4°C.

First strand buffer (5x)

250mM Tris-HCl (pH8.5), 375mM KCl and 15mM $MgCl_2$. The 5x stock was made up in RNase free water and used at a final concentration of 1x.

Hybridisation buffer for microarrays

Hybridisation buffer was prepared by filtering a mixture of de-ionised formamide (5ml), 50X Denhartd's (500μl), water (1ml), 10% SDS (500μl) though a 0.45μ syringe filter.

IKK kinase assay buffer

25mM HEPES (pH 7.4), 25mM β-glycerolphosphate, 25mM $MgCl_2$, 0.1mM Na_3VO_4, 0.5mM EDTA, made up to 200ml in distilled water and stored at 4°C. Immediately prior to use DTT was added to a final concentration of 0.5mM.

Nuclear protein buffer A

10mM HEPES (pH 7.8), 10mM KCl, 2mM $MgCl_2$, 1mM DTT, 0.1mM EDTA, 0.2mM NaF, 0.2mM Na_3VO_4 and 0.1% protease inhibitor cocktail added immediately prior to use. The stock was made up to 200ml in distilled water and stored at 4°C.

Nuclear protein buffer B

10% v/v Nonidet P40 made up to 100ml in distilled water and stored at 4°C.

Nuclear protein buffer C

50mM HEPES (pH 7.8), 50mM KCl, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 10% glycerol, 0.2mM NaF, 0.2mM Na_2VO_4 and 0.1% protease cocktail inhibitor added immediately prior to use. The stock was made up to 100ml in distilled water and stored at 4°C.

PI3K kinase assay buffer

20mM β-glycerolphosphate, 5mM sodium pyrophosphate, 30mM NaCl, 0.1mM DTT were dissolved in distilled water and stored at 4°C. Immediately prior to use,

phosphatidylinositol and phosphotidylcholine were added to give final concentrations of 0.3mg/ml.

PI3K wash buffers

1) Cell lysis buffer
2) 0.5M LiCl, 0.1M Tris, pH 8.0
3) 0.15M NaCl, 10mM tris, 1mM EDTA, pH7.6
4) 20mM HEPES, 1mM DTT, 5mM $MgCl_2$, pH 7.6

All wash buffers were made up in distilled water and stored at 4°C.

Polyacrylamide denaturing stacking gel (10ml)

6.8ml water, 1.7ml 30% acrylamide, 1.25ml 1M Tris (pH 6.8),100µl 10% SDS. Polymerisation was initiated upon addition of 100µl ammonium persulphate and 10µl TEMED.

Polyacrylamide denaturing running gel (10ml)

Volumes of water and acrylamide varied according to the percentage gel cast. The following remained the same: 2.5ml 1.5M Tris (pH 8.8), 100µl 10% SDS.

8% - 4.6ml water, 2.7ml 30% acrylamide

10% - 4ml water, 3.3ml 30% acrylamide

12% - 3.3ml water, 4ml 30% acrylamide

15% - 2.3ml water, 5ml 30% acrylamide

Polymerisation was initiated upon addition of 100µl ammonium persulphate and 10µl TEMED

Polyacrylamide non-denaturing gel for EMSA (50ml)

8ml 30% acrylamide. 49.75ml water, 3ml 5x TBE, 400µl ammonium persulphate and 100µl TEMED.

3x SDS loading buffer

187.5mM Tris-HCl (pH 6.8), 6% w/v SDS, 30% glycerol and 0.03 % w/v bromophenol blue made up to the appropriate volume in distilled water and stored at room temperature. Immediately prior to use, DTT was added to the buffer to a final concentration of 150mM.

SOC Medium

20g tryptone, 5g yeast extract, 85mM $NaCl_2$ and 2.5mM KCl were made up in 1l of distilled water and autoclaved. Following autoclaving, 10mM $MgCl_2$ was added, re-autoclaved and sterile glucose added to a final concentration of 20mM.

SSPE (20x)

3M NaCl, 1mM NaH_2PO_4 and 20mM EDTA were dissolved in RNase free water, and the pH adjusted to 7.4 using NaOH.

TBE buffer (5x stock)

The stock consisted of 445mM Tris Boric acid, 10mM EDTA (pH 8.0), made up to 1l in distilled water and stored at room temperature. TBE buffer was diluted to a 1x working solution prior to use.

TBST

20mM Tris (pH 7.5), 150mM sodium chloride and 0.1% Tween-20. The stock was made up to the appropriate volume in distilled water and stored at room temperature.

Tris EDTA buffer

10mM tris/HCl (pH 8.0), 1mM EDTA made up to an appropriate volume in distilled water and stored at room temperature.

Western running buffer (10x)

25mM Tris, 250mM glycine and 0.1% SDS, pH 8.3. The 10x stock was made up to 1l in distilled water and stored at room temperature. Dilute to a 1x working solution prior to use.

Western stripping buffer

62mM Tris and 2% SDS. The stock was made up in 500ml distilled water and stored at room temperature. Immediately prior to use, 0.8% v/v 2-mercaptoethanol was added.

Western transfer buffer

47mM Tris, 37mM glycine, 20% methanol and 0.04% SDS. The stock was made up to the appropriate volume in distilled water and stored at room temperature.

2.3 CELL LINES

All breast cell lines were kindly provided by Prof. R. A. Walker (Breast Cancer Research Unit, Glenfield General Hospital, Leicester, U.K.).

DU145 and LNCaP prostate cell lines were purchased from American Type Culture Collection (ATCC), VA, USA.

HBL 100: An SV40 immortalised cell line isolated from the milk of a nursing mother with no evidence of breast lesion.

T47D: Tumour derived, isolated from a pleural effusion from a patient with infiltrating ductal carcinoma.

MDA MB468: Tumour derived, isolated from a pleural effusion from a patient with metastatic adenocarcinoma.

MCF7: Tumour derived, isolated from a pleural effusion from a patient with metastatic adenocarcinoma.

All cell culture was undertaken in a class II laminar flow cabinet. Cell lines tested negative for mycoplasma infection.

HBL 100 and MDA MB468 cells were analysed for the presence of a Y chromosome due to a doubt in female origin of the HBL 100 cells announced on the ATCC website. Amelogenin testing was carried out by Dr. R. C. Barber (Dept. Genetics, University of Leicester) following the protocol originally described by Sullivan *et al.* 1993. Both cell lines were established to possess two X chromosomes (see appendix).

2.3.1 Maintenance of cell lines

HBL 100 and T47D cells were maintained in DMEM cell culture medium containing glutamax, sodium pyruvate and 1g/l glucose, supplemented with 10% FCS.

MCF7 and MDA MB468 cells were maintained in RPMI cell culture medium supplemented with 10% FCS and 2mM glutamax.

DU145 cells were maintained in Eagles MEM cell culture medium containing Earles salts supplemented with 0.2% non essential amino acids, 1mM sodium pyruvate and 10% FCS. LNCaP cells were maintained in RPMI cell culture medium supplemented with 2mM glutamax, 1mM sodium pyruvate, 2.5g/l glucose and 10% FCS.
All cell lines were maintained in a Sanyo incubator at 37°C, 5% CO_2 and 100% humidity.

2.3.1.1 Passaging of cells

Cells were routinely passaged when approximately 70% confluence had been reached. Following resurrection from storage, cells were not subcultured more than thirty times.
Cells were gently washed twice in PBS to remove all medium, and 5ml of 1x T/E added. The cells were incubated at 37°C for approximately 5 minutes, or until all the cells had just lifted off, whereupon the T/E was neutralised with the addition of 5ml of medium containing 10% FCS. Cells were pelleted at 200xg for 3 minutes and resuspended in 10 ml medium containing 10% FCS, before the addition of 2ml of cell suspension to a fresh T125 flask containing 30ml of medium. Cells were not re-passaged within 2 days of subculturing.

2.3.2 Treatment of cells

Cells were seeded at the required density and allowed to recover for at least 5 hours prior to treatment. The LNCaP prostate cells were seeded in a similar manner, but required a period of 48 hours to adhere before commencement of any treatment.

A stock solution of I3C was made up for each treatment such that all samples contained an equivalent volume of DMSO that did not exceed 0.1%. An untreated control and an equivalent percentage DMSO control were included in all experiments.

2.4 ASSESSMENT OF CELL PROLIFERATION AND CELL DEATH

2.4.1 Cell proliferation assay

Cells were seeded on 6-well plates at $5x10^4$ cells/well and allowed to adhere for 5 hours before the following treatment regime commenced in duplicate. To estimate the effect on proliferation of each cell line, samples consisted of an untreated control, DMSO control, 100, 250, 500, 750 and 1000μM I3C treatment, with additional treatments for the MDA-MB468 cells of, 10, 25, 50 and 75μM I3C for times up to 168 hours.

Cells were then harvested by trypsinisation, and 200μl of a single cell suspension was diluted with 9.8ml of isoton buffer. Cells were counted using a ZM particle counter (Beckman Coulter, High Wycombe, U.K.). Growth curves were performed in triplicate on 3 separate occasions.

2.4.2 Cell recoverability following treatment with I3C

Cells were seeded at $1x10^4$ per well on 12-well plates and allowed to adhere for 5 hours before treatment with I3C for 24 hours. The cells were then maintained in treated medium, or washed and replenished with fresh untreated medium and cultured for 144 hours, whereupon they were harvested and counted as for the growth curves. The cell proliferation rate was expressed as fold increase in cell number following the initial treatment period.

2.4.3 Cell cycle analysis

(Based on the method described by Ormerod, 1990).

Flow cytometry of propidium iodide (PI) stained cells gives a measure of the number of cells within each phase of the cell cycle. Propidium iodide intercalates with DNA, and the fluorescent staining of cells is therefore directly proportional to the total amount of DNA present, which will double during S phase.

Cells were plated onto 9cm dishes at $2.5x10^5$ cells/plate, left to adhere overnight and then treated with appropriate concentrations of I3C for 48 hours. Adherent cells were trypsinised and washed x 2 in PBS before being resuspended as a single cell suspension in 200μl PBS. Cells were fixed by the addition of 2ml ice cold 70% ethanol whilst vortexing vigorously and incubated at 4°C for a minimum of 2 hours. The fixed cells could be stored at 4°C for

up to 1 week prior to analysis. Cells were pelleted by centrifugation at 600xg for 10 minutes and resuspended in 800μl PBS, whereupon RNase and PI were added to final concentrations of 0.1mg/ml and 5μg/ml respectively. The cells were incubated at 4°C overnight before analysis of DNA content was carried out using the Becton Dickinson FACscan apparatus and Cell Quest software, with subsequent data analysis performed using Modfit LT software.

2.4.4 Annexin V staining for apoptosis

(Based on the method described by Vermes *et al.,* 1995).

This protocol allows the determination of live, apoptotic and necrotic populations of cells using PI and an annexin V FITC conjugate. Live cells that have not been committed to the apoptotic process take up neither annexin V nor PI. When cells begin to apoptose, they undergo cell membrane perturbations that result in the inner leaflet, which is rich in phosphatidylserine, flipping to the outside of the membrane. Annexin V staining allows apoptotic and necrotic cells to be distinguished from live cells, due to its ability to bind to phosphatidylserine. Necrotic cells are distinguished from apoptotic cells via PI uptake into the nucleus.

Cells were plated onto 9cm dishes at concentrations between $2.5x10^5$ cells/plate and $1.0x10^6$cells/plate depending upon the duration of treatment, and treated with the appropriate concentration of I3C/LY294002.

Medium containing floating cells was reserved, whilst adherent cells were trypsinised for as brief a time as possible before being combined with the floating cell fraction. The cells were pelleted for 5 minutes at 350xg at 4°C, and resuspended in 10ml of fresh medium containing 10% FCS. The cells were incubated at 37°C for 30 minutes before approximately $5x10^5$ cells were pelleted and resuspended in 1ml of annexin buffer. 1μl of annexin V FITC conjugate was added to the cell suspension and incubated at room temperature for 8 minutes before the addition of PI to a final concentration of 1.5μg/ml. After 1 minute incubation at room temperature, the cells were placed on ice and the apoptotic status of the cells determined on the FACscan, using the Cell Quest software.

2.5 IMMUNOCYTOCHEMISTRY FOR CYTOCHROME C RELEASE

Cells were seeded at $2x10^4$ cells/well on a Nunc 8-well chamber slide and left to adhere for 24 hours. Cells were treated with varying concentrations of I3C for 8 hours, with the mitochondrial staining mitotracker dye being added to a final concentration of 100nM 1 hour prior to the end of the treatment time. The medium was removed from the chambers and the cells fixed in 3.8% formaldehyde/PBS for 20 minutes at room temperature. The fixed cells were gently washed 3 times in PBS and permeabilised with 0.1% triton X-100 for 4 minutes before undergoing another 3 PBS washes. Before the addition of the primary antibody to the slides, the cells were blocked overnight at 4°C in 3% BSA in PBS. The antibody against cytochrome c was diluted 1 in 150 in 3% BSA/PBS and incubated with the cells at room temperature for 3 hours. The chambers were then washed 3 times in PBS before the addition of the goat anti-mouse secondary antibody at a concentration of 1 in 300 in 3% BSA/PBS for 45 minutes at room temperature. The chambers underwent a final 3 washes with PBS before counterstaining with the nuclear stain Hoechst 33258 at a final concentration of 500ng/ml for 15 minutes. The slides were then mounted using Vectashield and the mitochondria and cytochrome c release visualised by confocal microscopy using a Leica TCS4D confocal imaging system.

2.6 PREPARATION OF CELL FRACTIONS

Cells were seeded at $2.5x10^6$ cells/9cm dish or $6.0\ x10^6$ cells/15cm dish, and allowed to adhere before commencing treatment. Following treatment, the cells were harvested in one of the following ways depending upon which fractions were required.

2.6.1 Whole cell lysate preparation

Dishes were placed on ice, the medium removed and cells were washed twice with ice cold PBS. All residual liquid was removed, 300µl of cell lysis buffer was added and the dishes incubated on ice for 10 minutes before scraping the contents into an eppendorf. The cell lysates were then cleared by centrifugation at 13000xg for 5 minutes and stored at -20°C.

2.6.2 Nuclear protein preparation

Dishes were placed on ice, the medium removed and cells washed twice with ice cold PBS, before the whole cells were scraped into eppendorf tubes. The cells were pelleted by centrifugation at 1200xg for 10 minutes at 4°C and the pellet washed in ice cold PBS

before resuspension in 0.4ml of nuclear protein buffer A. The mixture was incubated on ice for 15 minutes, then 25μl of nuclear protein buffer B was added and vortexed vigorously for 15 seconds before centrifugation for 30 seconds at 13000xg, 4°C. The nuclear pellet was then resuspended in nuclear protein buffer C and mixed on a rotating platform for 20 minutes at 4°C. The nuclear lysate was cleared by centrifugation for 5 minutes at 13000xg, 4°C and the supernatant stored at -20°C .

2.6.3 Mitochondrial and cytosolic protein preparation

Cells were harvested by scraping as above, pelleted at 200xg for 5 minutes at 4°C, resuspended in 300μl of A113 buffer and incubated on ice for 30 minutes. Following incubation, the cells were lysed using a Dounce homogeniser B pestle for 25 strokes and centrifuged at 1000xg for 10 minutes at 4°C to give a membrane pellet. The supernatant was then centrifuged at 14,000xg for 15 minutes at 4°C, to give a cytosolic fraction and a mitochondrial enriched pellet. The mitochondria were then lysed with 35μl of cell lysis buffer and the suspension cleared by centrifugation for 5 minutes at 13000xg. Fractions were stored at -20°C.

2.6.4 Preparation of samples for PTEN determination

Cells were harvested by scraping in PBS, pelleted at 200xg for 5 minutes at 4°C, lysed directly in 100μl of loading buffer and cleared by centrifugation for 5 minutes at 13000xg. Lysates were stored at -20°C. Immediately prior to use, lysates were sonicated for 5x5-second bursts at 5 microns on ice.

2.6.5 Preparation of samples for PARP cleavage determination

Cells were treated in the same manner as for PTEN, except that all floating cells in the media were combined with adherent cells prior to pelleting at 200xg.

2.6.6 Bio-Rad protein assay

Prior to use, protein concentration of cell lysates was determined in order that equal protein loading on gels could be achieved within each set of experiments.

Bio-Rad protein assay reagent (200μl) was added to 800μl of sample (diluted in distilled water as appropriate), vortexed, and the absorbance at 595nm determined using a Perkin Elmer λ2 UV/VIS spectrophotometer. Protein concentrations of samples were determined

from a standard curve prepared in a similar manner using known concentrations of bovine serum albumin (BSA).

2.7 WESTERN BLOTTING

(Based on the method described by Shapiro and Maizel, 1969).

Samples of known protein concentration were combined with SDS loading buffer (unless stated otherwise) to give a final 1x concentration of SDS. They were then boiled for 5 minutes prior to loading onto a polyacrylamide gel consisting of a 5% denaturing stacking gel and an 8, 10, 12 or 15% denaturing running gel, dependent upon the molecular weight of the protein of interest. The samples were electrophoresed in 1x western running buffer at 120V for approximately 1 hour using the Hoeffer 'mighty small' vertical gel system before being transferred onto nitrocellulose membrane.

The proteins were transferred from the gel in western transfer buffer, using a Bio-Rad wet blotting system onto hybond-N nitrocellulose at 100V for 2 hours, 4°C, or at 30V overnight at room temperature. Once transferred, the membrane was washed in TBST and blocked, gently rocking in 5% non fat milk or 5% BSA for 2 hours. The membrane was then washed in TBST and the primary antibody added for 2 hours at room temperature or overnight at 4°C. Following 5 x 5 minute washes in TBST, the appropriate secondary antibody was added for 1 hour at room temperature, and the membrane was then washed again for 5 x 5 minutes in TBST.

Proteins could then be visualised via chemiluminescence using ECL reagent. The membrane was developed in ECL reagent for 1 minute, and the excess liquid drained before the blot was wrapped in Saran wrap and placed protein side up into an autoradiographic cassette. The membrane was exposed to ECL-hyperfilm and the blot developed in the dark using an X-ograph automated developer.

The density of all bands was in the linear range of the film as determined via a standard curve. This was constructed using increasing concentrations of secondary antibody and visualising via ECL. Pixel density (as determined using the Syngene imaging system) was plotted against antibody concentration.

To re-probe blots with a loading control, membranes were first placed in 50ml stripping buffer at 60°C in a shaking water bath for 45 minutes. The membranes were then washed x2 in TBST prior to re-blocking and addition of an appropriate primary antibody

2.7.1 Antibody conditions

Unless otherwise stated, all membranes were blocked in 5% milk in TBST, with primary antibodies diluted to 1:1000 in 5% powdered milk/TBST, and secondary antibodies diluted to 1:1000 in 5% milk/TBST.

Table 2.1 Antibodies: conditions for use.

1° and 2° ANTIBODY	CONDITIONS	MOLECULAR WEIGHT	% RUNNING GEL
p-Akt (1,2,3) (ser473); anti-rabbit	Whole cell lysates (30μg). Dilute primary antibody 1:1000 in 5% BSA.	60KDa	10%
p-Akt (1,2,3) (thr308); anti-rabbit	Whole cell lysates (30μg). Dilute primary antibody 1:1000 in 5% BSA.	60KDa	10%
Total Akt (1,2,3); anti-rabbit	Whole cell lysates (30μg). Dilute primary antibody 1:1000 in 5% BSA.	60KDa	10%
Total Akt 1; anti-goat	Whole cell lysates (30μg). Dilute secondary antibody 1:2000 in 5% milk	60KDa	10%
Total Akt 2:	Suitable for immunoprecipitation of whole cell lysates only (500μg). Westerns performed using p-Akt (1,2,3) ser473.	60KDa	10%
Total Akt 3;	Suitable for immunoprecipitation of whole cell lysates only (500μg).	60KDa	10%

	Westerns performed using p-Akt (1,2,3) ser473.		
p-Bad (ser136); anti-rabbit	Whole cells lysed in loading buffer. Dilute primary antibody 1:500 in 5% BSA.	27KDa	12%
Total Bad; anti-goat	Whole cell lysates (60μg). Block in 5% milk. Dilute secondary antibody 1:2000 in 5% milk	27KDa	12%
Bax; Anti-rabbit	Cytosolic/mitochondrial lysates (30μg/20μg). Dilute primary antibody 1:500 in 5% milk.	21KDa	12%
Bcl-2; anti-mouse	Whole cell lysates (30μg) Dilute primary antibody 1:500 in 5% milk.	27KDa	12%
Bcl-x; anti-mouse	Whole cell lysates (30μg). Dilute primary antibody 1:2000 in 5% milk	29KDa	12%
Cox-2; anti-goat	Whole cell lysates (200μg). Dilute primary antibody 1:2000 in 5% milk. Dilute secondary antibody 1:2000 in 5% milk.	70KDa	10%
Cyclin D1; anti-mouse	Nuclear lysates (30μg). Dilute primary antibody 1:500 in 5% milk.	30KDa	12%

Total 4EBP1; anti-goat	Whole cell lysates (30μg). Dilute primary antibody 1:2000 in 5% milk. Dilute secondary antibody 1:2000 in 5% milk	12.4Kda	15%
EGFR: anti-rabbit	Whole cell lysates (30μg). Dilute primary antibody 1:2000 in 5% milk. Dilute secondary antibody 1:2000 in 5% milk	170kDa	10%
Total eIF4E; anti-mouse	Whole cell lysates (30μg).	46KDa	12%
p-FKHR (ser256); anti-rabbit	Nuclear lysates (30μg). Dilute primary antibody 1:1000 in 5% BSA. Dilute secondary antibody 1:1000 in 5% milk.	76KDa	10%
p-FKHRL1; anti-rabbit	Nuclear lysates (30μg). Dilute primary antibody 1:500 in 5% milk.	100KDa	10%
p-GSK-3α/β (ser21/9); anti-rabbit	Whole cell lysates (30μg). Dilute primary antibody 1:1000 in 5% BSA.	52/46KDa	12%
Total GSK-3β; anti-mouse	Whole cell lysates (30μg). Dilute primary antibody 1:2500 in 5% milk. Dilute secondary antibody 1:2000 in 5% milk.	46KDa	12%
Hsp70/Hsc70 anti-mouse	Whole cell lysates (30μg). Dilute primary antibody 1:2000 in 5% milk. Dilute secondary antibody 1:2000 in 5% milk.	70KDa	10%
Total IKKα;	IP using 500μg of whole cell lysate. Used same antibody for primary.	84KDa	10%

PARP; anti-mouse	Whole cells lysed in loading buffer and whole sample loaded. Dilute primary antibody 1:10000 in TBST.	Uncleaved: 116KDa Cleaved: 84KDa	8%
PTEN; anti-rabbit	Whole cell lysates (100μg). Dilute primary antibody 1:1000 in 5% BSA.	54KDa	10%
p85 subunit of PI3Kinase; anti-mouse	For use on p-Tyr immunoprecipitated samples only. Dilute secondary antibody 1:2000 in 5% milk.	85KDa	10%
p65 NF-κB; anti-mouse	Nuclear lysates (20μg). Block in 5% BSA. Dilute primary antibody 1:1000 in 5% BSA. Dilute secondary antibody 1:2000 in 5% BSA.	65KDa	10%
p-p70S6K (ser411); anti-mouse	Whole cell lysates (30μg). Dilute secondary antibody 1:2000 in 5% milk.	70KDa	10%
p-Tyrosine; anti-mouse	For use with immunoprecipitated proteins. Dilute secondary antibody 1:2000 in 5% milk.	Dependent upon IP antigen	Dependent upon IP antigen

2.7.2 Immunoprecipitation of proteins

Twenty μl of protein-A agarose beads were placed into eppendorf tubes and washed in PBS to remove any sodium azide present. 5μl of the immunoprecipitating antibody was mixed with the beads and incubated at room temperature for 1 hour, after which, excess unbound antibody was removed by washing the beads twice in PBS. Protein (500μg) from whole cell lysates was added to the beads and tumbled overnight at 4°C. Unbound protein was removed by washing the beads twice with PBS containing 350mM sodium chloride and twice with PBS. Samples were then resuspended in 40μl of 3x SDS sample buffer, boiled for 5 minutes and centrifuged to remove the beads before loading all of the supernatant on to a polyacrylamide gel and processing as for standard western blotting.

2.8 KINASE ASSAYS

These assays rely upon the ability of the immunoprecipitated kinase of interest to phosphorylate a substrate as a direct measure of kinase activity. Activity can be visualised either using an antibody directed to the phosphorylated substrate, or via the incorporation of radiolabelled phosphate into the substrate.

2.8.1 Akt Kinase Assay

The Akt kinase assay was undertaken using a non-radioactive kinase assay kit supplied by Cell Signalling Technology (NEB).

In brief, the assay was as follows. Treated cells were harvested by the whole cell lysate method. Resuspended immobilised Akt antibody slurry was washed in 1 x cell lysis buffer, and 200μg protein from the whole cell lysates added prior to tumbling overnight at 4°C. The antibody slurry was then washed twice with 1x lysis buffer, twice in kinase assay buffer and the slurry pelleted. To the precipitated Akt/antibody pellet, 40μl of 1x kinase assay buffer, 200 μM ATP and 1μg GSK-3 fusion protein (substrate) were added and incubated at 30°C for 30 minutes. The reaction was terminated by addition of 20μl of 3x SDS loading buffer, the mix boiled for 5 minutes and the beads centrifuged out before loading all of the supernatant onto a 15% polyacrylamide gel. The gel was then analysed as for western blotting using an anti-phospho-GSK3α/β primary antibody. To determine whether I3C could directly inhibit the kinase, I3C was added directly into the kinase assay

buffer prior to the addition of substrate, and pre-incubated on ice for 30 minutes. The assay was then continued as described above.

2.8.2 IKK activity assay

Five µl of anti-IKKα antibody was added to protein A agarose beads and incubated at room temperature for 1 hour. The beads were then washed x2 in PBS, 500µg of whole cell lysate protein added and the mix tumbled overnight at 4°C. The beads were then washed twice in 1x cell lysis buffer and resuspended in 1ml of kinase assay buffer. For western blotting, 250µl of the bead suspension was pelleted and resuspended in 40µl of loading buffer, boiled for 5 minutes and the beads centrifuged out. The supernatant was loaded onto a 10% denaturing polyacrylamide gel and analysed by western blotting to assess whether there was equivalent loading for kinase assay samples.

For the kinase assay, the remaining beads were pelleted and resuspended in 34µl kinase assay buffer containing 2.5µl IκBα, 50µM cold ATP and 0.3MBq γ^{32}P-ATP, and the mix incubated for 30 minutes at 30°C. The reaction was terminated with 10µl of loading buffer. The samples were then boiled for 5 minutes, the beads centrifuged out and the whole supernatant loaded onto a 10% denaturing polyacrylamide gel and run back to back with the western samples. The radioactive dye front was allowed to run off the end of the gel before the gel was dried and visualised using the Molecular Dynamics phosphorimaging system.

2.8.3 PI3Kinase assay

(Based on the method described by Hawkins *et al.*, 1998).

Proteins from whole cell lysates were immunoprecipitated as for the IKK protocol (*2.3.2*), except that an anti- phosphotyrosine antibody was used.

After immunoprecipitation, the beads were washed in a series of buffers (3x in cell lysis buffer, 2x in LiCl buffer, 1x in NaCl buffer and 1x in HEPES buffer (*2.2*) and resuspended in 40µl of kinase assay buffer followed by the addition of 20µl 3mg/ml phosphatidylyserine/phosphatidylinositol mix, prior to incubation at 37°C for 5 minutes. Forty µl of ATP mix containing 3µM cold ATP, 7.5mM $MgCl_2$ and 0.37MBq γ^{32}P-ATP was then added to each sample and incubated for 30 minutes at 37°C. The reaction was

terminated by the addition of 450μl of a 1:2 chloroform/methanol mix, followed by 150μl chloroform and 150μl 0.1M HCl. The organic (lower) phase was extracted, and a further 150μl chloroform and 150μl 0.1M HCl added. The organic phase was extracted once more and then dried using a speed vac before resuspension in 50μl of a chloroform/methanol/0.1M HCl mix (200:100:1) containing 0.46KBq ^{3}H phosphatidyl–3-inositol.

The TLC tank was allowed to equilibrate with running buffer consisting of methanol/chloroform/concentrated ammonia/water (20:14:3:5), prior to spotting of samples onto the TLC plate. The plate was run in the solvent for approximately 2.5 hours before being removed, air dried and the samples detected using autoradiographic film. Quantification and identification of products was via dual scintillation counting of marked areas from the TLC plate using a Beckman Coulter scintillation counter.

2.9 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

(Based on the method described in Plummer *et. al.*, 1999).

2.9.1 Labelling of probe for EMSA

Five μl NF-κB consensus oligonucleotide, 1μl T4 polynucleotide kinase, 2μl T4 polynucleotide kinase 10x buffer and 1.85MBq $\gamma^{32}P$-ATP were mixed to a final volume of 20μl and incubated at 37°C for 30 minutes. The labelling reaction was terminated by the addition of 80μl of TE buffer, and unincorporated nucleotides removed by elution through a NAP '5' column with 100μl aliquots of TE buffer. Twelve fractions were collected and the 3 containing the most radioactivity combined. The labelled oligonucleotide was then precipitated overnight at -20°C with 750μl ethanol and 30μl 5M NaCl. The probe was pelleted for 30 minutes at 13000xg and resuspended in 30μl water.

2.9.2 Sample preparation for EMSA and running of the gel

Five μg nuclear protein was added to 12μl binding buffer containing 1μg poly dIdC, 0.2mM $MgCl_2$, 0.2mM DTT and 1μl of ^{32}P-labelled consensus oligonucleotide and incubated at room temperature for 30 minutes. To check the specificity of the banding patterns obtained, the following control samples were included: one sample containing a 100 fold excess of cold NF-κB consensus oligonucleotide to show competitive inhibition,

and one containing a 100 fold excess of cold AP-1 consensus oligonucleotide to show non-competitive inhibition.

The consensus oligonucleotide sequences were as follows:

NF-κB: 5' AGT TGA GGG GAC TTT CCC AGG C

3' TCA ACT CCC CTG AAA GGG TCC G

AP-1: 5' CGC TTG ATG AGT CAG CCG GAA

3' GCG AAC TAC TCA GTC GGC CTT

During the 30 minute incubation, a 4% non-denaturing polyacrylamide gel was pre-run at 120V. Termination of the reaction was via the addition of 2μl of DNA loading buffer, and the whole sample was loaded onto the gel. The samples were run into the gel, using 0.25x TBE cooled running buffer at 240V for 10 minutes, and the gel electrophoresed for a further 2.5 hours at 120V.

The gels were then dried at 70°C for 1 hour on to Whatman filter paper and visualised using the Molecular Dynamics phosphorimaging system.

2.10 PREPARATION AND TRANSFECTION OF PLASMID DNA

(Based on methods described in Molecular Cloning. Ed. Sambrook and Russell)

2.10.1 Transformation of competent bacteria

A 20μl aliquot of DH5α competent cells was thawed on ice, 1μg of plasmid DNA was added and incubated on ice for 30 minutes. The cells were then heat shocked for 45 seconds at 42°C and placed on ice for 2 minutes. Eighty μl of SOC medium was added, and the mix shaken at 225 r.p.m. for 1 hour at 37°C. The whole mix was then applied to, and spread evenly over an ampicillin/X-gal agar plate, and incubated at 37°C overnight, or until bacterial growth had occurred.

Plates for bacterial cell transformation were prepared by the addition of 60μg/ml ampicillin to liquid L-agar, and approximately 10ml applied to sterile petri dishes. Once set, the dishes were inverted and allowed to dry at 37°C for 1 hour. Twenty mg/ml X-gal in dimethyl

formamide (DMF) was then applied to the surface of the plates and allowed to dry before immediate use.

All constructs used for transient transfection contained an ampicillin resistance gene, conferring survival upon transformed cells when cultured in the presence of ampicillin. Bacterial colonies that contained recombinant plasmids were easily identifiable via β-galactosidase selection. Most vectors possess a partial coding sequence for β-galactosidase (lacZ) that is able to interact with the host's coding sequence, forming blue colonies in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). However, if a foreign piece of DNA has successfully inserted into the polycloning site of the vector (in the middle of the Lac Z sequence), then this interaction is prevented, resulting in the formation of white colonies containing recombinant plasmids.

2.10.2 Preparation of plasmid DNA

(Based on the methods described in Ausubel *et al.,* 1991).

2.10.2.1 Small scale preparation of plasmid DNA

From each transformation, a single white colony was picked under sterile conditions and added to 10ml of L-broth containing 60μg/ml ampicillin. This was repeated with 5 separate single colonies, and the broth incubated with shaking at 225 r.p.m. and at 37°C overnight. Small scale isolation of plasmid DNA was carried out using the Promega Wizard Miniprep DNA Purification System.

In brief, 3ml cell suspension was pelleted, and 200μl cell resuspension buffer added to create a single cell suspension. Cell lysis buffer (200μl) was then added and the suspension mixed prior to the addition of 200μl neutralisation buffer. The mixture was centrifuged and the cleared supernatant decanted into a fresh microcentrifuge tube. One ml purification resin was added to the supernatant, and mixed before applying to a minicolumn using a syringe and plunger. The minicolumn was then washed using column wash solution, and dried by centrifugation. Plasmid DNA was then eluted from the minicolumn using 50μl TE buffer heated to 50°C.

A restriction digest was performed on the purified plasmid to confirm its identity (see *2.10.2.3*).

2.10.2.2 Large scale preparation of plasmid DNA

Fifty µl cell suspension found to contain the plasmid of interest by small scale DNA preparation, was added to 250ml of L-broth containing 60µg/ml ampicillin in a 2L flask under sterile conditions. The flask was then shaken at 225 r.p.m at 37°C overnight. Large scale plasmid DNA preparation was undertaken using the Qiagen EndoFree Plasmid Maxi Prep Kit.

In brief, all bacterial cells were pelleted and resuspended in 10ml resuspension buffer (P1). Ten ml lysis buffer (P2) was then added, the suspension gently mixed and incubated at room temperature for 5 minutes. Ten ml of neutralisation buffer (P3) was added, the suspension mixed and applied directly to the barrel of a QIAfilter Maxi Cartridge in order to remove chromosomal DNA, salt, detergent and protein complexes. Following a 10 minute incubation at room temperature, a cleared lysate was produced, filtered through the column into a fresh tube and 2.5ml ER buffer added. The mixture was then inverted 10 times and incubated on ice for 30 minutes. Whilst incubating, a Qiagen-tip was equilibrated with QBT buffer, and then the DNA mixture applied to the tip and allowed to enter by gravity flow. The tip was washed with wash buffer (QC buffer), the DNA eluted with 15ml high salt buffer (QN) and precipitated with 10.5ml (room temperature) isopropanol. The precipitated DNA was centrifuged at 15000xg for 30 minutes at 4°C and the supernatant discarded. Pelleted DNA was washed with 2.5ml endotoxin free 70% ethanol and centrifuged at 15000xg for 10 minutes at 4°C and allowed to air dry before resuspension in 300µl TE buffer.

The plasmid DNA obtained again underwent a restriction digest followed by separation of the fragments on an agarose gel. Visualisation was by ethidium bromide DNA intercalation.

2.10.2.3 Restriction digest of plasmid DNA

The DNA insert of interest was excised from the vector using appropriate restriction enzymes, with separation and visualisation via agarose gel electrophoresis (*table 2.2*).

Restriction digests were prepared containing 0.5μg of plasmid DNA, 1μl of restriction enzyme/s, 1μl of appropriate SureCut buffer and made up to 10μl using water before incubation at 37°C. Uncut samples were prepared alongside each cut sample omitting only the enzyme. Following incubation, 1μl of DNA loading buffer was added, and the samples plus molecular weight marker run on a 1% high melting point agarose gel containing 0.01μl/ml of 10mg/ml ethidium bromide at 100V for 45 minutes. DNA bands were then visualised with a UV transilluminator and photographed using a Polaroid camera system.

Plasmid	**Restriction Enzyme**	**Cut size**
pTk	BamH1	Linearised at 5560bp
pCMVβ	Xho1	Linearised at 7200bp
p6NF-κB	BamH1	Linearised at 6060bp
pHA-Akt1	EcoR1/KPN1	Insert = 2400bp
pHA-Akt1 kinase dead	EcoR1/KPN1	Insert = 2400bp
pvgRXR	HindIII	Linearised at 8800bp

Table 2.2 Identification of plasmids by restriction digest (for futher details refer to appendix).

2.10.3 Transient transfection of plasmid DNA into mammalian cells lines

Plasmids containing the reporter genes for β-galactosidase and luciferase were transiently transfected into cells using the lipid based transfection reagent FuGene.

Cells were seeded at $2x10^6$ cells/well onto 6 well plates and allowed to adhere overnight. Eppendorfs containing 0.5μg of pCMVβ plasmid DNA (the pCMVβ vector expresses the reporter gene β-galactosidase from the human cytomegalovirus early gene promoter, and was used as a control plasmid for monitoring of transfection efficiency) and 0.5μg of pTK-6NF-κB (containing 6 NF-κB consensus sequences upstream of the luciferase reporter gene), or 0.5μg of pCMVβ and 0.5μg of pTK (empty vector containing luciferase reporter gene only) were prepared, along with an equivalent number of eppendorfs containing 97μl of serum free medium and 3 μl of FuGene transfection reagent. This mix was incubated at room temperature for 5 minutes before adding to the DNA mix and incubating at room

temperature for 15 minutes. The cell cultures were replenished with 2ml fresh medium containing 10% FCS, and the appropriate DNA/FuGene mix was added dropwise. The cells were incubated at 37°C for 24 hours before commencing any treatment regime.

2.10.4 Reporter gene assays

Transfected cells were washed x2 in PBS before the addition of 300μl β-galactosidase lysis buffer, harvested by scraping and the debris pelleted by centrifugation.

2.10.4.1 Measurement of β-galactosidase activity.

β-galactosidase activity was measured using the β-galactosidase enzyme assay system supplied by Promega. Cells from the transfected 6-well plates were harvested as above, and 25μl cell lysate mixed with 25μl assay buffer containing the substrate o-nitrophenol β-d - galactopyranosidase in a 96-well plate. The reaction was incubated at 37°C for 30 minutes and terminated by the addition of 75μl 1M sodium carbonate. β-galactosidase activity resulted in development of a yellow colour, the absorbance of which was determined on an iEMS Lab Systems plate reader (414nm).

2.10.4.2 Measurement of luciferase activity.

Luciferase activity was measured using the Luciferase assay kit supplied by Promega. The assay relies on the emission of light due to ATP-dependent oxidation of beetle luciferin which is proportional to the luciferase reporter activity in the sample. Ten μl cell lysate was added to 50μl luciferase assay buffer, and the light emission measured using a Wallac 1450 Microbeta Plus plate reader. Luciferase values were then corrected for transfection efficiency by normalising against β-galactosidase values.

2.10.5 Stable transfection of plasmid DNA into mammalian cell lines.

Cells were seeded in 6-well plates at $1x10^5$ cells per well and allowed to adhere overnight. Two wells were then transfected with the Akt1-HA vector containing the kinase dead insert and the pvgRXR construct containing the gene for zeocin resistance, and 2 with the Akt1-HA vector containing the active insert and pvgRXR construct using the Fugene transfection reagent. The same amount of transfection reagent was added to 2 further wells as a control. The transfectants were incubated overnight before the addition of medium containing 50μg/ml zeocin, in which they were maintained until all cells in the 'untransfected' wells

were dead. A number of single colonies were then picked and subcultured on in zeocin-containing medium until western blots for the presence of the HA tag could be performed.

2.11 PROSTAGLANDIN E2 ASSAY

(Based on the method described by Maclouf *et al.,* 1987).

Assessment of cellular prostaglandin E2 (PGE2) output was undertaken as an indirect measure of Cox-2 activity. The assay was carried out using a kit supplied by Cayman Chemicals, and the protocol supplied was adhered to. The principle behind the assay relies on the competition between cell-produced PGE2 and a PGE2-acetylcholinesterase tracer binding to a PGE2 monoclonal antibody. The substrate of acetylcholinesterase is then added and a colour change occurs. The colour intensity is proportional to the amount of PGE2 tracer present, which is inversely proportional to the amount of PGE2 present.

In brief, cells were seeded at 2.5×10^5 cells/well on six well plates, and allowed to adhere overnight. Medium was replenished prior to a 5 hour I3C treatment, after which time the medium was removed and reserved for the assay. 50μl of each medium sample was added to duplicate wells of a PGE2-antibody-coated 96-well plate, and 50μl of PGE2 acetylcholinesterase tracer added, prior to an 18 hour incubation at 4°C. The wells were then washed 5 times and 200μl of the acetylcholinesterase substrate added. Following a 60 minute incubation in the dark, the absorption of each well was measured at 415nm and PGE2 levels determined.

2.12 MICROARRAY ANALYSIS OF CHANGES IN GENE EXPRESSION IN RESPONSE TO I3C

(Based on the method described in Turton *et al.*, 2001).

2.12.1 Preparation of cells and labelling of RNA.

Seven large flasks of HBL 100 and MDA MB468 cells were required for each treatment, at a final confluence of no more than 60%. Cells were treated with 50μM I3C or DMSO alone for 5 or 24 hours and harvested by trypsinisation. Cells from the 7 flasks were combined and duplicate samples of 3×10^7 cells for each treatment were pelleted at 300xg for 3 minutes for RNA isolation.

2.12.1.1 Preparation of RNA

RNA was isolated using the Qiagen RNeasy kits, and a brief summary of the methodology is as follows:

Two ml of lysis buffer (RLT) containing 20μl of β-mercaptoethanol was added per $3x10^7$ cells, and the suspension sonicated for 6 times on ice for 5 second bursts at 10 microns, with 10 second intervals. An equal volume of 70% ethanol was added, and the cell suspension shaken vigorously. All subsequent stages were carried out at room temperature.

The 4ml cell aliquot was applied to the RNeasy columns contained in 15ml tubes, centrifuged at 3000xg for 5 minutes and washed with 3.8ml of RW1 guanidium-containing wash buffer in the same manner. A 2.5ml aliquot of equilibrating RPE buffer was centrifuged through the column, and this step was repeated before transferring the column to a fresh tube. RNA was eluted from the column by centrifugation using 2 aliquots of 150μl RNase free water, and the eluates combined with 1ml Tri reagent and 200μl chloroform. The mix was vortexed, the aqueous layer recovered following centrifugation and the RNA precipitated by adding 0.5 volumes of isopropanol and allowed to stand at room temperature for 10 minutes. The RNA was pelleted at 13,000xg for 10 minutes, washed twice in 70% ethanol and resuspended in 200μl RNase free water. A further purification step was undertaken by re-precipitation of the RNA with 20μl 3M sodium acetate and 600μl ethanol and incubation overnight at -70°C. RNA concentration and purity was determined by measuring the absorbance at 260, 280 and 320nm, and was resuspended in RNase free water to a concentration of 5μg/μl.

2.12.1.2 Labelling of RNA for hybridisation to microarrays

Oligo dT_{25} (0.5μl) was added to 50μg RNA in 10μl RNase free water and incubated at 70°C for 8 min, reducing to 42°C over 30 minutes. Whilst the RNA was annealing, a master mix was prepared containing the following reagents (concentrations are final concentrations after addition to the sample):

1x first strand buffer, 0.01M DTT and dNTP mix containing 0.5mM dATP, dCTP, dGTP and 0.2mM dTTP. For each array, a test and control sample were set up. This master mix (6.5μl) was then added to 2μl of the Cy3 (pink-control) dUTP and 2μl of Cy5 (blue-test) dUTP dyes in separate tubes, together with 0.5μl each of RNAsin and superscript II reverse

transcriptase. This final mix was then added directly to the RNA sample which was maintained in the PCR block at 42°C and incubated for 1 hour. A further 0.5μl of superscript II reverse transcriptase was added and incubated for an hour, prior to the addition of 20.5μl of RNase free water, bringing the total volume to 41μl.

2.12.1.3 Hydrolysation of RNA

The following reagents were then added sequentially:

0.5M EDTA (1μl)

10%w/v SDS (1μl)

3M NaOH (3μl)

The samples were incubated (10minutes, 70°C) to hydrolyse residual RNA, and to neutralise the samples 3μl 2M HCl and 10μl 1M Tris/HCl, (pH7.5) were added, followed by 1μl tRNA (4μg/ml) to act as a carrier.

2.12.1.4 Purification of the probe

TE buffer (140μl) was added to each dye tube and the test and control probes from each corresponding experiment (i.e. control versus test) were then combined when loaded onto a centricon purification column. The columns were then microfuged at 10,000xg for 7 minutes and the flow-through discarded. TE buffer (450μl) was added, the column microfuged at 10,000xg for 7 minutes, and again the flow-through discarded. TE buffer (450μl), 1μl human cot1 DNA (10μg/μl) and 1μl polyA (1μg/μl) were then added to each column prior to microfugation as before. The columns were microfuged at 10000xg for 2 minutes until the centricon filter was dry in the middle but the probe was still visible as a liquid ring. The column was finally inverted into a fresh tube, microfuged (10000xg, 2 minutes) and eluant containing the labelled mRNA dried in a Speedvac (15-20 minutes).

Probes were resuspended in 14μl hybridisation buffer and 6μl 20x SSPE, after which they were denatured at 100°C for 2 minutes, and then incubated at 42°C for 30-60 minutes.

2.12.2 Hybridisation of microarray slides

The slides (containing 4600 clones each) were heated at 100°C for 2 minutes and washed in distilled water to remove salts. After centrifuging to dryness the slides were prescanned in order to eliminate any badly printed slides. To reduce non-specific binding of material

to the arrays, and the inherent green fluorescence of the printed array, a prehybridisation step was included.

Prehybridisation buffer was prepared using 5ml de-ionised formamide, 1ml 50x Denhartd's, 400µl water, 500µl 10% SDS, 3ml 20x SSPE, 100µl 10mg/ml sonicated salmon sperm DNA and filtered through a 0.45µ syringe filter. The arrays were incubated with 20µl prehybridisation buffer at 42°C for 45-60 minutes and then underwent a series of washes. The slides were placed in 1x SSC, 0.03% SDS for 10 minutes and the cover slips allowed to slide off. Washing of the slides was facilitated by plunging several times. The slides were then transferred into 0.2x SSC, plunged several times and left for 5 minutes to soak. This was repeated in 0.05x SSC and the slides dried by centrifugation.

The probe (20µl) was placed onto the array and covered with a cover slip, and incubated at 42° C overnight. Prior to scanning, the slides underwent sequential washing as described for the pre-hybridisation step, and were centrifuged to dryness. The slides were then scanned using the GenePix 3.0 (version 3.0.0.85) software (Axon Instruments), and analysed using Treeview and Microsoft Excel.

2.13 STATISTICAL ANALYSIS

All statistical analysis was undertaken using Minitab 13. Data were assessed for statistical significance using a Balanced ANOVA or an ANOVA General Linear Model as appropriate, followed by Fisher's least significant difference post hoc test. Results were deemed significant if a p value of ≤ 0.05 was reached.

CHAPTER 3

EFFECT OF I3C ON PROLIFERATION AND APOPTOSIS

INTRODUCTION

Cell cycle and glucosinolates

Cover *et al.,* (1998) were the first to show that I3C, but not DIM or ICZ was able to cause a G1 cell cycle arrest in an ER-independent manner, via decreasing CDK6 mRNA levels, protein and activity in the MDA MB231 and MCF7 cells. I3C was also able to increase p21 protein levels and decrease phosphorylated Rb levels in MCF7 cells (Cover *et al.,* 1999). Similarly, in HepG2 cells, I3C was able to inhibit expression of CDK6 and cyclin D1 whilst inducing p21 levels (Kim, D.W. *et al.,* 2001). The CDK6 inhibition by I3C has been reported to be caused by the specific action of I3C upon the transcription factor Sp1, preventing its binding to and interaction with the CDK6 promoter region (Cram *et al.*, 2001, Hong *et al.,* 2002). In PC-3 prostate cells, I3C upregulated expression of the cell cycle inhibitors p21 and p27 independently of p53, and also decreased NF-κB DNA binding (Chinni *et al.*, 2001).

These data suggest an alternate mechanism of chemopreventive action for I3C that is not dependent on its ability to induce 2-hydroxylation of estrogen, and so may confer protection against aggressive, treatment resistant estrogen-independent tumours.

Apoptosis and glucosinolates

I3C treatment of benzo(a)pyrene-initiated cells derived from reduction mammoplasty has been shown to result in cell cycle arrest and p53-dependent apoptosis (Katdare *et al.*, 1998), whilst p53-independent apoptosis has been observed in DIM-treated MCF7 cells (Ge *et al.,* 1996, 1999). The induction of apoptosis by dietary indoles is not limited to breast cancer cells, as both I3C and DIM have also been observed to induce cell death in cervical epithelial cells *in vivo* and colon/prostate cell lines in *vitro* (Chen *et al.*, 2001, Bonnesen *et al.*, 2001, Chinni *et al.*, 2003).

I3C may be able to modulate key signalling molecules within the apoptotic cascade, and several candidates have so far been identified. Induction of apoptosis by I3C has been observed in the MDA MB435 breast cell line in conjunction with increased translocation of Bax to the mitochondria, and increased Bax and decreased Bcl-2 protein expression (Rahman *et al.,* 2000). These changes resulted in an overall increase in the Bax:Bcl-2 ratio,

so promoting induction of apoptosis. Similar changes to members of the Bcl-2 family have also been observed following DIM treatment of MCF7 cells (Hong *et al.*, 2002). In the PC-3 prostate cell line, I3C was shown to inhibit EGFR-mediated Akt phosphorylation, which was accompanied by concomitant downregulation of the anti-apoptotic Bcl-xL protein (Chinni and Sarkar, 2002).

The apoptotic pathway therefore provides numerous potential targets for I3C, investigation of which may further elucidate the antiproliferative properties of this agent. Studies described in this chapter examined the effect of I3C on cell cycle, apoptosis and apoptosis-regulating proteins in order to provide further insight into its mechanisms of action.

RESULTS

3.1 Cell proliferation

Cell growth and proliferation assays were performed over a period of one week in a panel of breast cell lines as described in *2.4.1. Figure 3.1* illustrates inhibition of proliferation by I3C in the HBL 100, MDA MB468, MCF7 and T47D breast cell lines, and shows a clear difference in sensitivity to I3C among the cell lines.

The MDA MB468 cells exhibited approximately 4 fold greater sensitivity to the effects of I3C than did the HBL 100 cells, with the T47D and MCF7 cell lines exhibiting approximately 2 fold higher sensitivity than the HBL 100 cells. In the MDA MB468 cells, there was significant inhibition of proliferation from 144 hours at 10μM I3C, and at earlier time points with higher concentrations. None of the MDA MB468 cells survived a 100μM I3C treatment over a period of 168 hours. In contrast to this, the HBL 100 cells underwent significant (but not complete) inhibition of proliferation in response to 100μM I3C treatment, with no surviving cells following treatment with 250μM I3C for 168 hour. Similarly to the HBL 100 cells, both the MCF7 and T47D cell lines underwent significant growth inhibition from 120 and 96 hours respectively following a 100μM I3C treatment, with no surviving cells following a 250μM treatment.

Approximate IC_{50} values were calculated for all 4 cell lines by plotting the number of cells as a percent of the DMSO control at 168 hours, against increasing I3C concentrations (*table 3.1*).

Cell Line	IC_{50} (μM)
HBL 100	122 ± 8.6
MDA MB468	33 ± 3.6
MCF7	67 ± 3.7
T47D	73 ± 1.5

Table 3.1 IC_{50} values for a panel of breast cell lines. *Values were determined following a 168 hour I3C treatment (n=6, ± SEM) .*

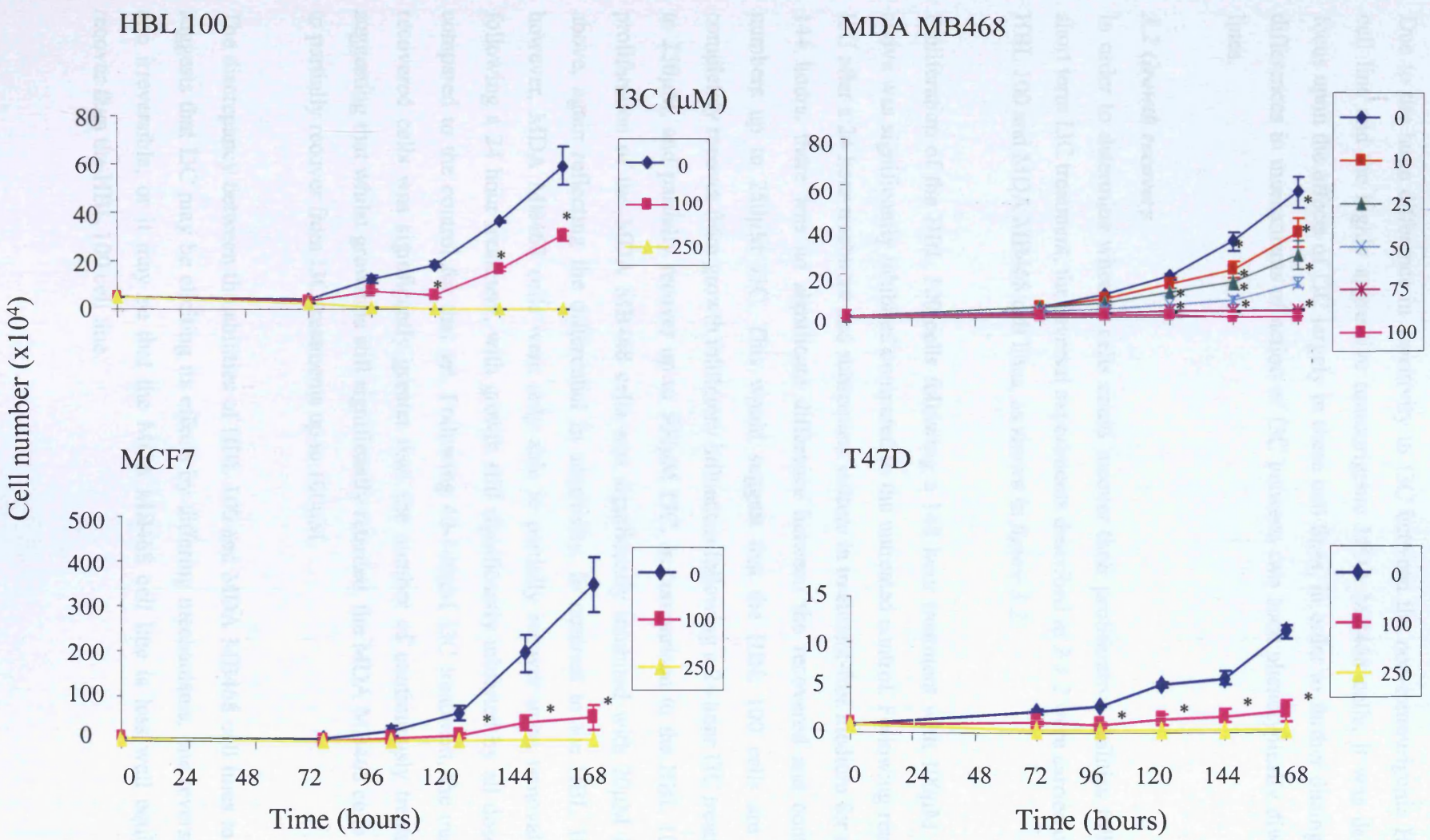


Figure 3.1 Effect of I3C on the proliferation of a panel of human breast cell lines. *Cells were cultured in the presence of I3C for times up to 168 hours. Data shown are means ± SEM .* denotes a significant difference from the DMSO control at that time point ($p \leq 0.05$, $n=6$, using an ANOVA General Linear Model followed by Fisher's post hoc test).*

Due to the large difference in sensitivity to I3C between the non-tumourigenic HBL 100 cell line and the highly aggressive tumourigenic MDA MB468 cells, it was decided to focus upon the effects of I3C largely in these cell lines, in order to further distinguish the differences in mechanisms of action of I3C between two such phenotypically diverse cell lines.

3.2 Growth recovery

In order to determine whether cells could recover their proliferative abilities following a short term I3C treatment, the reversal experiments described in *2.4.2* were carried out in the HBL 100 and MDA MB468 cell lines, as shown in *figure 3.2.*

Proliferation of the HBL 100 cells following a 168 hour treatment with 100μM I3C and above was significantly inhibited compared to the untreated control. Following removal of I3C after a 24 hour treatment and subsequent culture in treatment-free medium for a further 144 hours, there was no significant difference between the recovered and control cell numbers up to 250μM I3C. This would suggest that the HBL 100 cells are able to completely recover from growth inhibitory influences following a 24 hour I3C treatment up to 250μM, and partially recover up to 500μM I3C. In comparison to the HBL 100 cells, proliferation of the MDA MB468 cells was significantly inhibited with 20μM I3C and above, again reflecting the differential in sensitivity. In contrast to the HBL 100 cells however, MDA MB468 cells were only able to partially recover upon removal of I3C following a 24 hour treatment, with growth still significantly inhibited by all doses when compared to the control for that set. Following 40-100μM I3C treatment, the number of recovered cells was significantly greater than the number of continuously treated cells, suggesting that whilst growth is still significantly retarded, the MDA MB468 cells are able to partially recover from I3C treatments up to 100μM.

The discrepancy between the abilities of HBL 100 and MDA MB468 cell lines to recover suggests that I3C may be eliciting its effects by differing mechanisms, one reversible and one irreversible, or it may be that the MDA MB468 cell line is less well equipped to recover than the HBL 100 cell line.

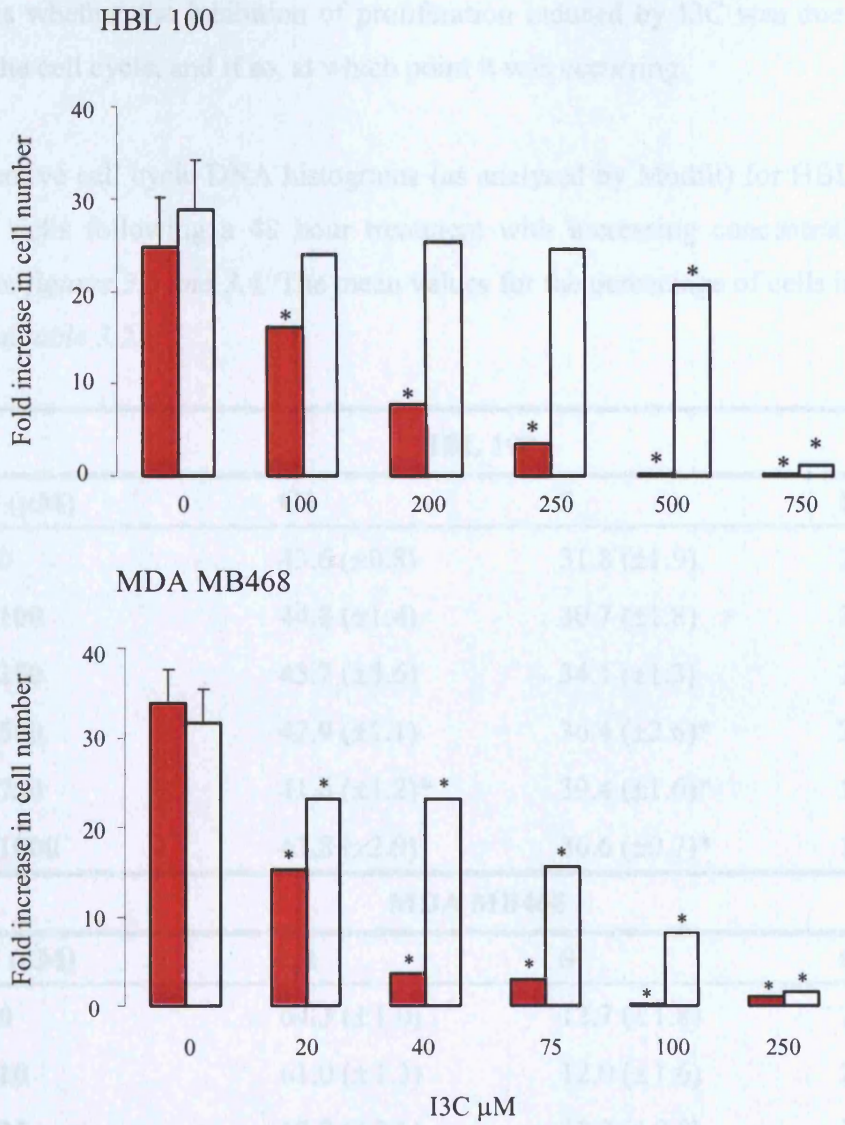


Figure 3.2 Growth recovery study for HBL 100 and MDA MB468 cells. *This followed a 168 hour treatment with I3C (red bars), or a 24 hour treatment with I3C , after which time the cells were washed and maintained in fresh untreated medium until 168 hours (white bars). Cell growth is expressed as a fold increase in cell number following the initial 24 hour treatment period. * denotes a significant difference from DMSO control ($p \leq 0.05$, $n=3$, ± pooled S.D.), using a Balanced ANOVA and Fisher's least significant difference post hoc test). All data acquired by Dr. A. Hudson.*

3.3 Cell cycle analysis

Flow cytometric analysis was used to determine DNA content in the HBL 100 and MDA MB468 cells following a 48 hour I3C treatment as described in *2.4.3.* This analysis indicates whether the inhibition of proliferation induced by I3C was due to disturbances within the cell cycle, and if so, at which point it was occurring.

Representive cell cycle DNA histograms (as analysed by Modfit) for HBL 100 and MDA MB468 cells following a 48 hour treatment with increasing concentrations of I3C are shown in *figures 3.3 and 3.4.* The mean values for the percentage of cells in each phase are shown in *table 3.2.*

HBL 100			
I3C (μM)	**G1**	**S**	**G2/M**
0	43.6 (±0.8)	31.8 (±1.9)	24.6 (±1.2)
100	44.8 (±1.4)	30.7 (±1.8)	24.5 (±0.4)
250	43.7 (±3.6)	34.1 (±1.3)	22.2 (±2.4)
500	42.9 (±1.1)	36.4 (±2.6)*	20.7 (±1.6)*
750	41.6 (±1.2)*	39.4 (±1.6)*	19.0 (±1.7)*
1000	43.8 (±2.9)	36.6 (±0.7)*	19.7 (±2.3)*
MDA MB468			
I3C (μM)	**G1**	**S**	**G2/M**
0	64.3 (±1.0)	12.7 (±1.8)	20.2 (±3.3)
10	61.0 (±1.3)	12.0 (±1.6)	20.3 (±2.7)
25	63.2 (±2.6)	13.2 (±0.8)	21.0 (±2.2)
50	64.3 (±1.1)	13.0 (±1.4)	22.1 (±1.5)
100	61.1 (±2.6)	18.2 (±4.6)*	16.8 (±8.9)
200	54.8 (±39)	25.6 (±21)	14.6 (±18)

Table 3.2. Percentage of cells in each phase of the cell cycle in the HBL 100 and MDA MB468 cell lines following a 48 hour I3C treatment. * denotes a significant difference from DMSO control *(n=3, ± S.D., p<0.05). nb. Figures in table do not include cells in sub G1 peak and represent data for remaining live cells only.*

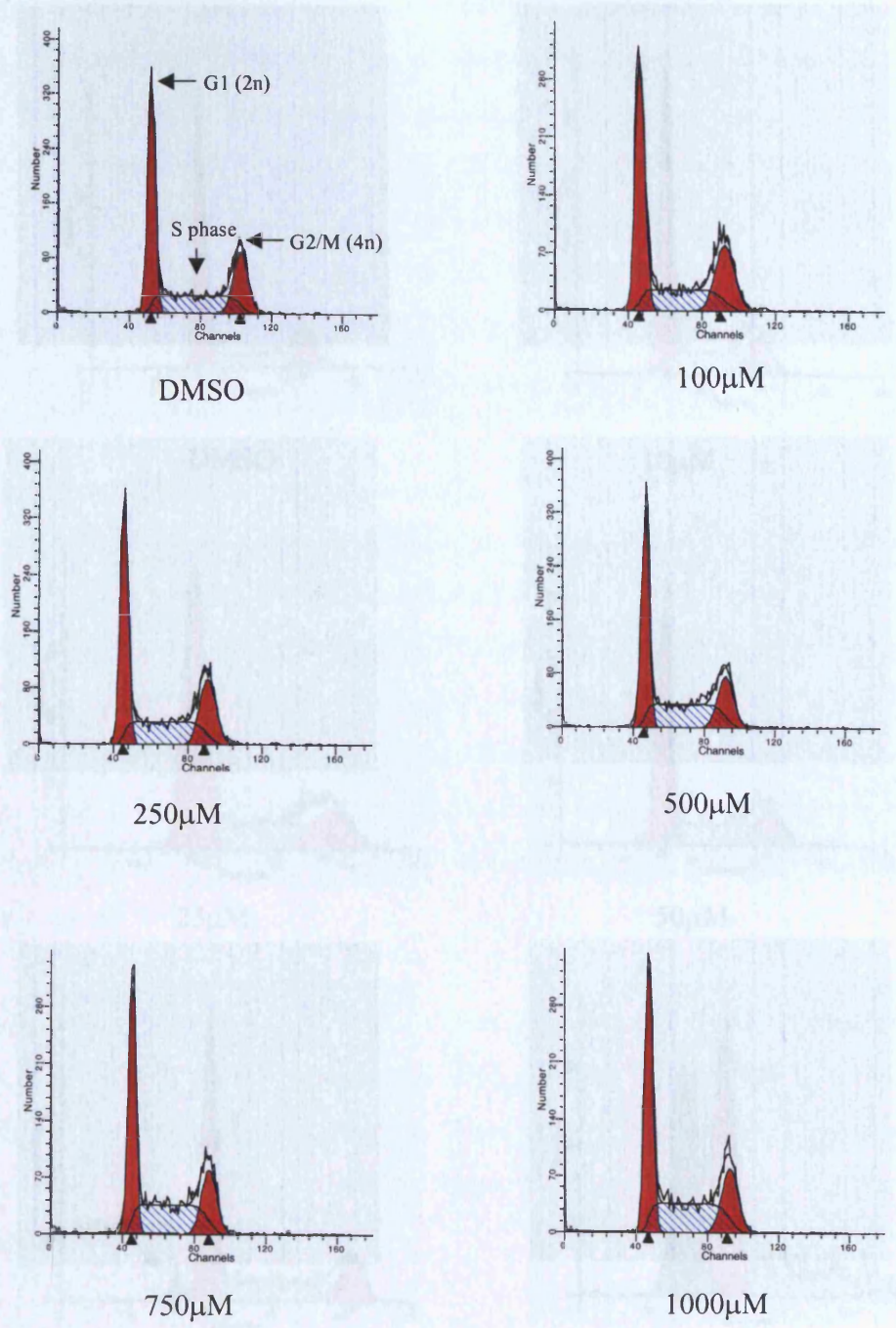


Figure 3.3 Flow cytometric analysis of prodium iodide stained HBL 100 cells following a 48 hour I3C treatment. *Analysis was performed upon 3 separate occasions and data analysed using Modfit L.T. software.*

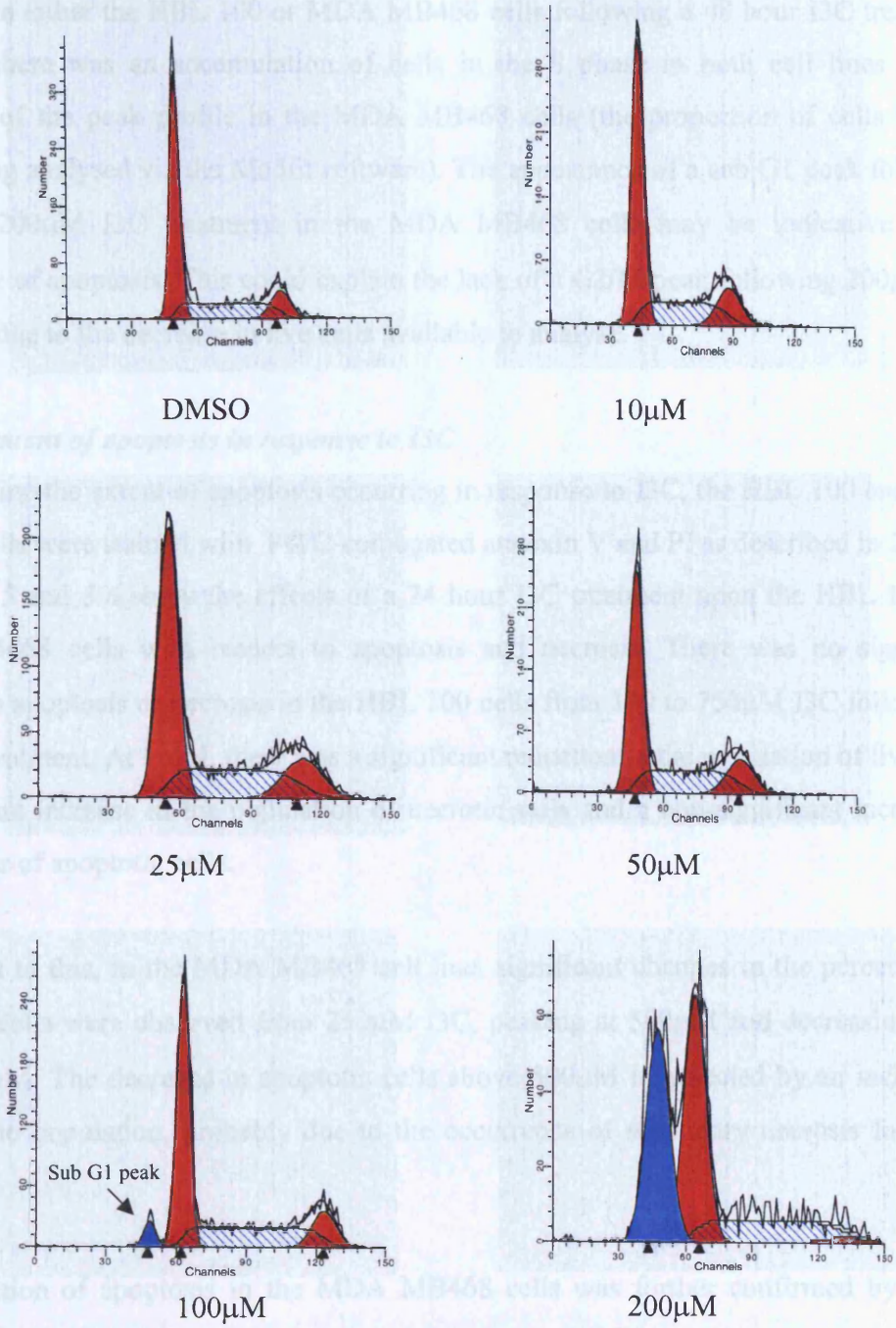


Figure 3.4 Flow cytometric analysis of prodium iodide stained MDA MB468 cells following a 48 hour I3C treatment. *Analysis was performed upon 3 separate occasions and data analysed using Modfit L.T. software.*

Cell cycle arrest is characterised by an accumulation of cells within specific areas of the cycle, which can be visualised by an alteration to the peak areas obtained in DNA histograms compared to that of the controls. No clear phase-specific cell cycle arrest was observed in either the HBL 100 or MDA MB468 cells following a 48 hour I3C treatment, although there was an accumulation of cells in the S phase in both cell lines and an alteration of the peak profile in the MDA MB468 cells (the proportion of cells in each phase being analysed via the Modfit software). The appearance of a sub G1 peak following 100 and 200μM I3C treatment in the MDA MB468 cells may be indicative of the occurrence of apoptosis. This could explain the lack of a G2/M peak following 200μM I3C treatment due to the decrease in live cells available to analyse.

3.4 Assessment of apoptosis in response to I3C

To determine the extent of apoptosis occurring in response to I3C, the HBL 100 and MDA MB468 cells were stained with FITC-conjugated annexin V and PI as described in *2.4.4.* *Figures 3.5* and *3.6* show the effects of a 24 hour I3C treatment upon the HBL 100 and MDA MB468 cells with respect to apoptosis and necrosis. There was no significant increase in apoptosis or necrosis in the HBL 100 cells from 100 to 750μM I3C following a 24 hour treatment. At 1mM, there was a significant reduction in the population of live cells, a significant increase in the population of necrotic cells and a non-significant increase in the number of apoptotic cells.

In contrast to this, in the MDA MB468 cell line, significant changes in the percentage of apoptotic cells were observed from 250μM I3C, peaking at 500μM and decreasing again from 750μM. The decrease in apoptotic cells above 500μM is reflected by an increase in the necrotic population, probably due to the occurrence of secondary necrosis following apoptosis.

The induction of apoptosis in the MDA MB468 cells was further confirmed by a time course with 500μM I3C treatment over 24 hours. *Figure 3.7* shows a time-dependent increase in apoptosis from as early as 2 hours, reaching significance by 8 hours, and the highest levels observed at 24 hours. Another measure of apoptosis is via cleavage of the caspase 3/7 substrate poly (ADP ribose) polymerase (PARP). *Figure 3.8* shows significant

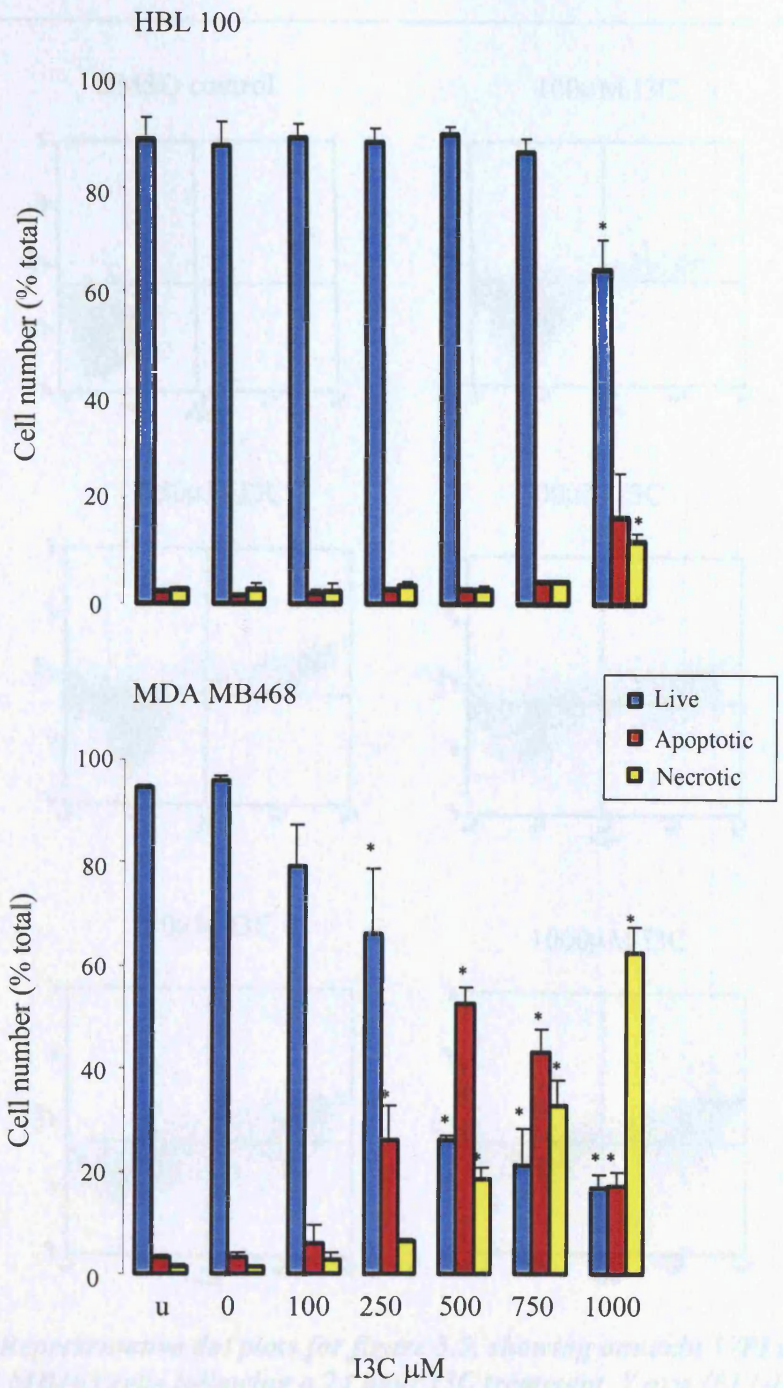


Figure 3.5 Assessment of apoptosis and necrosis by annexin V and PI staining in HBL 100 and MDA MB468 cells following a 24 hour I3C treatment. ** denotes a significant difference from DMSO control ($p \leq 0.05$, $n=3$, $\pm$ SEM) using a Balanced ANOVA followed by Fisher's least significant difference post hoc test. u = untreated cells, 0 = DMSO control.*

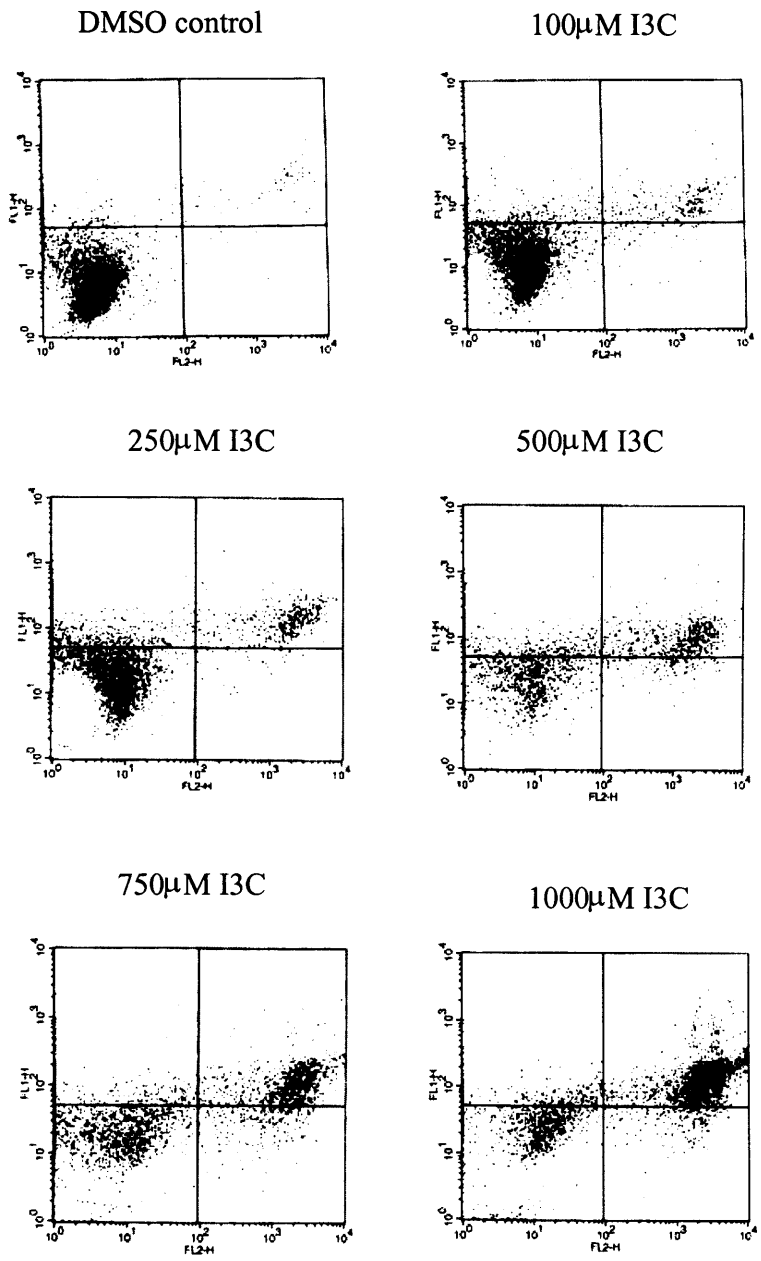


Figure 3.6. Representative dot plots for figure 3.5, showing annexin V/PI staining in the MDA MB468 cells following a 24 hour I3C treatment. *Y axis (FL1-H) represents annexin V staining, and X axis (FL2-H) represents PI staining. Live cells are present in the bottom left corner, apoptotic cells in the top left corner, and necrotic cells in the top right corner.*

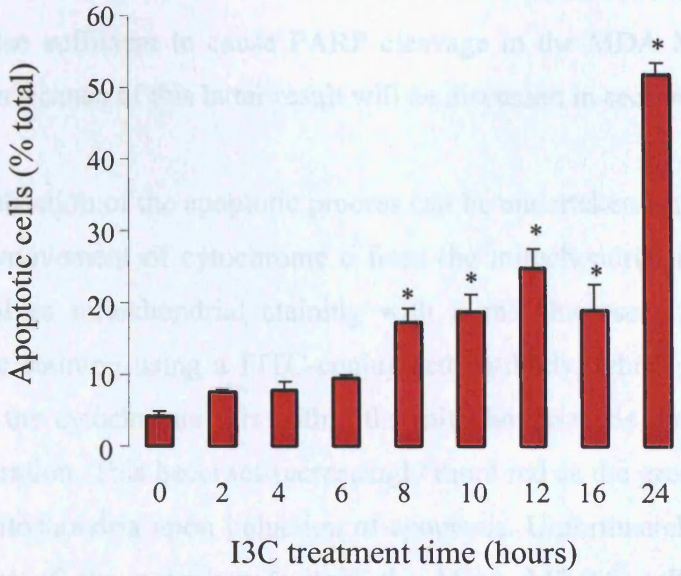


Figure 3.7 The effect of 500μM I3C upon apoptosis in the MDA MB468 cells over a period of 24 hours as determined by annexin V staining. ** denotes a significant difference from DMSO control ($p \leq 0.05$, $n=3$, ± SEM) using a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

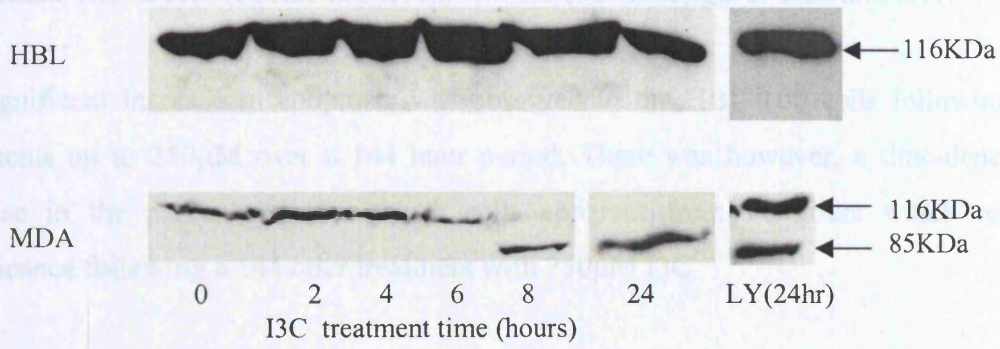


Figure 3.8 Western blot showing the effect of 500μM I3C or 50μM LY294002 upon PARP cleavage in the HBL 100 and MDA MB468 cells over a 24 hour time period. (The significance of the results with LY (an inhibitor of PI3K) will discussed in chapter 4).

PARP cleavage by 8 hours following a 500μM I3C treatment in the MDA MB468 cells, but not in the HBL 100 cells. The addition of the PI3K inhibitor LY294002 at 50μM for 24 hours was also sufficient to cause PARP cleavage in the MDA MB468 cell line *(figure 3.8)*. The significance of this latter result will be discussed in section *4.1*

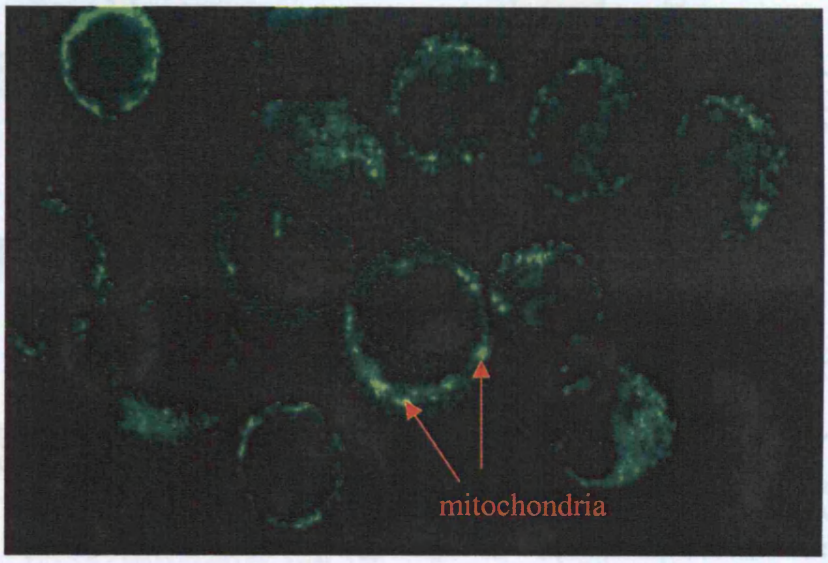
Further visualisation of the apoptotic process can be undertaken using confocal microscopy to track the movement of cytochrome c from the mitochondria into the cytoplasm. This process involves mitochondrial staining with a red fluorescent dye (mitotracker) and cytochrome c staining using a FITC-conjugated antibody, which has green fluorescence. When all of the cytochrome c is within the mitochondria, the dual staining results in an orange colouration. This becomes increasingly more red as the green stained cytochrome c leaves the mitochondria upon induction of apoptosis. Unfortunately, due to the inherently small amount of the cytoplasm within the MDA MB468 cells, this method proved unreliable, with any changes difficult to track as shown in *figure 3.9.*

Cells were then treated with lower doses of I3C for longer periods of time, allowing a more accurate assessment of doses that initiated apoptosis in the MDA MB468 cells, and whether or not the HBL 100 cells would undergo apoptosis following more prolonged treatments. The effects of these I3C treatments are shown in *figures 3.10 and 3.11.*

No significant increase in apoptosis was observed in the HBL 100 cells following I3C treatments up to 250μM over a 144 hour period. There was however, a time-dependent increase in the percentage of necrotic cells apparent from 96 hours which reached significance following a 144 hour treatment with 250μM I3C.

There was a significant increase in apoptosis in the MDA MB468 cells *(figure 3.11)* from 24 hours with 100μM I3C, and from 48 hours onwards with 50μM. An increase in apoptosis was also found with 10μM I3C from 72 hours which was bordering upon significance by 168 hours.

A)



B)

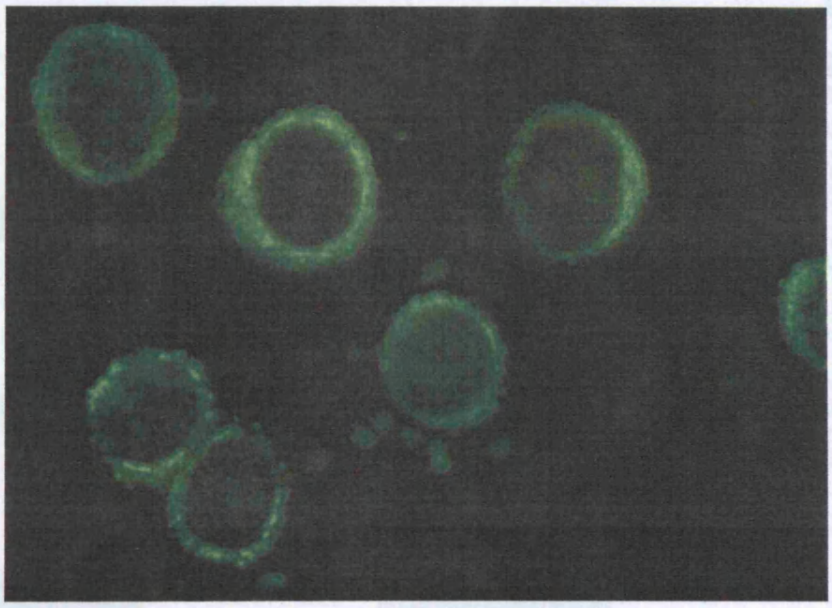


Figure 3.9 Cytochrome c staining and visualisation by confocal microscopy of MDA MB468 cells. *Panel A features control MDA MB468 cells, treated for 7 hours with DMSO. Panel B represents MDA MB468 cells treated for 7 hours with 100μM I3C.*

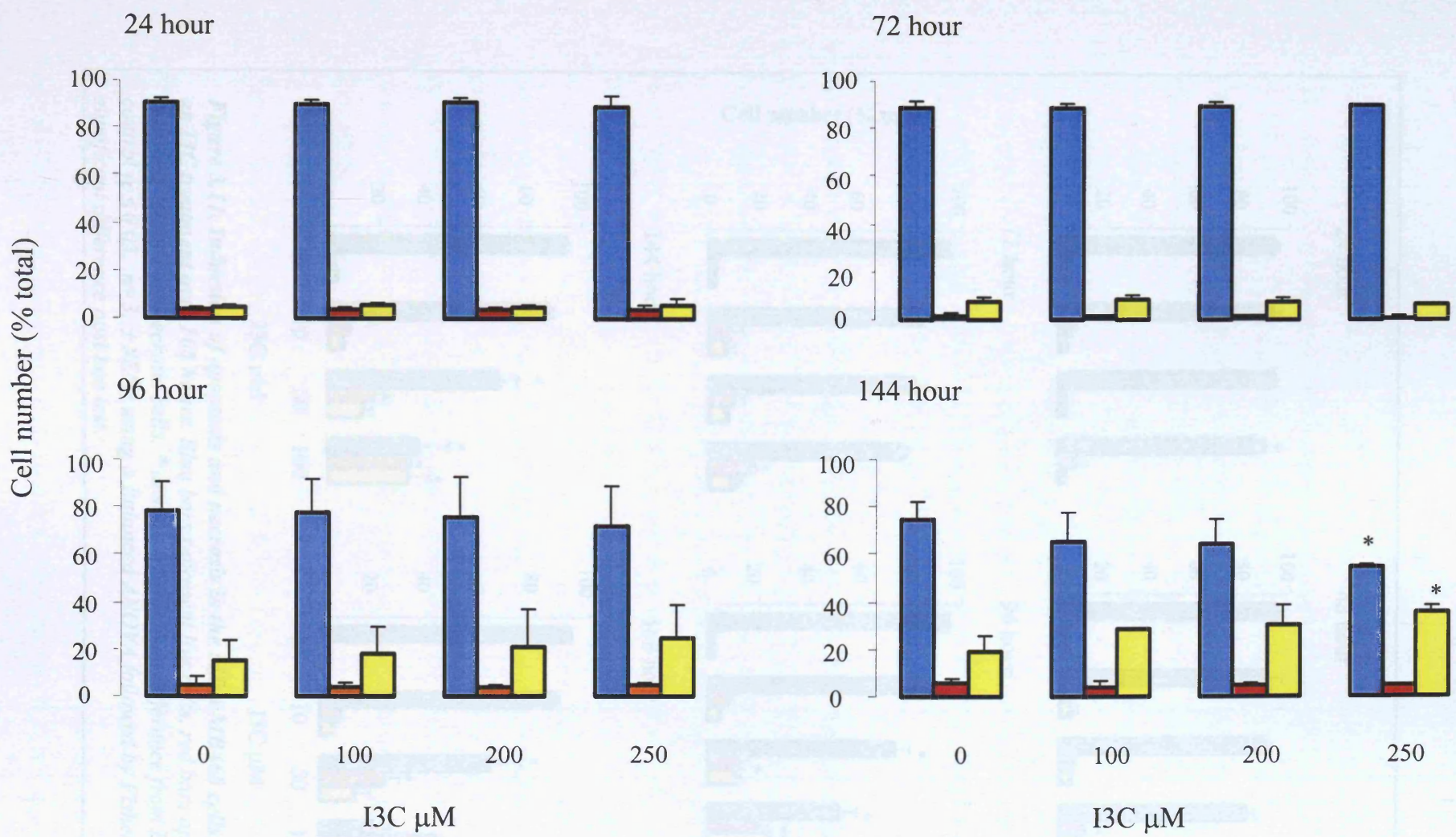


Figure 3.10. Lack of apoptosis but induction of necrosis in HBL 100 cells following I3C treatment over 144 hours. *Blue bars represent live cells, red bars represent apoptotic cells and yellow bars represent necrotic cells. * denotes a significant difference from DMSO control ($p \leq 0.05$, $n=3$, $\pm$SEM) using a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

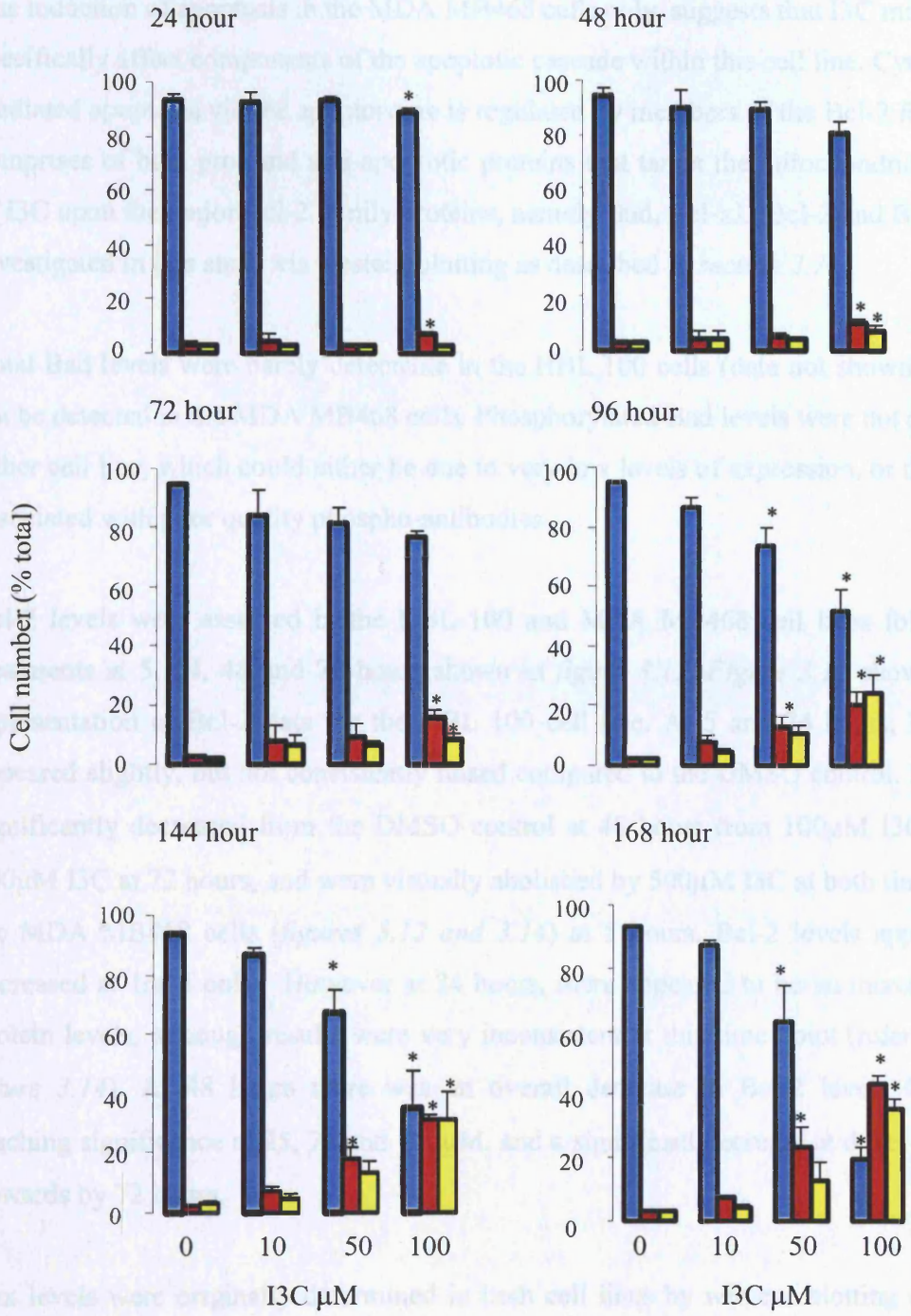


Figure 3.11. Induction of apoptosis and necrosis in the MDA MB468 cells following an I3C treatment over 168 hours. *Blue bars represent live cells, red bars apoptotic cells and yellow bars necrotic cells. * denotes a significant difference from DMSO control ($p \leq 0.05$, $n=3$, $\pm$ SEM) using a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

3.5 Effect of I3C on apoptosis-regulatory proteins of the Bcl-2 family.

The induction of apoptosis in the MDA MB468 cells only, suggests that I3C may be able to specifically affect components of the apoptotic cascade within this cell line. Cytochrome c-mediated apoptosis via the apoptosome is regulated by members of the Bcl-2 family which comprises of both pro- and anti-apoptotic proteins that target the mitochondria. The effect of I3C upon the major Bcl-2 family proteins, namely Bad, Bcl-xL, Bcl-2 and Bax, has been investigated in this study via western blotting as described in *section 2.7.*

Total Bad levels were barely detectable in the HBL 100 cells (data not shown), and could not be detected in the MDA MB468 cells. Phosphorylated Bad levels were not detectable in either cell line, which could either be due to very low levels of expression, or the problems associated with poor quality phospho-antibodies.

Bcl-2 levels were assessed in the HBL 100 and MDA MB468 cell lines following I3C treatments at 5, 24, 48 and 72 hours shown in *figure 3.12. Figure 3.13* shows graphical representation of Bcl-2 data for the HBL 100 cell line. At 5 and 24 hours, Bcl-2 levels appeared slightly, but not consistently raised compared to the DMSO control. Levels were significantly decreased from the DMSO control at 48 hours from 100μM I3C, and from 250μM I3C at 72 hours, and were virtually abolished by 500μM I3C at both time points. In the MDA MB468 cells (*figures 3.12 and 3.14*) at 5 hours, Bcl-2 levels appeared to be decreased at 1mM only. However at 24 hours, there appeared to be an increase in Bcl-2 protein levels, although results were very inconsistent at this time point (refer to SEM on *figure 3.14*). At 48 hours there was an overall decrease in Bcl-2 levels from 10μM, reaching significance at 25, 75 and 100μM, and a significant decrease at doses from 10μM upwards by 72 hours.

Bax levels were originally determined in both cell lines by western blotting using whole cell lysates, following an I3C treatment at 5, 24, 48 and 72 hours. There was no change to total cellular Bax levels in the HBL 100 or MDA MB468 cells following I3C treatment at any time point (*figure 3.15*). Further westerns were performed using mitochondrial rich pellets (refer to *section 2.6.3*) in order that any small changes to Bax levels might be visualised, but again, no reproducible changes were observed (data not shown).

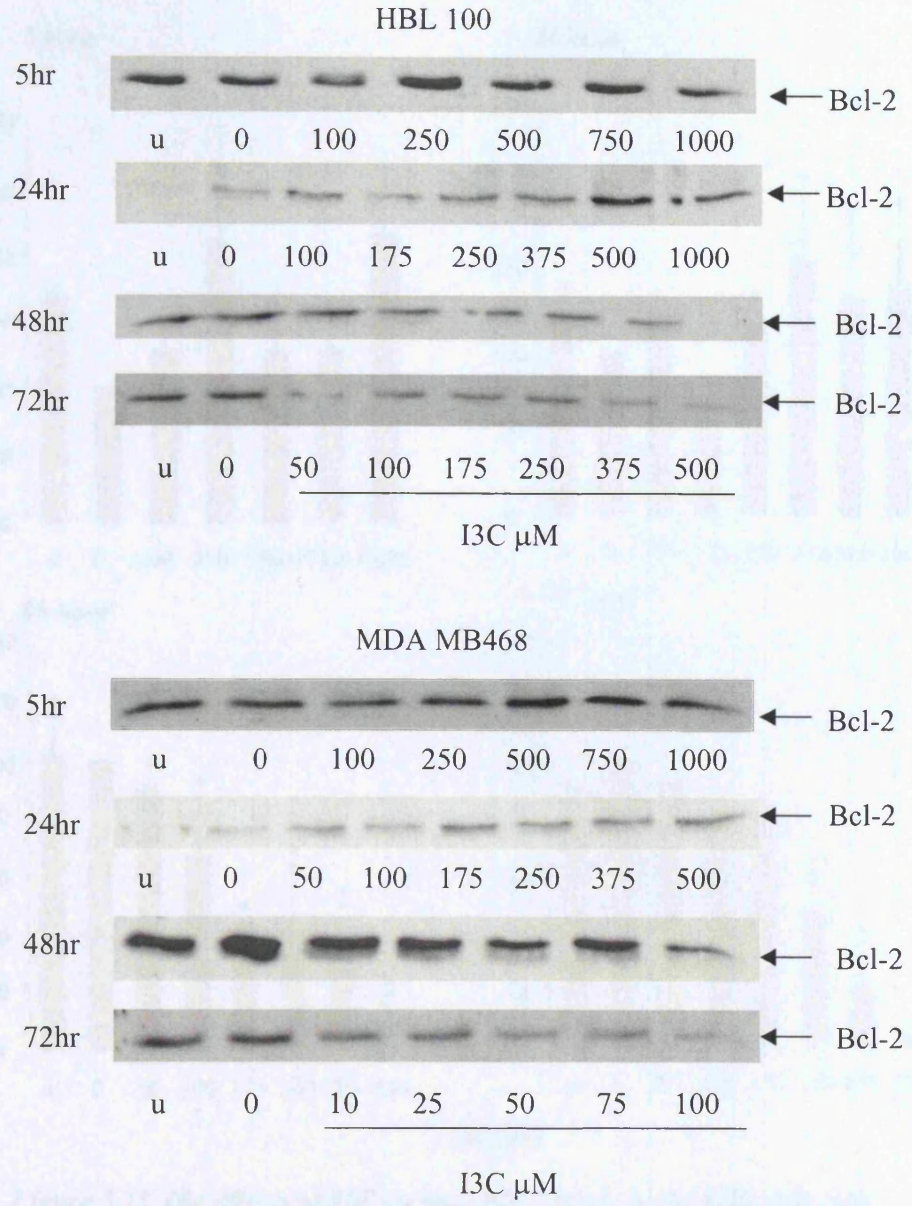


Figure 3.12 Representative western blots showing Bcl-2 levels in HBL 100 and MDA MB468 cells following an I3C treatment for time points up to 72 hours. *Each experiment was performed on at least 3 separate occasions, loading an equivalent amount of protein for each sample. The same samples were used to determine total Bax levels. Blots shown represent varying exposure times. u = untreated control, (24 hour data acquired by Dr. E. A. Hudson).*

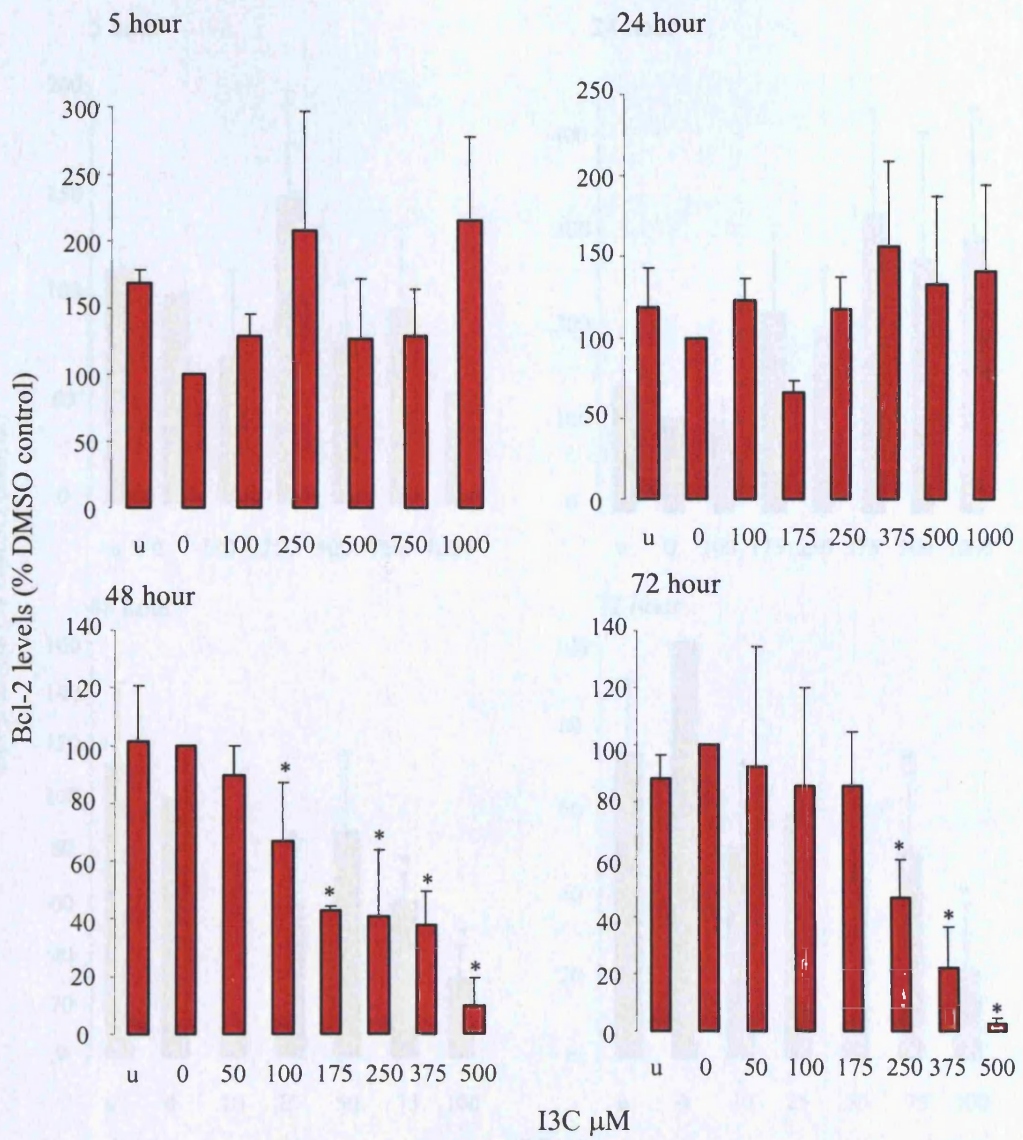


Figure 3.13 The effects of I3C on total Bcl-2 levels in the HBL 100 cells following 5 to 72 hour I3C treatments. *Western blot bands were analysed by densitometry.* * denotes a significant difference from DMSO control ($p \leq 0.05$, $n=3$, ±SEM) using a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

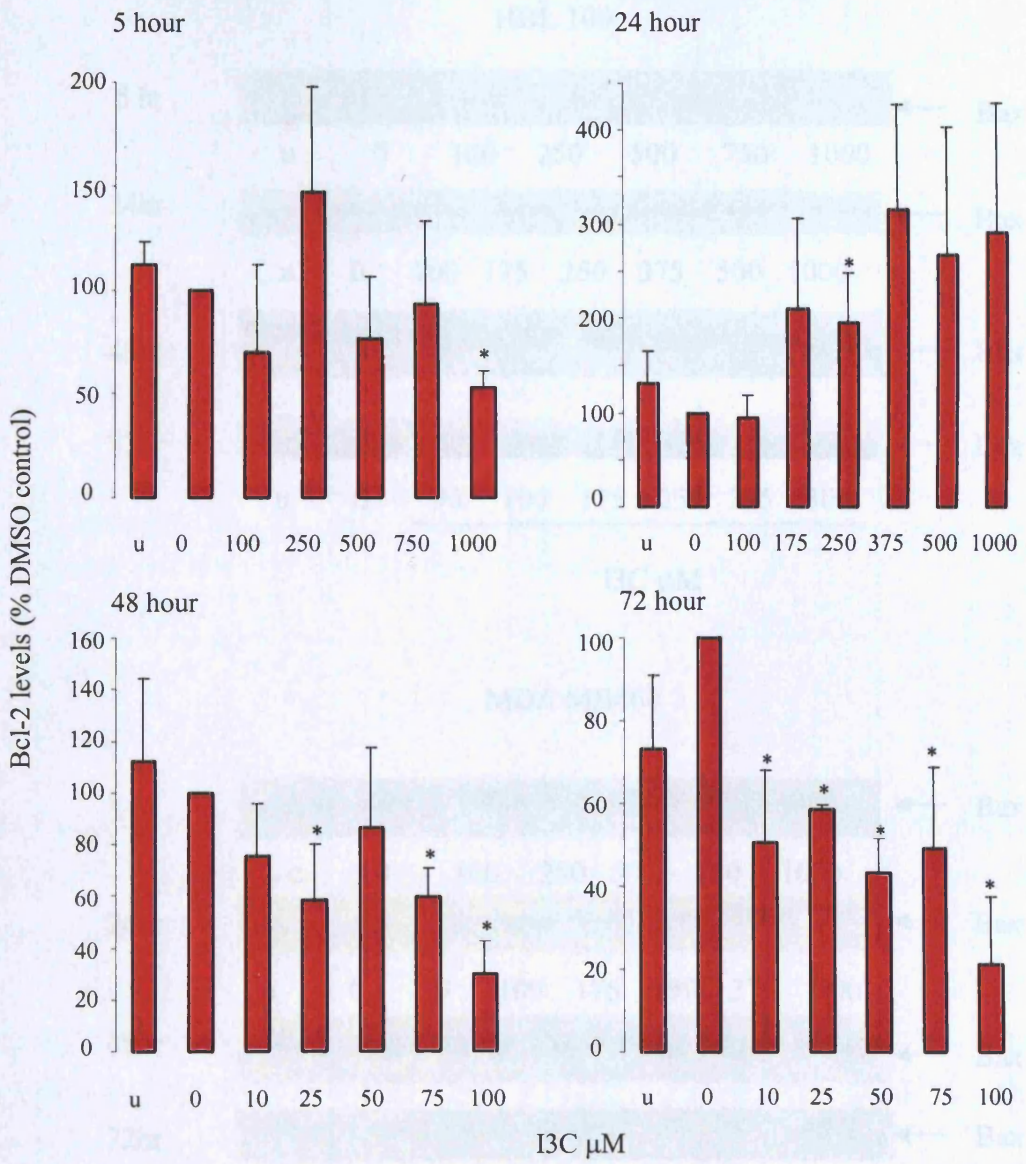


Figure 3.14 The effects of I3C on total Bcl-2 levels in the MDA MB468 cells following 5 to 72 hour I3C treatments. *Western blot bands were analysed by densitometry.* denotes a significant difference from DMSO control ($p \leq 0.05$, $n=5$, ±SEM) using a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

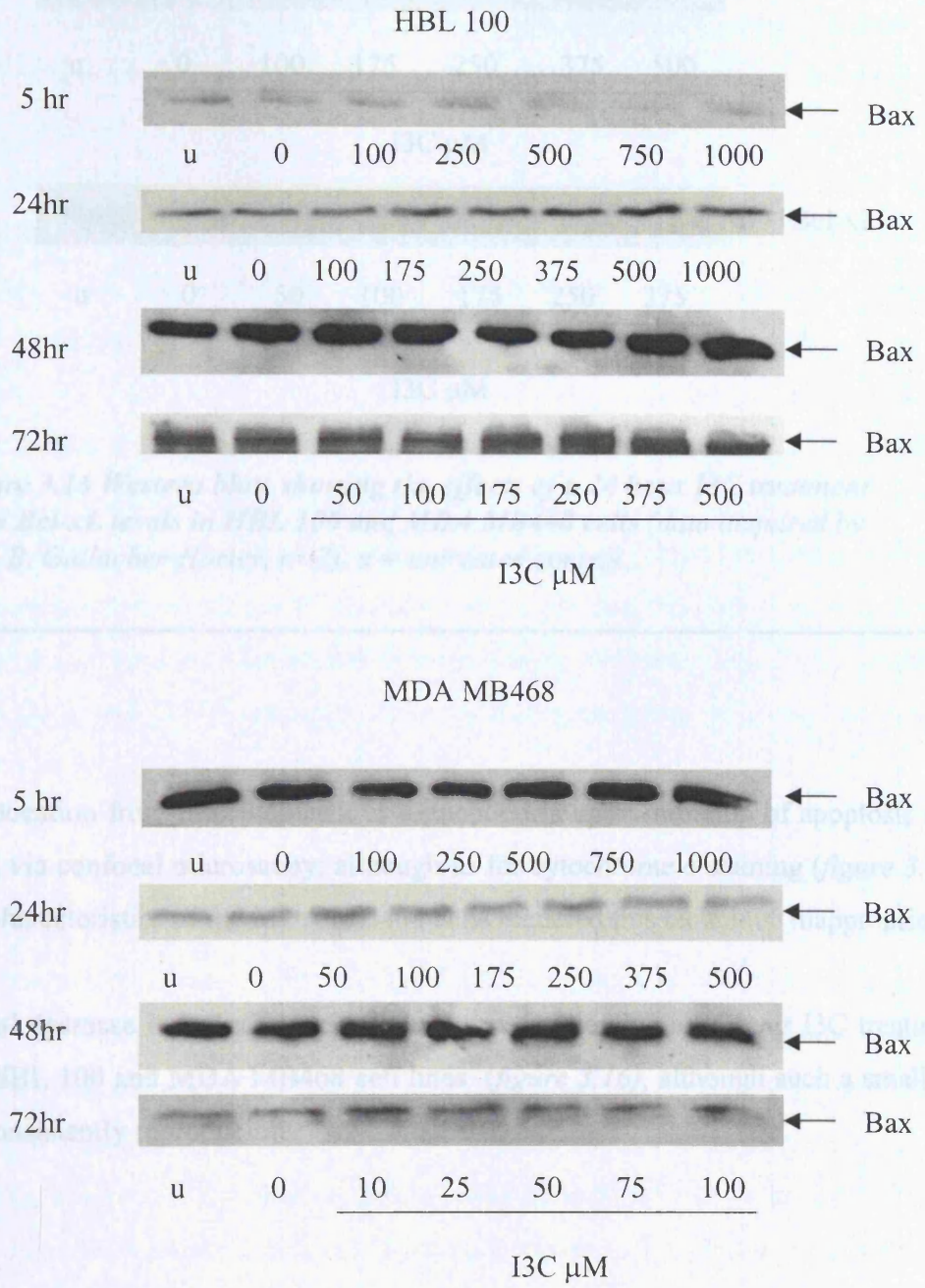


Figure 3.15 Representative western blots showing Bax levels in HBL 100 and MDA MB468 cells following an I3C treatment for time points up to 72 hours. *Each experiment was performed on 3 separate occasions, loading an equivalent amount of protein for each sample. The same samples were used to determine total Bcl-2 levels. Blots shown represent varying exposure times. u = untreated control.*

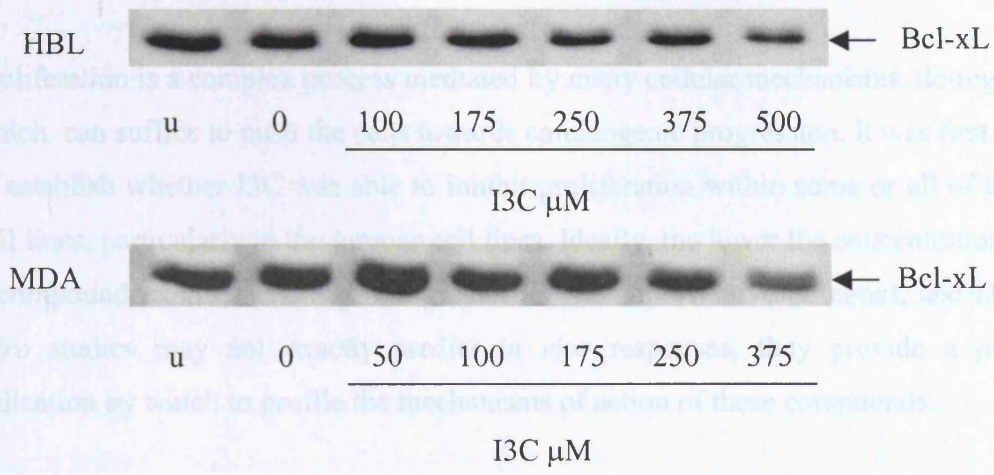


Figure 3.16 Western blots showing the effects of a 24 hour I3C treatment upon Bcl-xL levels in HBL 100 and MDA MB468 cells *(data acquired by Mrs. B. Gallacher-Horley, n=2). u = untreated control.*

Bax translocation from the cytoplasm to mitochondria upon initiation of apoptosis can be visualised via confocal microscopy, although as for cytochrome c staining (*figure 3.9)*, the physical characteristics of the MDA MB468 cells rendered this technique inappropriate.

A marginal decrease in Bcl-xL levels was observed following a 24 hour I3C treatment in both the HBL 100 and MDA MB468 cell lines (*figure 3.16)*, although such a small effect was not consistently reproducible.

DISCUSSION

Proliferation is a complex process mediated by many cellular mechanisms, de-regulation of which, can suffice to push the cells towards carcinogenic progression. It was first important to establish whether I3C was able to inhibit proliferation within some or all of the chosen cell lines, particularly in the tumour cell lines. Ideally, the lower the concentration at which a compound exhibits efficacy, the greater its chemopreventive potential, and although *in vitro* studies may not exactly predict *in vivo* responses, they provide a preliminary indication by which to profile the mechanisms of action of these compounds.

I3C was effective at inhibiting proliferation to varying degrees in all four breast cell lines. However, the differences in sensitivity between these cell lines, suggests that I3C may be exerting a cell-type specific effect, possibly involving divergent mechanisms of inhibition of proliferation. Several studies to date have demonstrated the ability of I3C to initiate growth arrest in a variety of breast cell lines including Rahman *et al.*, 2000, Cover et *al.,* 2000 and Ge *et al.*, 1999, although there is still speculation as to its more specific mechanisms of action both *in vitro* and *in vivo.*

The difference in sensitivity to I3C between cell lines was most marked between the normal-derived HBL 100 cells and the tumour-derived MDA MB468 cells, which are representative of an aggressive ERα-negative adenocarcinoma of the type that is clinically often found resistant to conventional therapies (Swain, 2001). The IC_{50}s for the HBL 100 and MDA MB468 cells were 122 and 33μM respectively, exhibiting a four fold difference in sensitivity between these two cell lines in response to I3C, compared to a 2 fold difference between the MDA MB468 cells and the tumour-derived T47D and MCF7 cells. It may be that this is purely a cell type specific response to I3C, but there is a possibility that aggressive tumour types may be more sensitive to growth inhibition by this compound. Growth inhibition of the MDA MB468 cells was observed following a low I3C treatment of 10μM over the period of one week, suggesting that I3C may be able to affect tumour cell growth at concentrations relevant to those seen in the diet. Low concentrations of I3C condensation products have previously been observed in livers of animals administered dietary I3C (Larsen-Su and Williams, 2001), although there is little pharmacokinetic data

currently available showing accurate concentrations in target tissues such as breast. However, within our laboratory, Anderton *et al.,* (unpublished) have revealed I3C to be rapidly absorbed in mice following a single oral dose, with the parent compound and acid condensation products appearing in plasma, liver, kidney, lung, heart and brain.

The ability of the HBL 100 cells to recover their proliferative ability completely after treatment with 250μM I3C, suggests that these cells do not undergo an irreversible inhibitory event in response to I3C exposure at this or lower concentrations. Indeed, HBL 100 cells were found not to readily undergo apoptosis following a 24 hour I3C treatment *(figure 3.5)*, even at concentrations up to 1mM, with only minimal necrosis at this concentration, which is compatible with the ability of these cells to recover. It is interesting to note, however, that growth of the HBL 100 cells was completely inhibited following a 96 hour treatment with 250μM I3C *(figure 3.1)*, whereas there was little effect upon relative percentages of live, apoptotic and necrotic cells as shown by the annexin V/PI staining *(figure 3.9)* at this time point. This discrepancy is likely to be due to the inability of the cells to overcome the inhibitory effects of I3C when seeded at very low densities such as those used for the cell proliferation assays (noted in Hudson *et al.*, 2000).

I3C has previously been shown to cause G1 cell cycle arrest in the MCF7 breast cells (Cover *et al.,* 2000), but in this study, there appeared to be no definitive arrest in the HBL 100 cells following treatment with any concentration of I3C. *Table 3.2* shows an increase in the percentage of cells in the S phase with increasing I3C, possibly indicating a prolonged cell cycling time due to a decrease in the rate of DNA synthesis. This effect could be further investigated via use of cell synchronisation agents (such as nocodazole or aphidicolin) in order to clarify any small changes that may be occurring, or via BrDu staining.

In contrast to the HBL 100 cells, the MDA MB468 cell line was able to only partially recover following a 20μM I3C treatment, suggesting that the effect of I3C upon this cell line may be occurring via a distinct mechanism that commits these cells irreversibly to growth inhibition. Cell cycle analysis of MDA MB468 cells following a 48 hour I3C treatment confirmed that decreased proliferation in these cells was not occurring due to a cell cycle arrest, but was most likely due to the induction of apoptosis. Studies in the MDA

MB468 cells show an increase in apoptotic cells following a 24 hour treatment with 100μM I3C, with over 50% of cells undergoing apoptosis after treatment with 500μM (*figure 3.7*). Longer time points confirm the susceptibility of these cells to I3C-induced apoptosis even at concentrations as low as 10μM. This susceptibility of the MDA MB468 cells to undergo apoptosis provides the most likely explanation as to why these cells are only able to partially recover from a short term I3C treatment, in that commitment to apoptosis is an irreversible procedure, ultimately resulting in cell death.

A study by Rahman *et al.,* (2000) showed induction of apoptosis in the MDA MB435 cells, and they suggested that I3C-induced upregulation of Bax protein, translocation of Bax to the mitochondria and downregulaion of Bcl-2 was responsible for this effect. The effects of I3C upon Bcl-2 family members Bad, Bcl-2, Bax and Bcl-xL were investigated in this thesis, in order to provide an insight as to how I3C may be inducing apoptosis in the MDA MB468 cells. Little information was obtained about the effects of I3C upon total or phosphorylated Bad protein levels in either cell line, which was disappointing due to the major role that Bad can play as a pro-apoptotic molecule. However, as levels were so difficult to detect (even using immunoprecipitation), it may be that Bad does not have a key pro-apoptotic role in these cell lines.

The interaction between the anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins depends upon their ratio within the cell, and therefore their ability to determine whether cells will be committed to the apoptotic process. There was no change in Bax total protein levels in response to I3C at times up to 72 hours (*figure 3.15*), whereas Bcl-2 levels were decreased significantly from 48 hours in both cell lines (*figure 3.12*). Although the Bcl-2:Bax ratio was altered in the favour of apoptosis at these later time points, it still does not provide an explanation for the induction of apoptosis in the MDA MB468 cells at times of 24 hours or earlier. It is therefore likely that this change in Bcl-2:Bax ratio is occurring as a consequence of cells undergoing apoptosis, rather than being an initiator of it. In this study, total Bax levels were investigated, whereas it may be that it is the translocation of Bax to the mitochondria from the cytosol that has greater importance at these earlier time points, rather than total protein levels. Observation of Bax mitochondrial translocation may be undertaken using confocal microscopy. However, as previously found when attempting to visualise cytochrome c release, MDA MB468 cells do not provide a suitable model for

such an experiment, and whilst the HBL 100 cells are morphologically ideally suited, they do not appear to undergo apoptosis in response to I3C. Detection of Bax translocation by laser scanning cytometry is an alternative technique that may be able to overcome problems associated with visualisation of cytosolic proteins within the MDA MB468 cells, as it is able to quantitatively distinguish the passage of labelled Bax from cytosol to mitochondria (Darzynkiewicz *et al.,* 2002). However, it is interesting to note that Ge *et al.,* (1999) found induction of apoptosis in the MCF7 breast cells in response to I3C was independent of an effect upon Bax.

Data shown in this chapter have illustrated the differences in I3C-mediated inhibition of proliferation between cell lines. Over a long-term treatment at low doses, the HBL 100 cells did not undergo any apoptosis, whereas in contrast, the MDA MB468 cells became apoptotic following a 10μM I3C treatment. The fact that such low doses can elicit this effect over time is encouraging, particularly if these doses are within a physiologically attainable range. There are currently very few studies describing the fate of I3C in cells in culture, but it has been suggested that the parent compound disappears from the culture media fairly quickly (Staub *et al.,* 2002). It should be noted then, that the effects discussed are seen following a single dose of I3C, whereas if treatments were continued for longer in a multi-dosing regime, then lower efficacious doses may be obtained through a cumulative effect.

The differences in response to I3C between the HBL 100 and MDA MB468 cell lines warrant further investigation in an attempt to elucidate these mechanistic disparities. Although no direct effect upon the Bcl-2 family and apoptosome driven apoptosis was observed (from confocal data) prior to phosphatidylserine externalisation, there are many signalling pathways that I3C might perturb or interact with in order to contribute to the apoptotic process, some of which will be discussed in the following chapters.

CHAPTER 4

EFFECT OF I3C ON THE PI3 KINASE SIGNALLING PATHWAY

INTRODUCTION

As previously discussed, the PI3K pathway plays an important role in cell survival and proliferation (refer to *section 1.6*). It may be that I3C is able to exert an effect upon this pathway and could result in the initiation of the apoptotic cascade. It was important to determine the status of PI3K signalling particularly in the HBL 100 and MDA MB468 cells, to see whether there could be a possible explanation of the differential sensitivity between the cell lines in response to I3C.

The protein kinase Akt has proved to be a major effector protein within the PI3K pathway (Datta *et al.*, 1999, El-Deiry, 2001). In this chapter, the effects of I3C on this kinase are discussed in an attempt to further elucidate whether I3C may be eliciting its effects within this signalling cascade.

To date, there have been relatively few studies investigating the PI3K pathway with respect to the mechanisms of action of chemopreventive agents. However, there is growing interest within this particular area, with research investigating the effects of I3C in breast cell lines and upon proteins involved in the apoptotic cascade that lie downstream of Akt, such as Bcl-2 and Bax (Rahman *et al.,* 2000). Work to date within the group has suggested that I3C may not exert its growth inhibitory effects via inhibition of the ERK, JNK or p38 MAPK cascades (Squires, 2000), although it may be able to decrease EGF-stimulated EGFR phosphorylation.

The PI3K cascade with its links to apoptosis-regulating molecules, therefore provides a novel potential target for I3C.

RESULTS

4.1 The effects of I3C upon Akt protein levels, phosphorylation status and activity

It was initially important to determine basal levels of Akt phosphorylation within the 4 breast cell lines when grown under normal conditions, in order to see whether levels were easily and reproducibly detectable, and significantly different between cell lines. *Figure 4.1* depicts basal levels of phosphorylated Akt (ser473) under normal growth conditions in the HBL 100, T47D, MDA MB468 and MCF7 breast cell lines. The highest level of phosphorylated Akt was found in the MDA MB468 cells, which expressed approximately 2.5 fold higher levels than the HBL 100 and T47D cells. Levels in the MCF7 cells were at the limits of detection of this assay.

The effects of I3C upon Akt phosphorylation status (using the p-Akt1,2,3 antibody) at concentrations up to 1mM were initially determined after 5 hours in all 4 breast cell lines and represented by western blot as shown in *figure 4.2A*. Cell lines varied in their response to I3C. I3C elicited no significant effect on levels of Akt phosphorylation under normal growth conditions in the HBL 100 cells. I3C had little consistent effect upon the T47D cells, particularly at the higher concentrations. Similarly, Akt phosphorylation levels in the MCF7 cells at the higher concentrations of I3C were variable, with some blots showing a large increase. The I3C-induced changes in Akt phosphorylation for the HBL 100, MCF7 and T47D cells are represented graphically in *figure 4.2B,* in order to show the SEM for these blots. It may be that high concentrations of I3C do have a stimulatory effect, particularly if basal levels are initially very low.

The most striking effect of I3C was upon the MDA MB468 cells, in which a dose-dependent decrease in Akt phosphorylation at the serine 473 phosphorylation site, was observed from 100μM treatment. As both serine and threonine phosphorylation are required for full activity of the kinase, the effect of I3C on the second phosphorylation site was determined, and a similar decrease in Akt phosphorylation was observed from 500μM. The MDA MB468 cell line underwent a DMSO-dependent stimulation of Akt

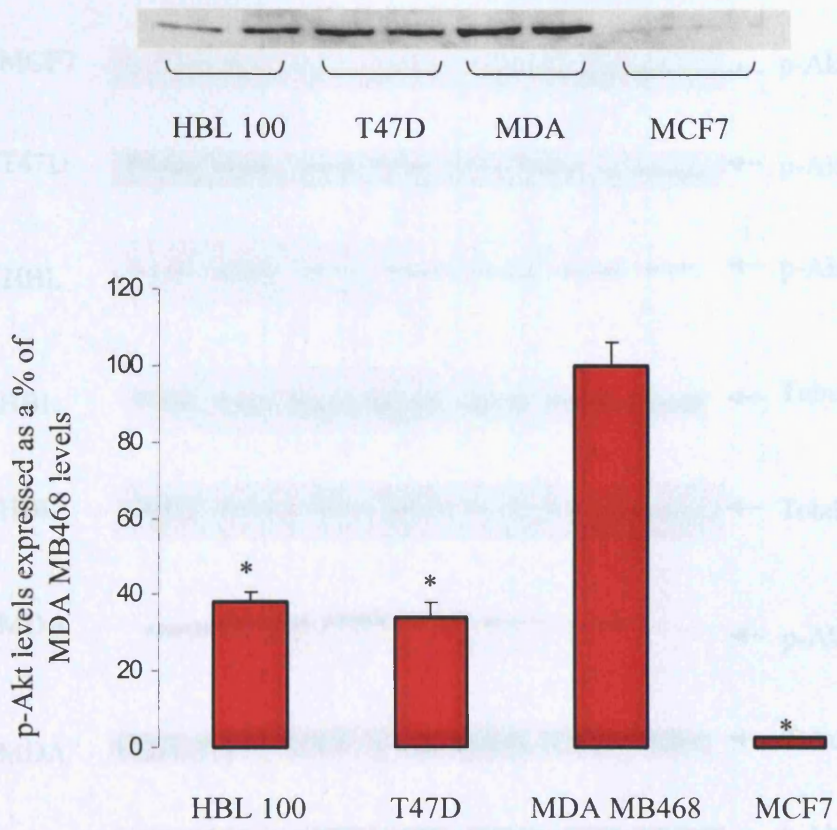


Figure 4.1 Basal levels of phosphorylated Akt in a panel of breast cell lines. *The top panel shows a representative western blot for basal levels of phosphorylated Akt (ser 473) in 4 breast cell lines using separate samples for each duplicate. The chart shows relative levels compared to MDA MB468 cells as determined via densitometric analysis of blots. * denotes a significant difference from p-Akt levels in the MDA MB468 cells (n=4, ± SEM, p≤0.05) as determined by a balanced ANOVA followed by Fisher's least significant difference post hoc test.*

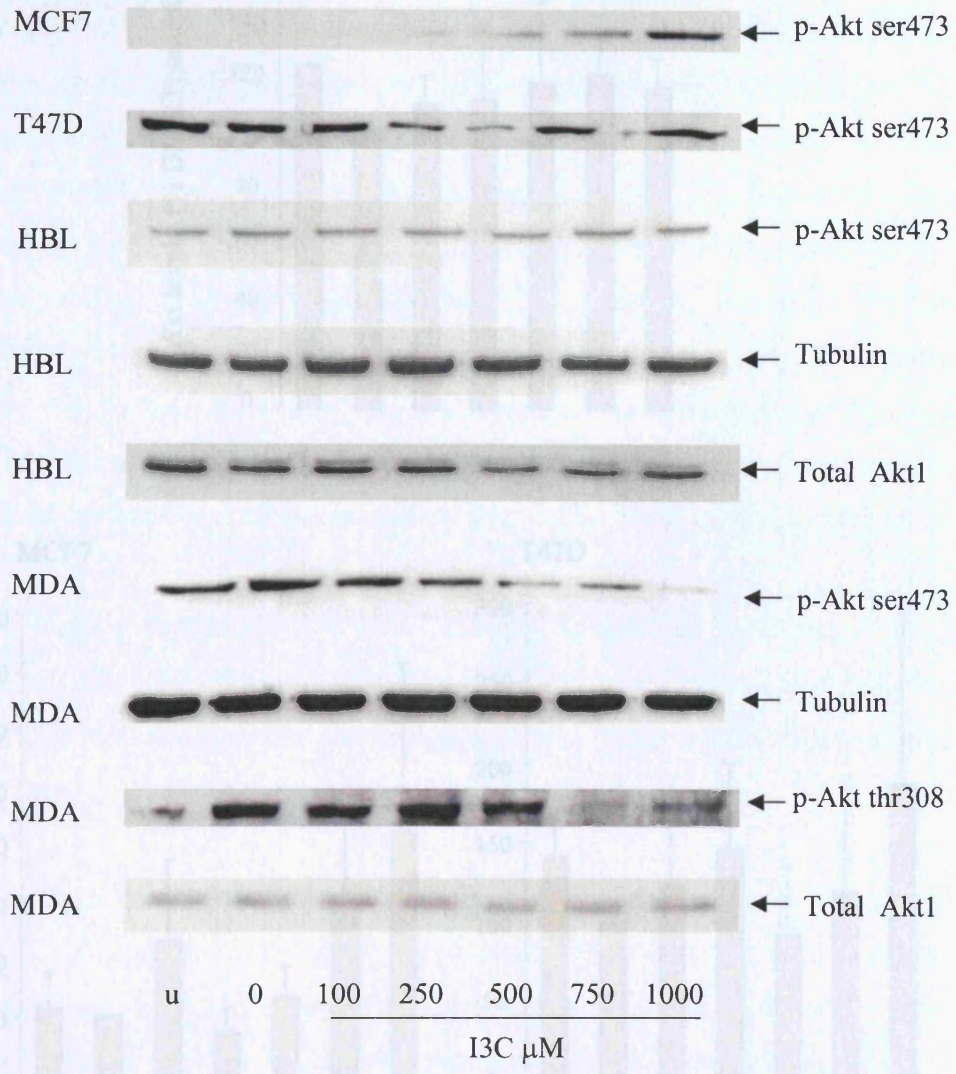


Figure 4.2A Representative western blots showing the effect of a 5 hour I3C treatment on p-Akt (ser473) levels in 4 breast cell lines. *Blots also show tubulin protein-loading controls, total Akt 1 levels (in the HBL 100 and MDA MB468 cells), and p-Akt (thr308) in the MDA MB468 cells only. Blots were performed on at least 3 separate occasions.*

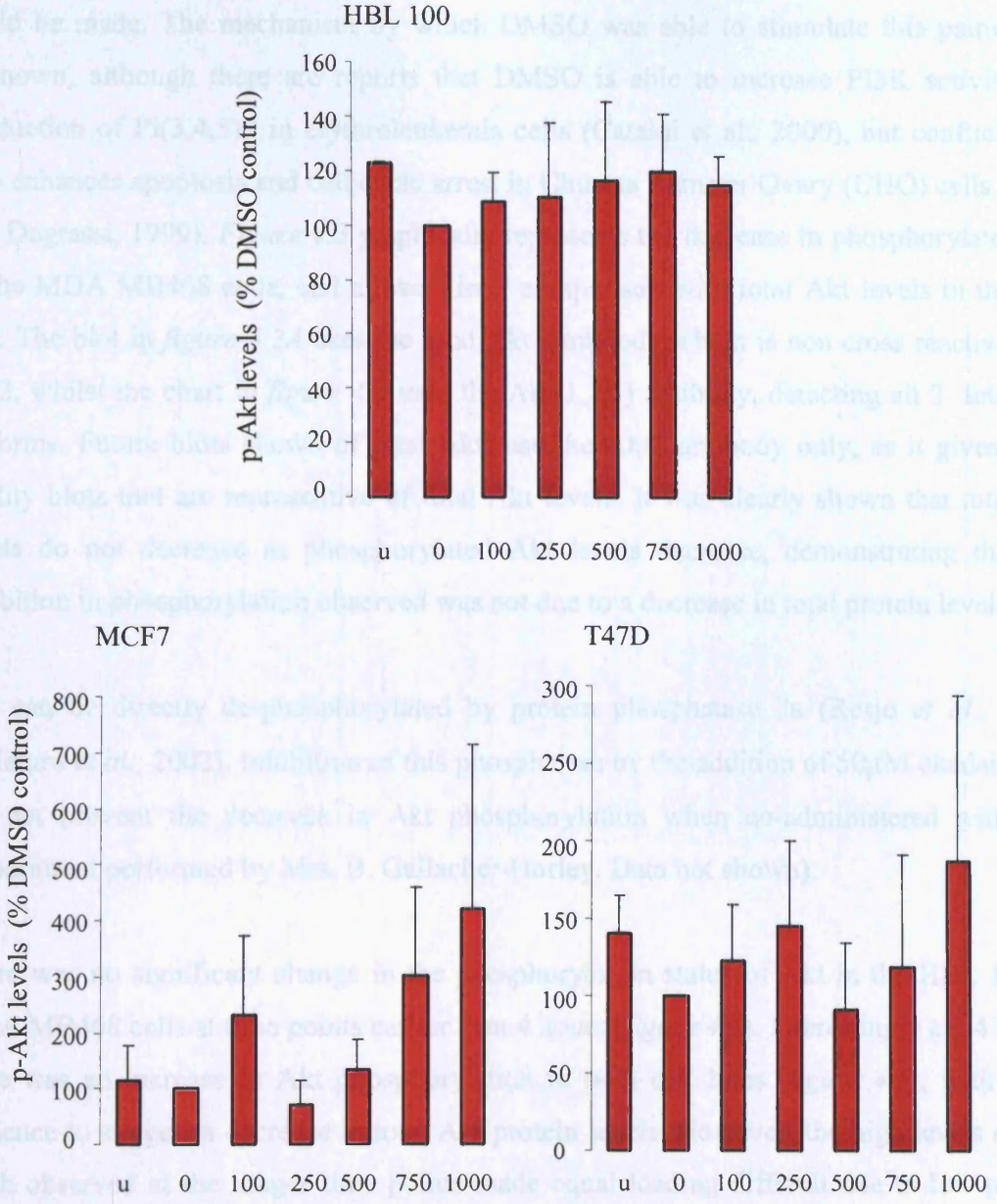


Figure 4.2B p-Akt (ser473) levels expressed as a percentage of the DMSO control following a 5 hour I3C treatment in MCF7, T47D and HBL 100 cells. *Levels were determined by densitometric analysis (n=3, ± SEM).*

phosphorylation, which occurred for these cells only (*figure 4.3)*. Within each experiment, treatments contained the same final DMSO concentration in order that direct comparisons could be made. The mechanism by which DMSO was able to stimulate this pathway is unknown, although there are reports that DMSO is able to increase PI3K activity and production of PI(3,4,5)P in erythroleukemia cells (Cataldi et al., 2000), but conflictingly, also enhances apoptosis and cell cycle arrest in Chinese Hamster Ovary (CHO) cells (Fiore and Degrassi, 1999). *Figure 4.3* graphically represents the decrease in phosphorylated Akt in the MDA MB468 cells, and allows direct comparison with total Akt levels in this cell line. The blot in *figure 4.2A* uses the total Akt1 antibody which is non cross reactive with Akt2, whilst the chart in *figure 4.3* uses the Akt(1,2,3) antibody, detecting all 3 total Akt isoforms. Future blots shown of total Akt, use the Akt1 antibody only, as it gives good quality blots that are representive of total Akt levels. It was clearly shown that total Akt levels do not decrease as phosphorylated Akt levels decrease, demonstrating that the inhibition in phosphorylation observed was not due to a decrease in total protein levels.

Akt can be directly de-phosphorylated by protein phosphatase 2a (Resjo *et al.*, 2002, Yellaturu *et al.,* 2002). Inhibition of this phosphatase by the addition of 50μM okadaic acid did not prevent the decrease in Akt phosphorylation when co-administered with I3C (experiment performed by Mrs. B. Gallacher-Horley. Data not shown).

There was no significant change in the phosphorylation status of Akt in the HBL 100 or MDA MB468 cells at time points earlier than 4 hours (*figure 4.4*). Interestingly at 24 hours, there was an increase in Akt phosphorylation in both cell lines (*figure 4.5)*, with some evidence to suggest a decrease in total Akt protein levels. However, the high levels of cell death observed at the longer time points made equal loading difficult due to low protein availability, so it was difficult to determine whether this was a true decrease.

Although the kinase activity of Akt is fully regulated by dual phosphorylation at the 2 specified sites, it was important to confirm that the decrease in phosphorylation observed in the MDA MB468 cells closely correlated with a decrease in kinase activity. There was a good correlation between kinase activity (shown in *figure 4.6)* and phosphorylation status in the MDA MB468 cells, which exhibited a decrease in kinase activity of approximately

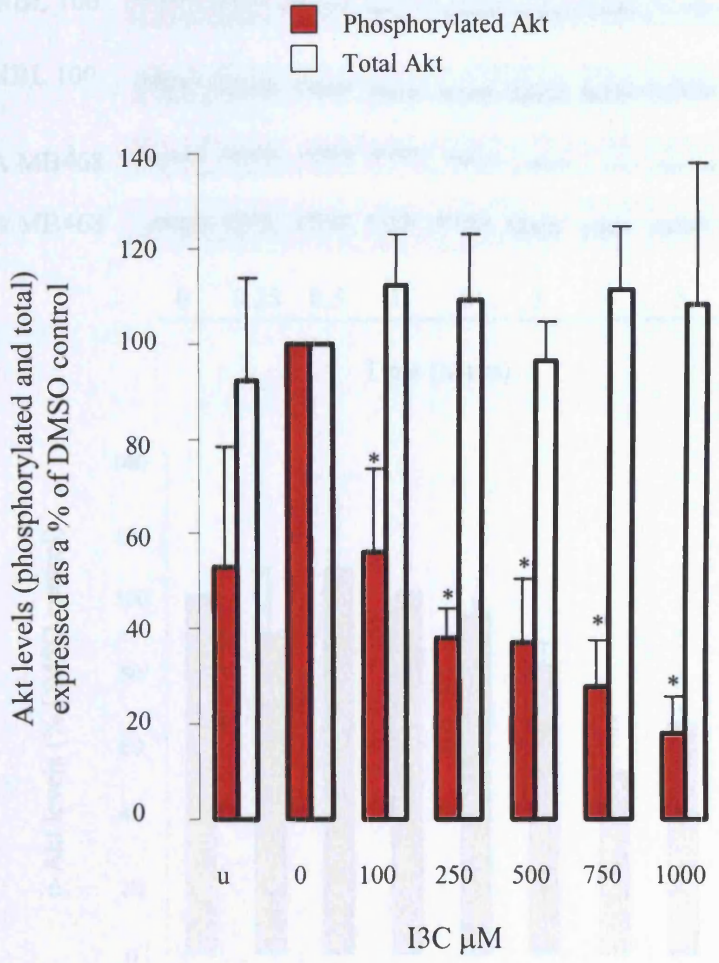


Figure 4.3 Total Akt (1,2,3) and p-Akt (1,2,3 ser473) levels expressed as a percentage of the DMSO control following a 5 hour I3C treatment in MDA MB468 cells. *Levels were determined by densitometric analysis. * denotes a significant difference from DMSO controls (n=3, ± SEM, p≤0.05) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

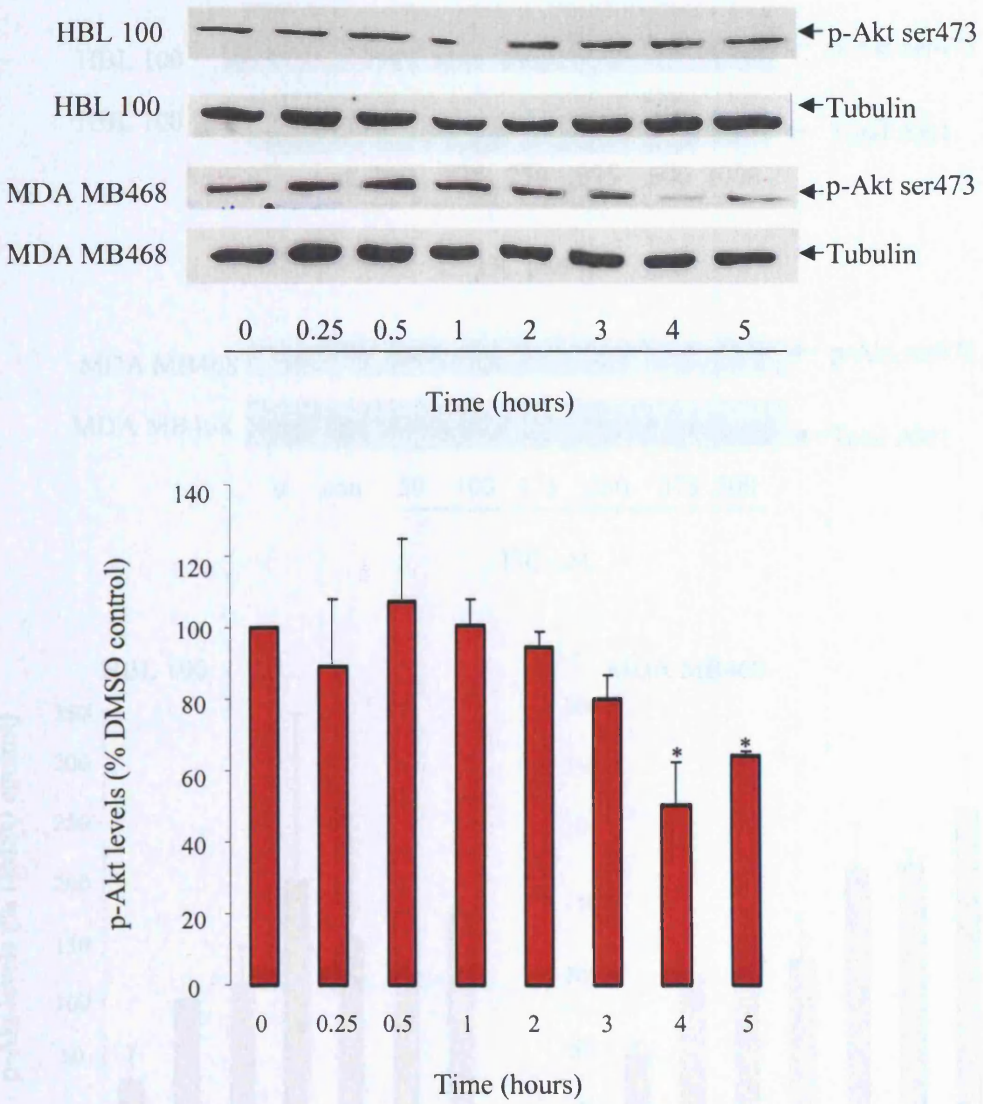


Figure 4.4 The effect of I3C on Akt phosphorylation pre- 5 hours. *Representative blots show p-Akt levels following an I3C treatment over 5 hours in HBL 100 (500μM) and MDA MB468 (100μM) cells. Blots were stripped and reprobed with a tubulin antibody as a loading control. Chart represents p-Akt levels in MDA MB468 cells following a 100μM I3C time course determined by densitometric analysis. * denotes a significant difference from DMSO controls (n=3, ± SEM, p≤0.05) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

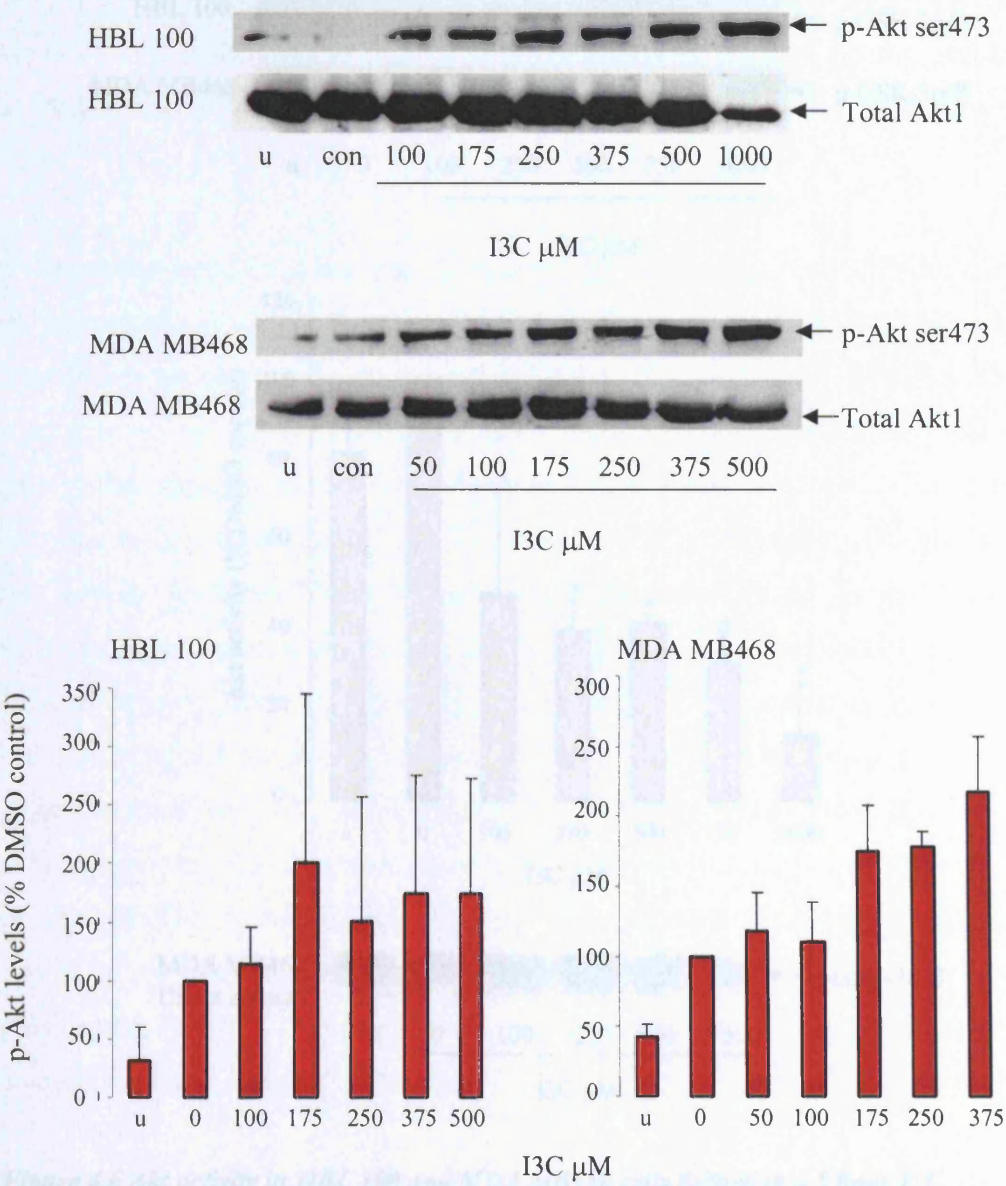


Figure 4.5 Effect of a 24 hour I3C treatment on Akt phosphorylation in the HBL 100 and MDA MB468 cell line. *Representative blots show p-Akt and total Akt1 levels in HBL 100 and MDA MB468 cells following a 24 hour treatment with increasing doses of I3C. Blots were performed on 3 separate occasions.*
Chart shows p-Akt levels in the HBL 100 and MDA MB468 cell line. Values were determined by densitometric analysis (n=3, ± SEM).

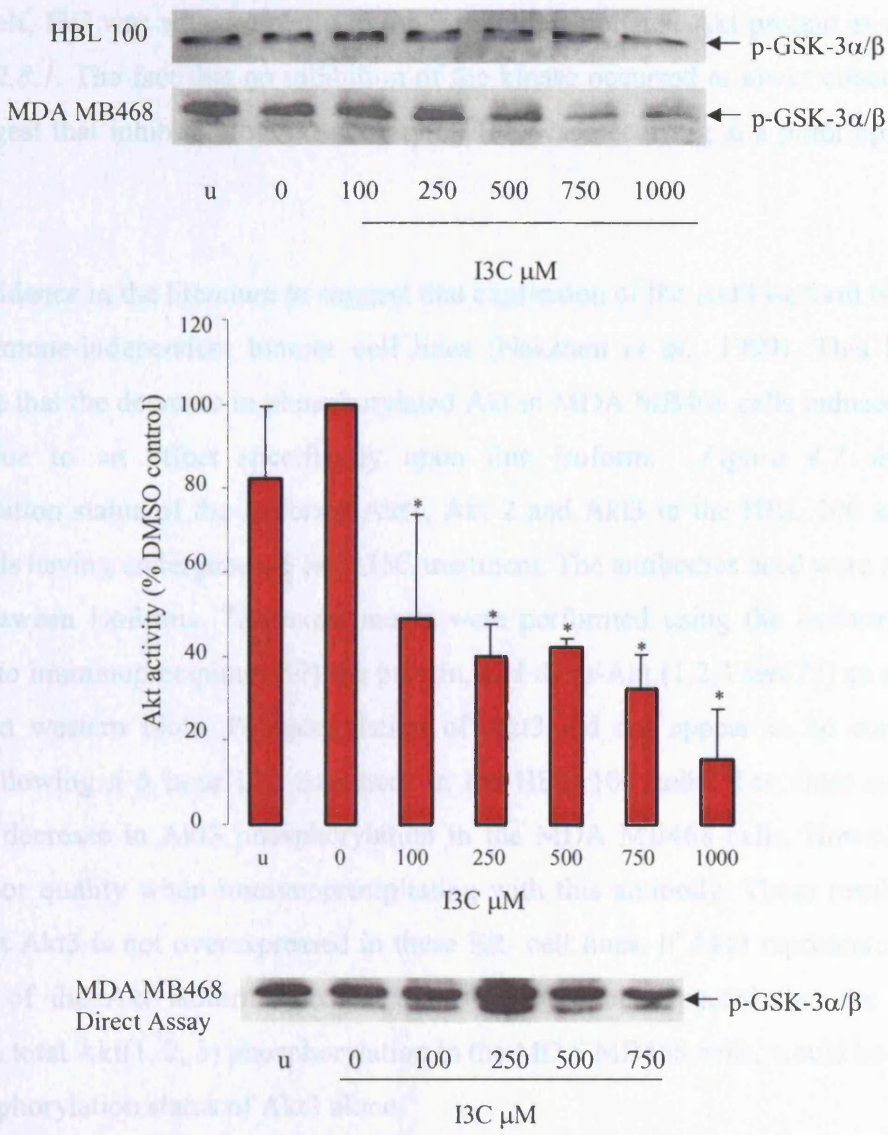


Figure 4.6 Akt activity in HBL 100 and MDA MB468 cells following a 5 hour I3C treatment. *Representative blots showing Akt kinase activity determined by phosphorylation of a GSK-3α/β substrate in the assay. In the 2 upper blots, cells were pretreated with I3C prior to immunoprecipitation, whereas in the lower 'direct assay' blot, I3C was added into the assay 30 minutes prior to substrate addition. The chart depicts Akt kinase activity in MDA MB468 cells following a 5 hour treatment with increasing doses of I3C determined by densitometric analysis.* denotes a significant difference from DMSO controls (n=3, ±SEM, p≤0.05) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

55% and phosphorylation of approximately 45% following a 100μM, 5 hour I3C treatment. To investigate the possibility that I3C could be having a direct effect upon the Akt kinase activity itself, I3C was added directly to the immunoprecipitated Akt protein as described in section *2.8.1*. The fact that no inhibition of the kinase occurred at lower concentrations would suggest that inhibition of Akt activity by I3C was occurring at a point upstream of the kinase.

There is evidence in the literature to suggest that expression of the Akt3 isoform is raised in certain hormone-independent tumour cell lines (Nakatani *et al.*, 1999). This led us to hypothesise that the decrease in phosphorylated Akt in MDA MB468 cells induced by I3C, may be due to an effect specifically upon this isoform. *Figure 4.7* shows the phosphorylation status of the isoforms Akt1, Akt 2 and Akt3 in the HBL 100 and MDA MB468 cells having undergone a 5 hour I3C treatment. The antibodies used were non-cross reactive between isoforms. The experiments were performed using the isoform-specific antibodies to immunoprecipitate (IP) the protein, and the p-Akt (1,2,3 ser473) as a primary antibody on western blots. Phosphorylation of Akt3 did not appear to be consistently affected following a 5 hour I3C treatment in the HBL 100 cells. The chart suggests a significant decrease in Akt3 phosphorylation in the MDA MB468 cells. However, blots were of poor quality when immunoprecipitating with this antibody. These results would suggest that Akt3 is not overexpressed in these ER- cell lines. If Akt3 represented a large proportion of the Akt isoforms present, then it would be expected that the decrease observed in total Akt(1, 2, 3) phosphorylation in the MDA MB468 cells, would be mirrored in the phosphorylation status of Akt3 alone.

It was then proposed that either Akt1 and/or Akt2 must make up a significant proportion of the Akt isoforms present in these cell lines. There was no overall change in phosphorylation of Akt 1 in the HBL 100 cell lines, whilst levels were significantly decreased in the MDA MB468 cells from 500μM in response to I3C. Akt 1 phosphorylation was almost completely inhibited in both cell lines following a 5 hour treatment with 50μM LY294002. In contrast to this however, Akt2 phosphorylation was consistently decreased following a 1mM I3C treatment in the MDA MB468 cell line only.

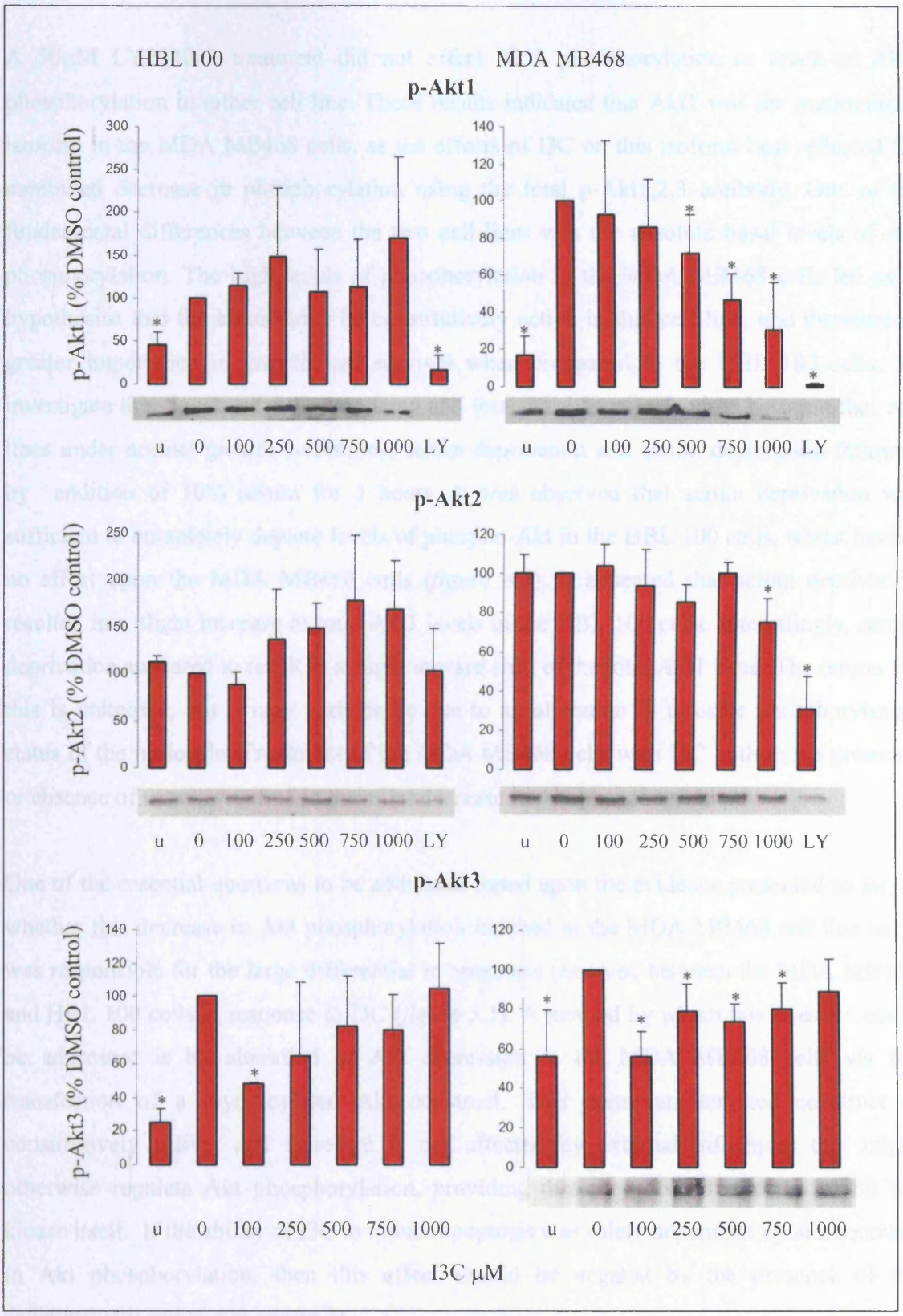


Figure 4.7 Effect of a 5 hour I3C treatment on the three Akt isoforms. *Representative western blots showing p-Akt in the HBL 100 and MDA MB468 cells.* The *charts show total p-Akt (ser473) for the Akt1, 2 and 3 isoforms. Levels are expressed as a percentage of the DMSO control as determined by densitometric analysis. * denotes a significant difference from DMSO controls (n=3, ± SEM, p≤0.05) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test. (n=2 for HBL Akt 3, ±SD. Blots could not be scanned).*

A 50μM LY294002 treatment did not affect Akt2 phosphorylation as much as Akt1 phosphorylation in either cell line. These results indicated that Akt1 was the predominant isoform in the MDA MB468 cells, as the effects of I3C on this isoform best reflected the combined decrease in phosphorylation using the total p-Akt1,2,3 antibody. One of the fundamental differences between the two cell lines was the absolute basal levels of Akt phosphorylation. The high levels of phosphorylation in the MDA MB468 cells led us to hypothesise that the kinase may be constitutively active in this cell line, and therefore of greater importance in growth and survival when compared to the HBL 100 cells. To investigate this, levels of phosphorylated and total Akt1 were compared between the cell lines under normal growth conditions, serum deprivation and serum deprivation followed by addition of 10% serum for 5 hours. It was observed that serum deprivation was sufficient to completely deplete levels of phospho-Akt in the HBL 100 cells, whilst having no effect upon the MDA MB468 cells (*figure 4.8*). It appeared that serum deprivation resulted in a slight increase in total Akt1 levels in the HBL 100 cells. Interestingly, serum deprivation appeared to result in a slight upward shift of the total Akt1 band. The reason for this is unknown, but it may perhaps be due to an alteration in tyrosine phosphorylation status of the molecule. Treatment of the MDA MB468 cells with I3C both in the presence or absence of serum, resulted in a similar decrease in Akt phosphorylation.

One of the essential questions to be addressed based upon the evidence presented so far, is whether this decrease in Akt phosphorylation induced in the MDA MB468 cell line only, was responsible for the large differential in apoptosis observed between the MDA MB468 and HBL 100 cells in response to I3C (*figure 3.5*). A method by which this question could be addressed is by alteration of Akt expression in the MDA MB468 cells via the transfection of a myristoylated Akt construct. This membrane-targeted construct is constitutively active, and therefore is not affected by external influences that might otherwise regulate Akt phosphorylation, providing that they do not directly inhibit the kinase itself. If the ability of I3C to induce apoptosis was solely dependent upon a decrease in Akt phosphorylation, then this effect should be negated by the presence of the constitutively active Akt construct.

In an attempt to address this, the MDA MB468 cells were transfected with one of two constructs: a HA-tagged myristylated Akt1 expression construct (HA-Akt1), or the

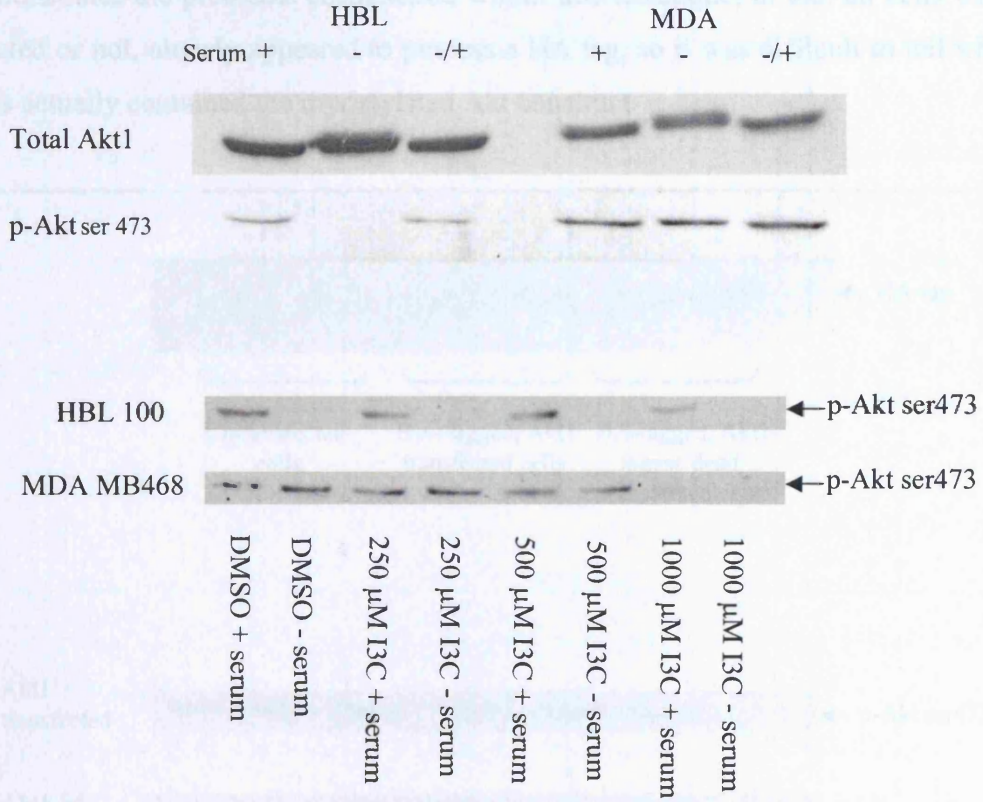


Figure 4.8 The effects of serum deprivation on Akt phosphorylation in the HBL 100 and MDA MB468 cell lines. *The top panel shows representative western blots for phosphorylated and total Akt1 levels in the HBL 100 and MDA MB468 cells following (+) normal growth conditions, (-) 24 hour serum deprivation and (-/+) 20 hours of serum deprivation followed by addition of 10% serum for 5 hours.*
The lower panel shows representative western blots depicting phosphorylated Akt levels in HBL 100 and MDA MB468 cells in response to I3C treatment in the presence of 10% serum (+ serum), or following overnight serum deprivation (- serum). Blots were performed on 3 separate occasions.

equivalent 'kinase dead' construct possessing a mutation at the ser473 position (HA-Akt1kd). These constructs were co-transfected with a pvgRXR construct, which conferred resistance against the potent antibiotic zeocin, in order to select transfected cells. *Figure 4.9* demonstrates the problems encountered within this technique, in that all cells whether transfected or not, already appeared to possess a HA tag, so it was difficult to tell whether the cells actually contained the myristylated Akt construct.

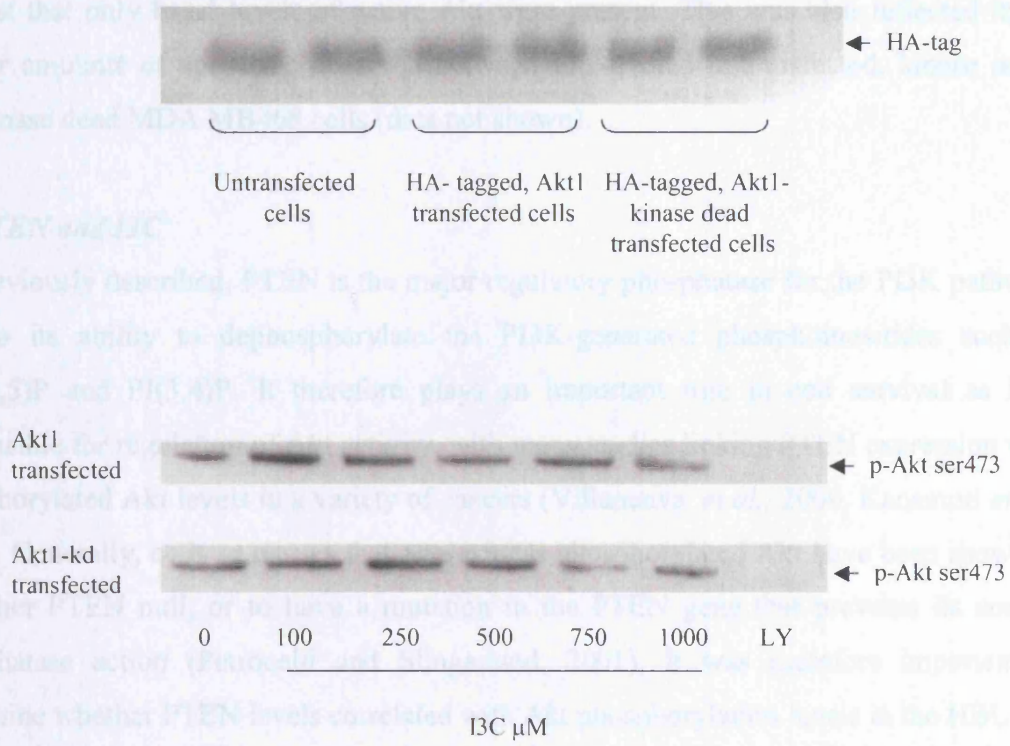


Figure 4.9 p-Akt levels in myristoylated Akt1-transfected cells.
Top panel shows a representative western blot for the MDA MB468 cells, in which non-transfected cells, HA-Akt1 and HA-Akt1-kd transfected cells were probed for the presence of an HA-tag (n=3).
The lower panel shows representative western blots for the MDA MB468 cells in which HA-Akt1 and HA-Akt1-kd transfected cells were probed for p-Akt, following a 5 hour I3C treatment, or 5 hour 50μM LY294002 treatment (n=2).

It may have been that these cells did not transfect efficiently, but a small number were able to become zeocin resistant, so continued to grow in zeocin-treated medium. If this was the case, then the HA-tag band in *figure 4.9* may represent a non-specific band of the predicted molecular weight which would have been discounted in the presence of a strong HA-tag signal. The lower panel in *figure 4.9* would further suggest that the Akt construct had not been efficiently transfected into the cells, as there was little difference between the effects of I3C upon Akt phosphorylation in the kinase active and kinase dead transfected cells. That Akt activity was completely abolished on treatment with LY294002 would also suggest that only basal levels of active Akt were present. This was also reflected in the similar amounts of apoptosis observed between I3C-treated untransfected, kinase active and kinase dead MDA MB468 cells (data not shown).

4.2 PTEN and I3C

As previously described, PTEN is the major regulatory phosphatase for the PI3K pathway, due to its ability to dephosphorylate the PI3K-generated phosphoinositides such as PI(3,4,5)P and PI(3,4)P. It therefore plays an important role in cell survival as it is responsible for regulation of Akt activity, with many studies linking PTEN expression with phosphorylated Akt levels in a variety of cancers (Villaneuva. *et al.,* 2000, Kanamori *et al.,* 2001). Generally, cells or tissues that overexpress phosphorylated Akt have been shown to be either PTEN null, or to have a mutation in the PTEN gene that prevents its normal phosphatase action (Petrocelli and Slingerland, 2001). It was therefore important to determine whether PTEN levels correlated with Akt phosphorylation levels in the HBL 100 and MDA MB468 cells, and to investigate whether I3C was able to inhibit Akt phosphorylation in the MDA MB468 cell line, through an ability to induce PTEN protein levels. Li *et al.,* (1997) have previously reported the MDA MB468 cells to possess a 44 base pair deletion in the PTEN gene, and predicted that it should undergo a frameshift mutation, suggesting that I3C may have no effect on PTEN in this cell line.

Figure 4.10 shows basal levels of PTEN in 4 cell lines under normal growth conditions. The T47D cells expressed the highest level of PTEN, with the HBL 100 and MCF7 cells expressing approximately 35% and 50% respectively, of T47D levels. PTEN was not detectable by western blot in the MDA MB468 cell line.

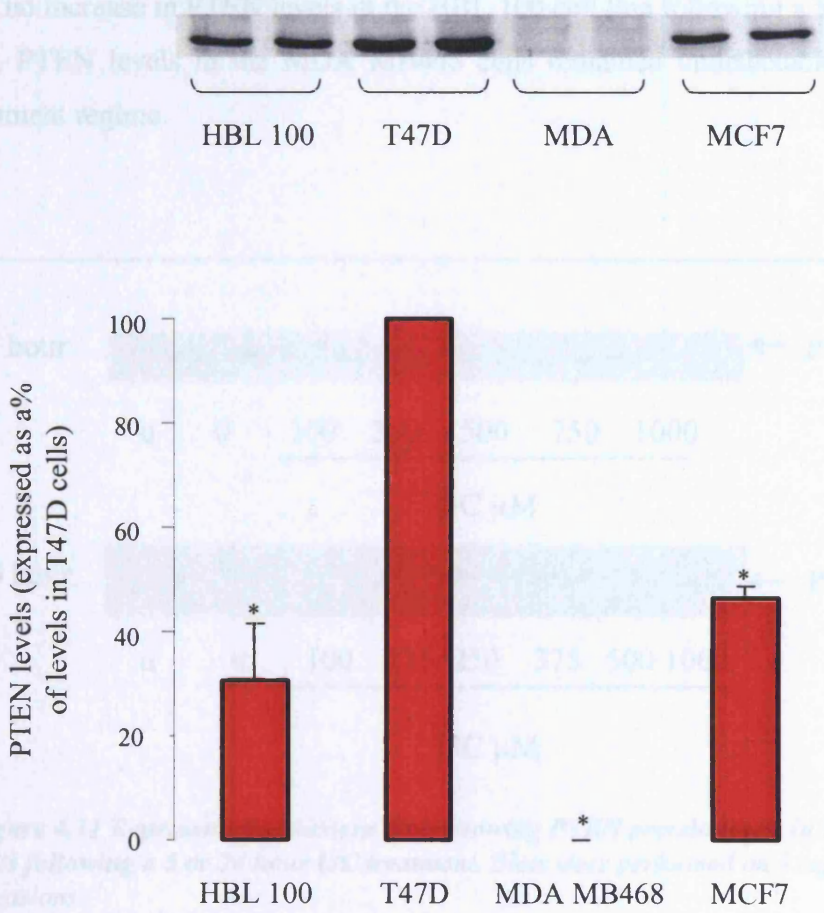


Figure 4.10 PTEN levels in a panel of breast cell lines.
The top panel shows a representative western blot for basal levels of the tumour suppressor PTEN in 4 breast cell lines (duplicates represent different samples).
*The chart shows relative levels as compared to T47D cells determined by densitometric analysis. * denotes a significant difference from PTEN levels in the T47D cells (n=4, ± SEM, $p \leq 0.05$) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

Meng *et al.*, (2000) have shown that I3C was able to induce PTEN levels over times up to 48 hours in the T47D breast cells. The effect of I3C upon PTEN levels in the HBL 100 and MDA MB468 cells over 24 hours was therefore investigated. *Figure 4.11* demonstrates that there was no increase in PTEN levels in the HBL 100 cell line following a 5 or 24 hour I3C treatment. PTEN levels in the MDA MB468 cells remained undetectable following the same treatment regime.

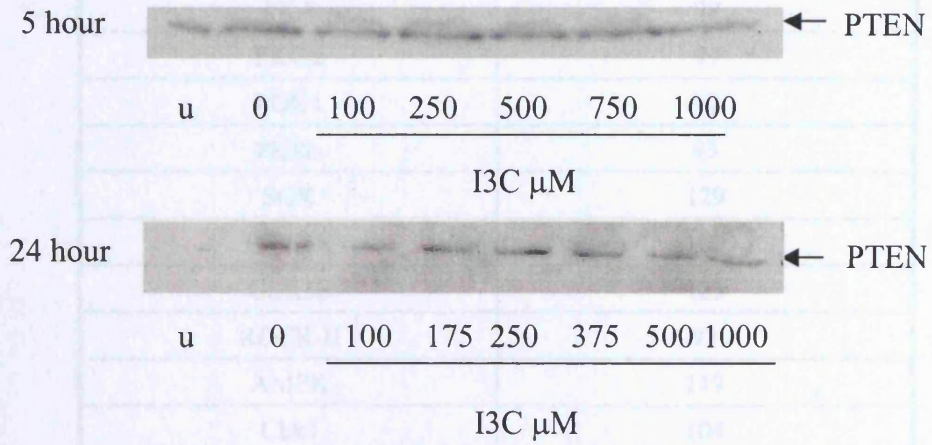


Figure 4.11 Representative western blots showing PTEN protein levels in HBL 100 cells following a 5 or 24 hour I3C treatment. *Blots were performed on 3 separate occasions.*

4.3 The effects of I3C on PI3 kinase activity

Data so far have demonstrated that I3C decreased Akt kinase activity and levels of phosphorylation in the MDA MB468 cells, suggesting that this effect is elicited upstream of the Akt kinase at a point somewhere between the level of receptor phosphorylation and Akt phosphorylation. One likely candidate for a target is the PI3 kinase, which can be regulated by direct phosphorylation, binding of the kinase to a phosphorylated receptor or inhibition of the enzyme itself.

Table 4.1 shows the results from an *in vitro* kinase screen (kindly provided by Prof. Cohen *et al.*, MRC Signalling Unit. Dundee) where 50μM I3C was added directly to the purified

Kinase	% activity remaining following I3C treatment
PI3K	**28**
MKK1	111
MAPK2/ERK2	100
JNK1	98
SAPK2a/p38	104
MAPKAP-K1b	117
MAPKAP-K2	133
MSK1	110
PRAK	87
PKA	98
PKCα	99
PDK1	99
PKBα	93
SGK	129
P70S6K	100
GSK3β	123
ROCK-II	93
AMPK	119
Chk1	104
CK2	104
PHK	104
Lck	115
CDK2/cyclinA	120
DYRK1A	81
SAPK2b/p38β2	105

Table 4.1 Table showing the effects of 50 μM I3C upon a variety of purified kinases. *Results are presented as kinase activity remaining, as a percentage of that in control incubations. ATP was present at 0.1mM in all assays with the exception of PI3-K (ATP 50μM). Kinase screen data was kindly provided by Prof. Sir Phil Cohen, MRC Signalling Unit., Dundee.*

Abbreviations: PI3K phosphatidylinositol-3-kinase; MKK1 MAPK kinase; MAPK2 mitogen activated protein kinase2; JNK1 c-jun N-terminal kinase; SAPK stress-activated protein kinase; MAPKAP-K MAPKinase activating protein kinase; MSK1 mitogen- and stress-activated protein kinase-1; PRAK=MAPKAPK5; PKA protein kinase A; PKC protein kinase C; PDK1 3-phosphoinositide-dependent kinase 1; PKB protein kinase B; SGK serum/glucocorticoid regulated kinase; GSK-3 glycogen synthase kinase-3; ROCK-II Rho-associated, coiled-coil containing protein kinase-II; AMPK AMP-activated protein kinase; Chk1 checkpoint homologue 1; CK2 casein kinase 2; PHK phosphorylase kinase; Lck lymphocyte-specific protein tyrosine kinase; CDK2 cyclin-dependent kinase 2; DYRK1A dual specificity tyrosine phosphorylation-regulated kinase 1A. (Methodology in Davies *et al.*, 2000).

kinases. PI3K was the only kinase in the screen that was significantly inhibited by I3C. These results were confirmed by using PI3K immunoprecipitated directly from the HBL 100 and MDA MB468 cells. *Figure 4.12* showed that both I3C (500μM) and LY294002 (50μM) inhibited PI3K activity when added directly into the kinase assay, using phospho-tyrosine immunoprecipitated protein from HBL 100 and MDA MB468 cells.

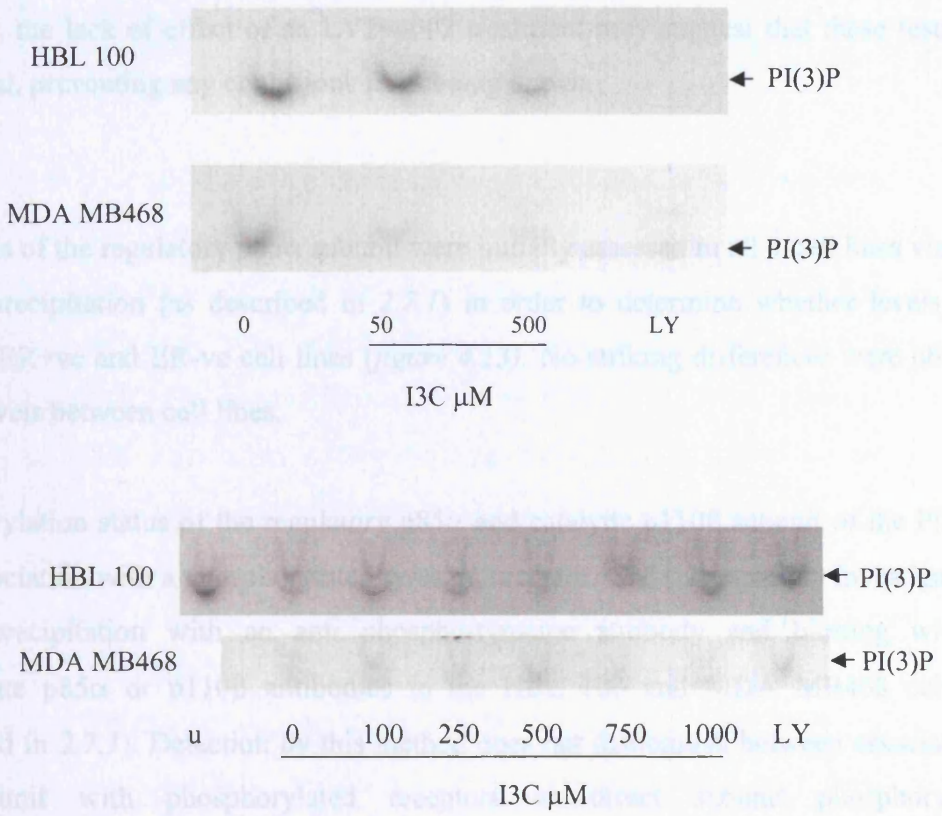


Figure 4.12 PI3 Kinase assay for HBL 100 and MDA MB468 cells.
Top panel shows representative PI3K assays for HBL 100 and MDA MB468 cells, in which protein was immunoprecipitated using an anti-phosphotyrosine antibody, and the compounds added directly into the kinase assay for 30 minutes prior to the addition of the phosphatidylinositol substrate.
Lower panel shows representative PI3K assay for HBL 100 and MDA MB468 cells following cell treatment with I3C or 50μM LY294002 for 5 hours prior to immunoprecipitation of tyrosine phosphorylated proteins (n=3).

The HBL 100 cells showed higher activity than the MDA MB468 cells, which may partly explain the difference in sensitivity to I3C between the two cell lines, with a 50μM I3C treatment inhibiting PI3K in the MDA MB468 cells only. We next wished to determine whether I3C was able to inhibit PI3K activity in cells in culture. *Figure 4.12* shows that when cells were treated with I3C or LY294002 for 5 hours prior to immunoprecipitation, no effect was observed in either cell line. One explanation for this could be that the very low micromolar concentrations of I3C that would be in the cell, may not be sufficient to overcome the competing or displacement effects of the relatively high ATP concentration for the PI3K ATP-binding site, as it would be when added directly to the kinase assay. However, the lack of effect of an LY294002 treatment may suggest that these results are artefactual, preventing any conlusions from being drawn.

The levels of the regulatory p85α subunit were initially assessed in all 4 cell lines via a p85 immunoprecipitation (as described in *2.7.1*) in order to determine whether levels differ between ER+ve and ER-ve cell lines (*figure 4.13).* No striking differences were observed in p85 levels between cell lines.

Phosphorylation status of the regulatory p85α and catalytic p110β subunit of the PI3K, or their association with a phosphorylated tyrosine receptor, was subsequently investigated by immunoprecipitation with an anti phospho-tyrosine antibody and blotting with the appropriate p85α or p110β antibodies in the HBL 100 and MDA MB468 cell lines (described in *2.7.1*). Detection by this method does not distinguish between association of the subunit with phosphorylated receptors or direct subunit phosphorylation. Phosphorylation status of p85α was found to be unaffected in the HBL 100 cells, but showed an increase at 1mM in the MDA MB468 cells, with a slight increase in p110β phosphorylation following a 5 hour I3C treatment in both cell lines (*figure 4.14*). This may suggest that I3C was not eliciting its inhibitory effects on Akt phosphorylation by preventing the activation of PI3K, as the blots illustrated no inhibition of binding of the subunits to a phosphorylated receptor or phosphorylation of the PI3K Ia subunits.

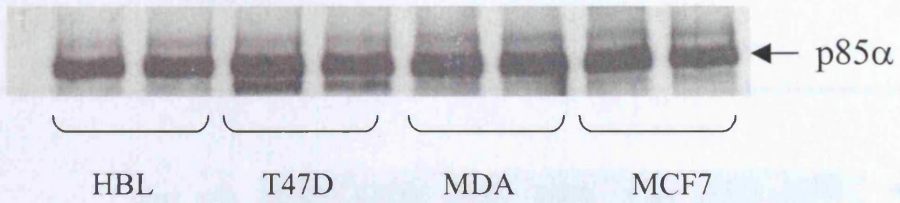


Figure 4.13. Representative western blot showing total p85α basal levels in all 4 cell lines. *Protein was immunoprecipitated using the p85α antibody, and blotted using the same antibody. Duplicates represent different samples. Blots were performed on 2 separate occasions.*

4.4 The effect of LY294002 on Akt phosphorylation and induction of apoptosis

In order to confirm that inhibition of phosphoinositide generation was able to inhibit Akt phosphorylation in these cell lines, the PI3K-specific inhibitor LY294002 was employed. LY294002 acts as a direct competitive inhibitor of PI3K by blocking the ATP-binding site of the p110 subunit at a concentration of 50μM (Vlahos *et al.,* 1994). *Figure 4.15* shows inhibition of PI3K activity by LY294002, represented by complete inhibition of Akt phosphorylation in both cell lines following a 50μM treatment for 1 hour. Induction of apoptosis following PARP cleavage after a 24 hour LY294002 treatment in the MDA MB468 cells only, is shown in *figure 4.16.* However, it should be noted that specificity of LY294002 for PI3K may vary between cell lines, as it has recently been shown within this laboratory that LY294002 can also inhibit ERK phosphorylation in these cell lines (Fox and Howells, Unpublished). Although widely used primarily as a PI3K inhibitor, LY294002 is also able to inhibit phosphorylation of a variety of other proteins in an *in vitro* kinase assay (Davies *et al.,* 2000).

From *figure 4.16*, it can be seen that an LY294002 treatment was sufficient to cause apoptosis in the MDA MB468 cells. LY294002 was then employed at increasing concentrations over 5 hours in order to determine a concentration which gave a similar degree of Akt inhibition as 500μM I3C (which caused 55% apoptosis at 24 hours). *Figure 4.17* demonstrates that 10μM LY294002 resulted in an inhibition of Akt phosphorylation of approximately 55%, similar to that caused by 500μM I3C. Treatment of MDA MB468

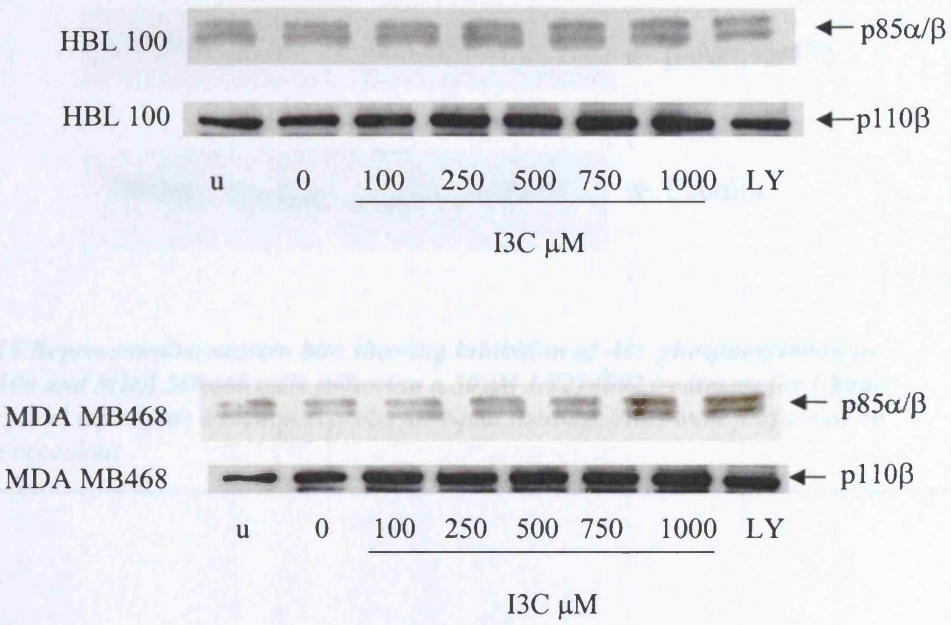


Figure 4.14 Representative western blots showing phosphorylated p85α and p110β levels in HBL 100 and MDA MB468 cells following a 5 hour treatment with I3C or 50μM LY294002. *Lysates were immunoprecipitated with an anti-phospho-tyrosine antibody and probed for p85α or p110β. Blots were performed on 3 separate occasions.*

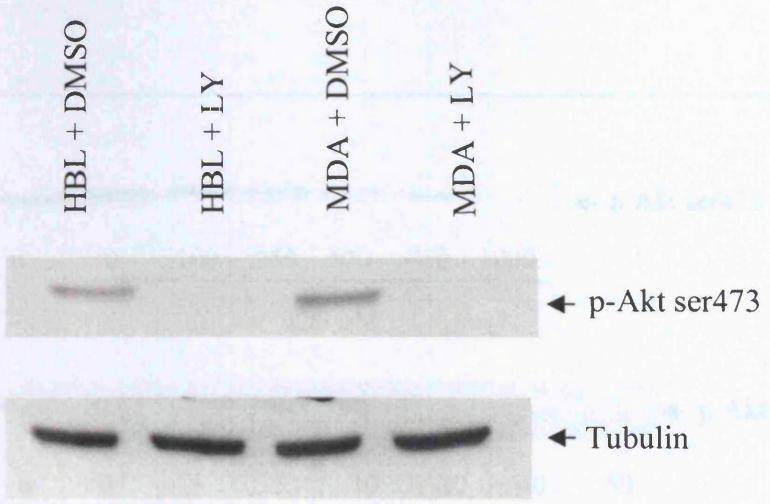


Figure 4.15 Representative western blot showing inhibition of Akt phosphorylation in the HBL 100 and MDA MB468 cells following a 50μM LY294002 treatment for 1 hour. *The lower panel represents a tubulin reprobe for equal loading. Blots were performed on 3 separate occasions*

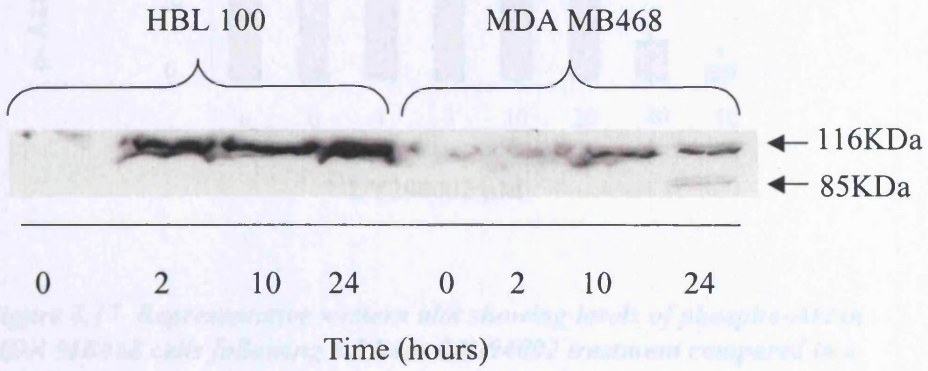


Figure 4.16 Representative western blot showing PARP cleavage following a 24 hour LY294002 treatment (50μM). *Samples were prepared from all attached and floating cells, sonicated, and the whole sample loaded. Blots were performed on 3 separate occasions.*

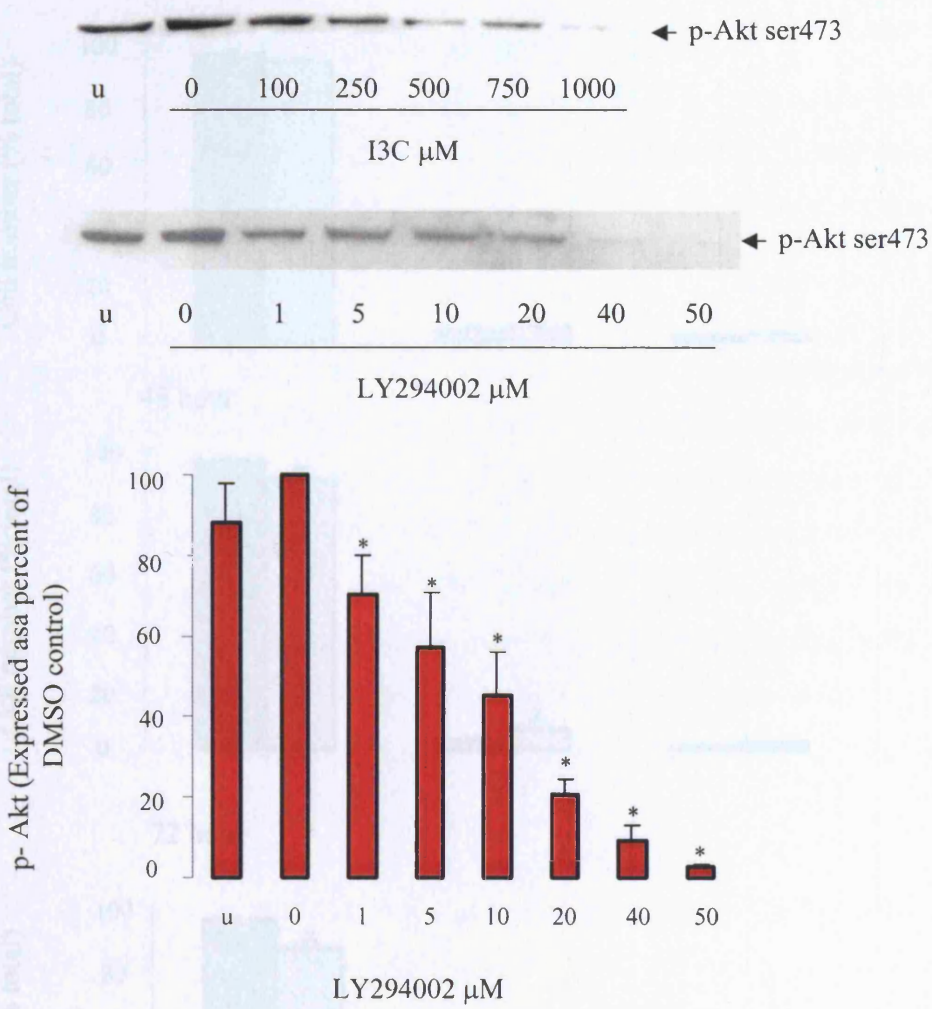


Figure 4.17 Representative western blot showing levels of phospho-Akt in MDA MB468 cells following a 5 hour LY294002 treatment compared to a 5hr I3C treatment.

*Chart shows levels of Akt phosphorylation following a 5 hour LY294002 treatment, expressed as a percentage of the DMSO control as determined by densitometric analysis. * denotes a significant difference from DMSO control (n=3, ±SEM, $p \leq 0.05$) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

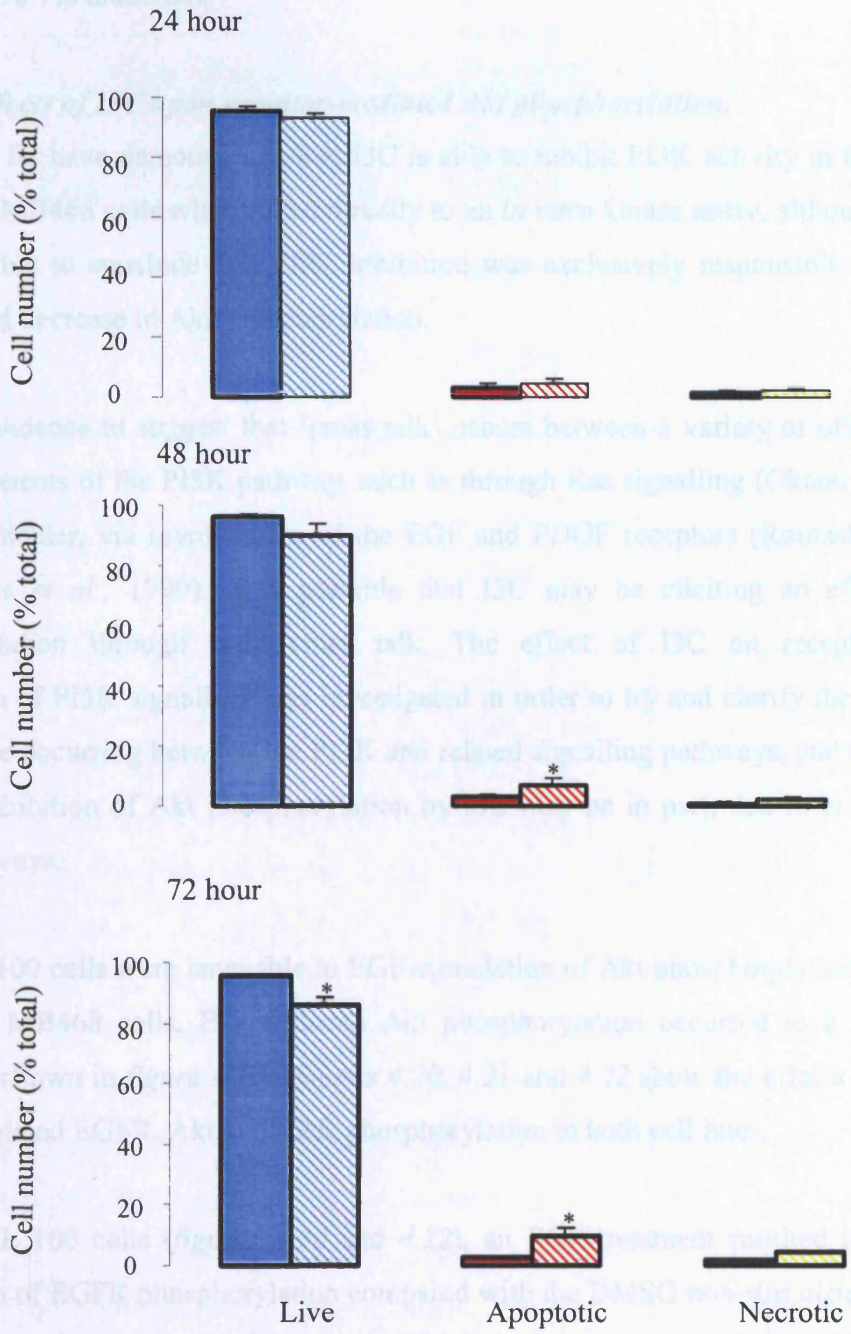


Figure 4.18 Induction of apoptosis as determined by annexin V and PI staining in the MDA MB468 cell line following a 10μM treatment with LY294002 over 72 hours. *Solid bars represent the percentage of cells present in the DMSO control. Hatched bars represent the percentage of cells present in the 10μM LY294002 treatment. * denotes a significant difference from DMSO controls (n=4, ± SEM, p≤0.05) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test*

cells with this concentration of LY294002 resulted in only a 4% induction of apoptosis at 24 hours (*figure 4.18*), which did not reach significance until 48 hours of treatment, resulting in a 7% induction.

4.5 The effects of I3C upon receptor-mediated Akt phosphorylation.

Results so far have demonstrated that I3C is able to inhibit PI3K activity in the HBL 100 and MDA MB468 cells when added directly to an *in vitro* kinase assay, although it has not been possible to conclude that PI3K inhibition was exclusively responsible for the I3C-precipitated decrease in Akt phosphorylation.

There is evidence to suggest that 'cross talk' occurs between a variety of other pathways and components of the PI3K pathway, such as through Ras signalling (Okano *et al.*, 2000) and in particular, via involvement of the EGF and PDGF receptors (Romashkova *et al.*, 1999, Ozes *et al.,* 1999). It is possible that I3C may be eliciting an effect on Akt phosphorylation through such cross talk. The effect of I3C on receptor-mediated stimulation of PI3K signalling was investigated in order to try and clarify the interactions that may be occurring between the PI3K and related signalling pathways, and to determine whether inhibition of Akt phosphorylation by I3C may be in part, due to an effect upon these pathways.

The HBL 100 cells were amenable to EGF-stimulation of Akt phosphorylation, whereas in the MDA MB468 cells, EGF-induced Akt phosphorylation occurred to a much lesser degree, as shown in *figure 4.19. Figures 4.20, 4.21* and *4.22* show the effects of I3C upon EGF-stimulated EGFR, Akt and ERK phosphorylation in both cell lines.

In the HBL 100 cells (*figures 4.20* and *4.22*), an EGF treatment resulted in significant stimulation of EGFR phosphorylation compared with the DMSO non-stimulated control. A 5 hour I3C pre-treatment did not prevent EGF-induced stimulation of the EGFR, although there was a decrease in EGFR phosphorylation when pre-treated with 50μM LY294002. Despite the lack of effect of an I3C pre-treatment on EGFR phosphorylation, there was a significant decrease in EGF-stimulated ERK phosphorylation from 250μM I3C and following an LY294002 treatment. Interestingly, whilst I3C could not prevent EGF-stimulated Akt phosphorylation in the HBL 100 cells, the 50μM LY294002 treatment

almost completely prevented Akt phosphorylation, showing levels similar to that of basal phosphorylation.

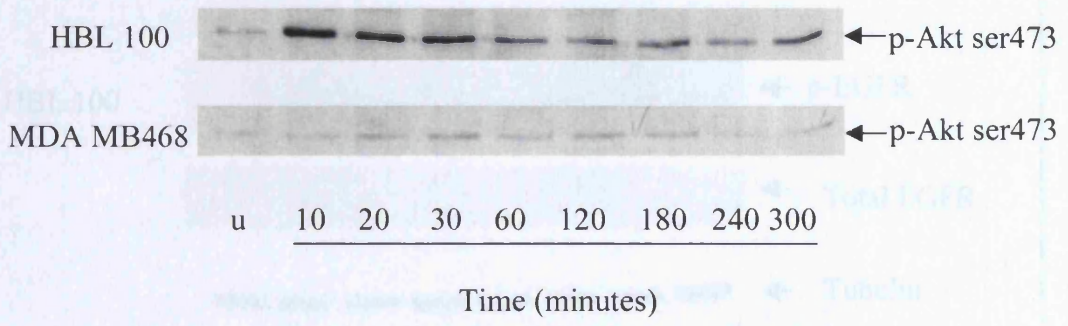


Figure 4.19 Representative western blots showing a time course over 5 hours for EGF-stimulated phospho-Akt levels in HBL 100 and MDA MB468 cells. *Cells were serum starved overnight, prior to stimulation with 15nM EGF. Blots were performed on 3 separate occasions. u = untreated.*

The MDA MB468 cells expressed approximately 10 times greater EGFR levels than the HBL 100 cells (Squires *et al.* 2003). Following a 5 hour I3C pre-treatment with subsequent EGF stimulation, there was no significant effect upon EGFR phosphorylation in the MDA MB468 cells (*figures 4.21* and *4.22*), which was reflected in the lack of effect upon ERK phosphorylation. However, I3C and LY294002 treatments appeared to cause a dose-dependent inhibition of induction of Akt phosphorylation from basal levels in this cell line at concentrations of 100μM and 50μM respectively. This would suggest that the effect of I3C on Akt phosphorylation was not mediated through the EGFR, perhaps suggesting a difference between cell lines in signalling and cross talk.

4.6 The effects of I3C on two prostate cell lines.

The effect of I3C on apoptosis and the PI3K pathway in the MDA MB468 cells provides exciting evidence for I3C as a potential inhibitor of Akt phosphorylation and inducer of apoptosis in phospho-Akt-overexpressing, PTEN-negative cells. To explore whether these effects were specific to the MDA MB468 cells, or whether the mechanism applies to other cells overexpressing phosphorylated Akt, further investigations were conducted using two prostate cell lines that possessed different levels of Akt phosphorylation.

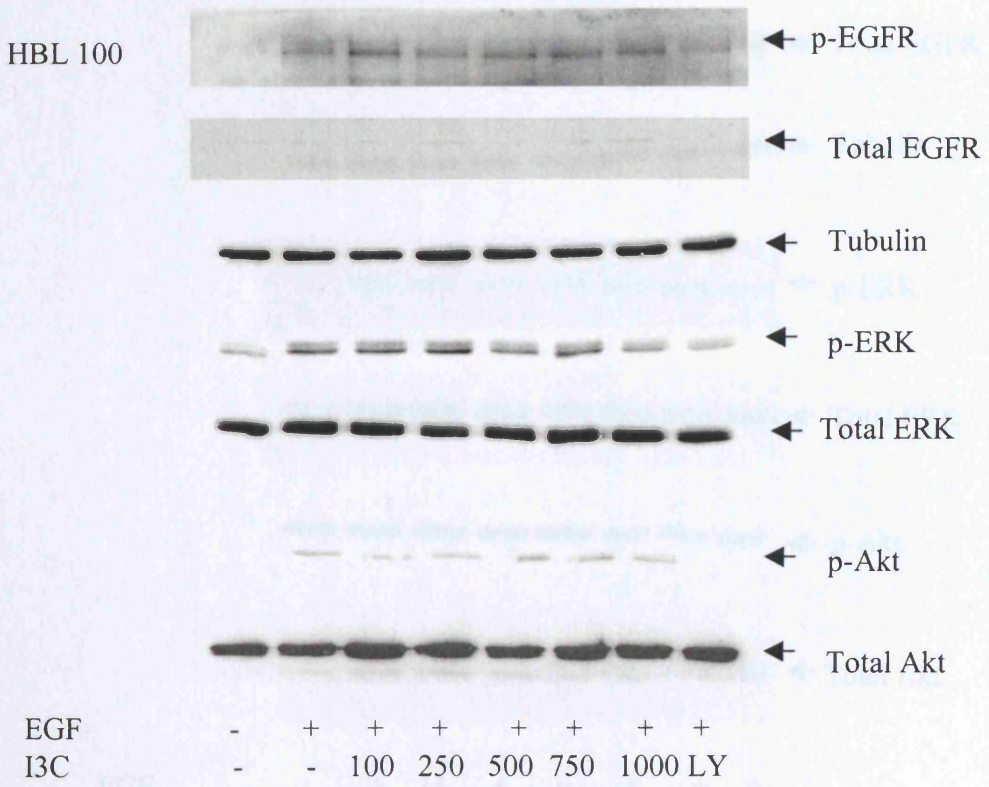


Figure 4.20 Representative western blots showing the effects of a 4.5 hour I3C pre-treatment followed by a 30 minute EGF stimulation on EGFR-related signalling molecules in the HBL 100 cells. *The vehicle for EGF was acetic acid and for I3C, DMSO, which were present in all treatments to the same concentration. LY=50 μM. Blots were performed on 3 separate occasions (with the exception of Akt blots n=2).*

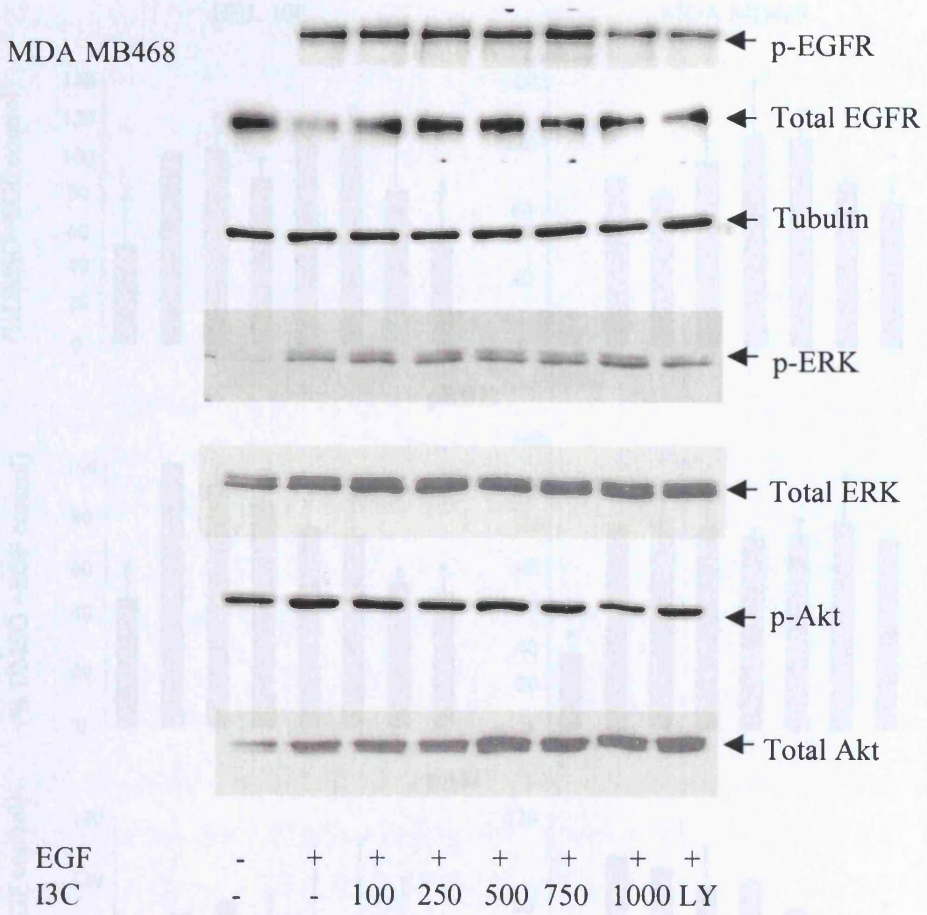


Figure 4.21 Representative western blots showing the effects of a 4.5 hour I3C pre-treatment followed by a 30 minute EGF stimulation on EGFR-related signalling molecules in the MDA MB468 cells. *The vehicle for EGF was acetic acid and for I3C, DMSO, which were present in all treatments to the same concentration. LY=50μM. Blots were performed on 3 separate occasions (with the exception of Akt blots n=2).*

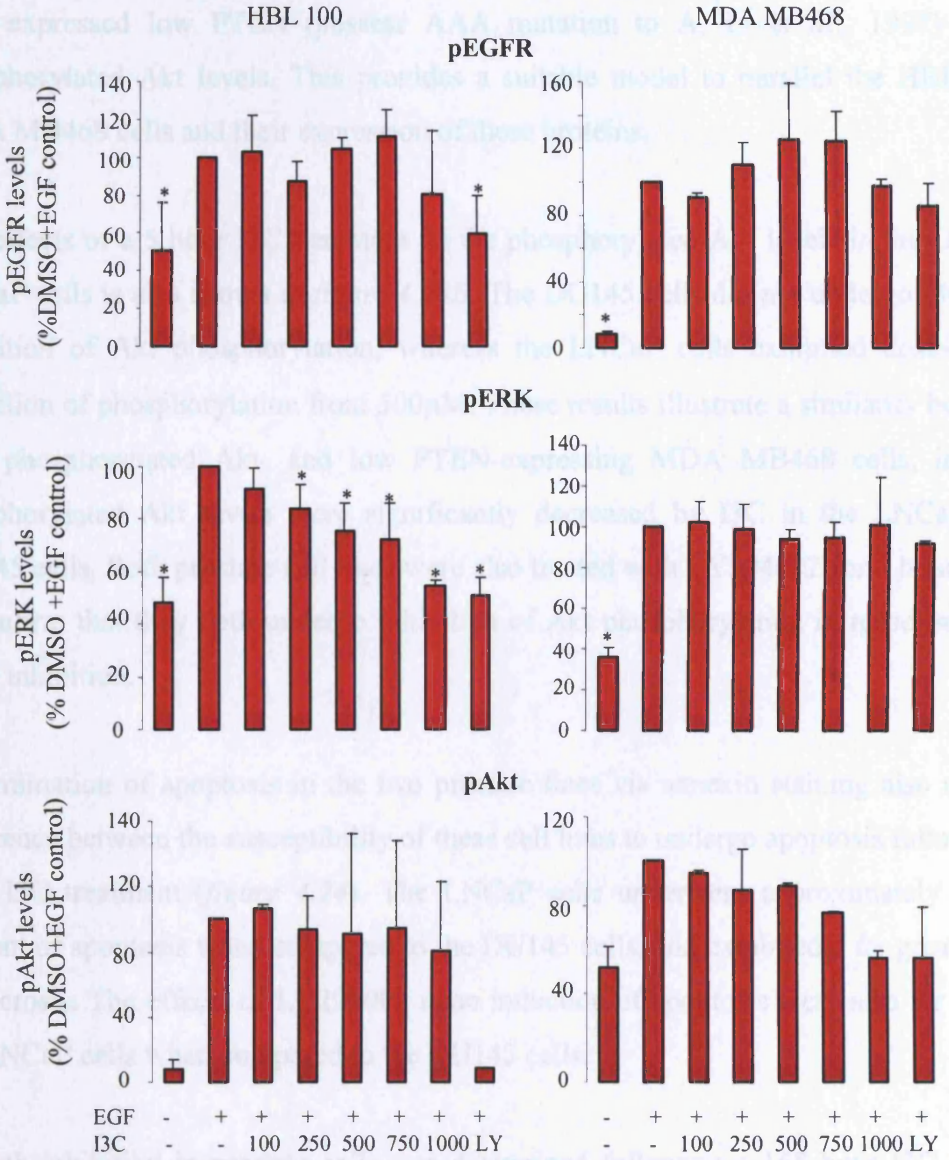


Figure 4.22 The effects of a 4.5 hour I3C pre-treatment followed by a 30 minute EGF stimulation on EGFR-related signalling molecules in the HBL 100 and MDA MB468 cells. *The vehicle for EGF was acetic acid and for I3C, DMSO, which were present in all treatments to the same concentration. * denotes a significant difference from DMSO + EGF controls (n=3, ± SEM, p≤0.05) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test (apart from Akt blots n=2).*

Figure 4.23A addresses the basal levels of PTEN and phosphorylated Akt in the LNCaP and DU145 prostate tumour cells compared to the HBL 100 and MDA MB468 breast cells. DU145 cells expressed high PTEN and low phosphorylated Akt levels, whereas the LNCaP cells expressed low PTEN (possess AAA mutation to A, Li *et al.*, 1997) and high phosphorylated Akt levels. This provides a suitable model to parallel the HBL 100 and MDA MB468 cells and their expression of these proteins.

The effects of a 5 hour I3C treatment on the phosphorylated Akt levels in the DU145 and LNCaP cells is also shown in *figure 4.23B.* The DU145 cells did not undergo I3C-induced inhibition of Akt phosphorylation, whereas the LNCaP cells exhibited dose-dependent inhibition of phosphorylation from 500μM. These results illustrate a similarity between the high phosphorylated Akt- and low PTEN-expressing MDA MB468 cells, in that the phosphorylated Akt levels were significantly decreased by I3C in the LNCaP but not DU145 cells. Both prostate cell lines were also treated with LY294002 for 5 hours in order to confirm that they both undergo inhibition of Akt phosphorylation in response to direct PI3K inhibition.

Determination of apoptosis in the two prostate lines via annexin staining also revealed a difference between the susceptibility of these cell lines to undergo apoptosis following a 24 hour I3C treatment (*figure 4.24*). The LNCaP cells underwent approximately twice the amount of apoptosis when compared to the DU145 cells, and exhibited a far greater degree of necrosis. The effects of LY294002 upon induction of apoptosis were also far greater in the LNCaP cells when compared to the DU145 cells.

Growth inhibition in prostate cells was determined following a 168 hour I3C treatment. The approximate IC_{50} at 168 hours for the LNCaP cells was 22μM, and for the DU145 cells 48μM (data not shown).

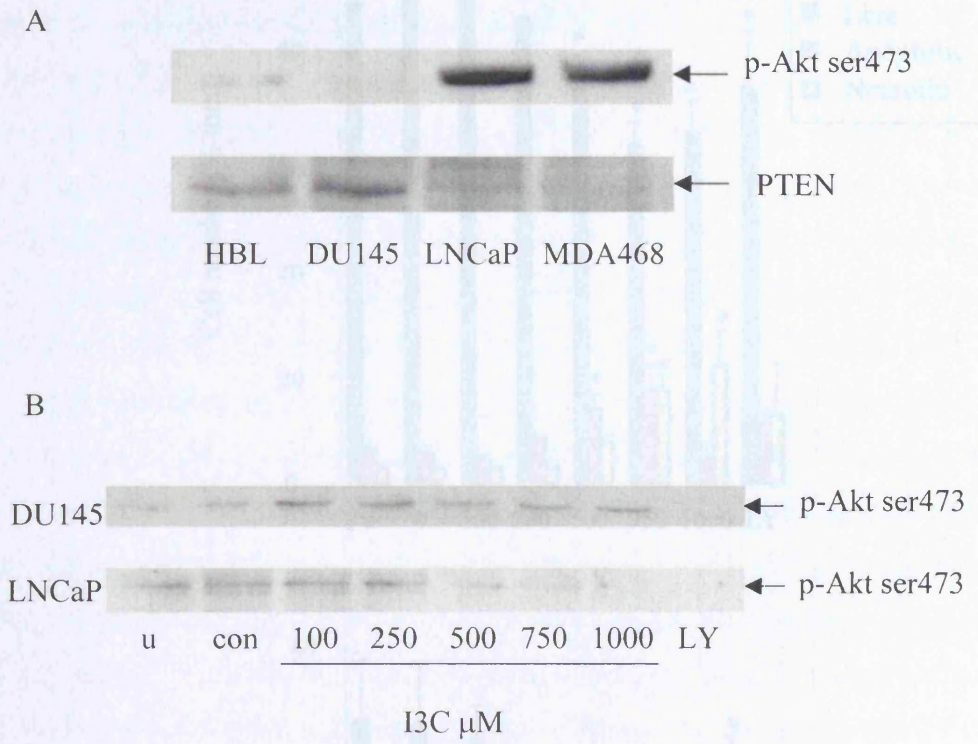


Figure 4.23 Basal PTEN levels and the effects of a 5 hour I3C treatment in 2 prostate cell lines.
Panel A - representative western blots showing basal levels of phosphorylated Akt and PTEN in the HBL 100, DU145, LNCaP and MDA MB468 cell lines. Blots were performed on 3 separate occasions Panel B shows representative western blots for phosphorylated Akt levels in DU145 and LNCaP cells following an I3C treatment for 5 hours. Blots were performed on 3 separate occasions.

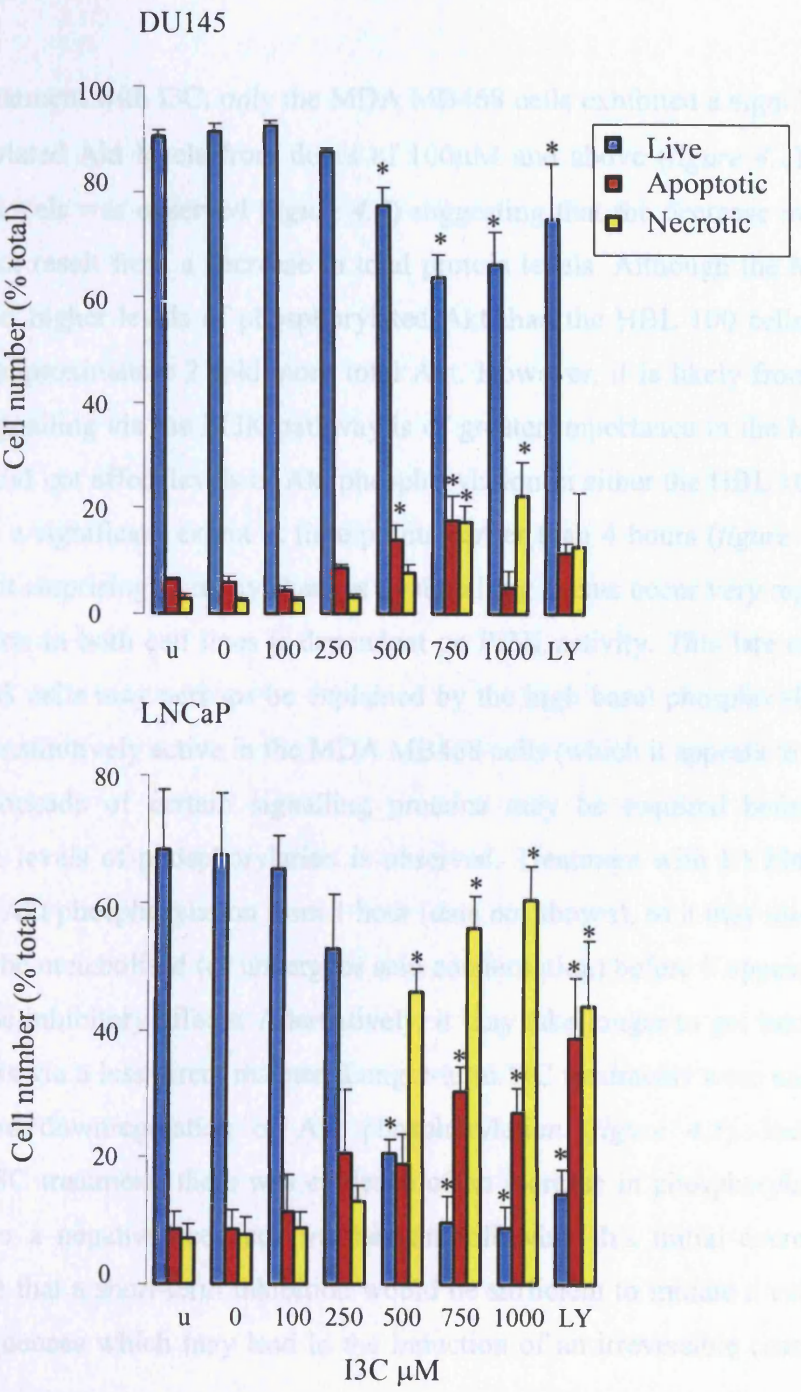


Figure 4.24 Assessment of apoptosis by annexin V and PI staining in DU145 and LNCaP cells following a 24 hour I3C treatment. ** denotes a significant difference from DMSO control ($p \leq 0.05$, $n=3$, $\pm$ SEM) using a Balanced ANOVA followed by Fisher's least significant difference post hoc test. u= untreated cells, 0 = DMSO control.*

DISCUSSION

Following a 5 hour treatment with I3C, only the MDA MB468 cells exhibited a significant decrease in phosphorylated Akt levels from doses of 100μM and above (*figure 4.2*). No effect upon total Akt levels was observed (*figure 4.3*) suggesting that the decrease in Akt phosphorylation did not result from a decrease in total protein levels. Although the MDA MB468 cells possessed higher levels of phosphorylated Akt than the HBL 100 cells, the latter cells possessed approximately 2 fold more total Akt. However, it is likely from the data presented, that signalling via the PI3K pathway is of greater importance in the MDA MB468 cell line. I3C did not affect levels of Akt phosphorylation in either the HBL 100 or MDA MB468 cells to a significant extent at time points earlier than 4 hours (*figure 4.4*), which is to some extent surprising as many changes to signalling events occur very rapidly and Akt phosphorylation in both cell lines is dependent on PI3K activity. This late effect upon the MDA MB468 cells may perhaps be explained by the high basal phosphorylated Akt levels. If Akt is constitutively active in the MDA MB468 cells (which it appears to be), then a continuous blockade of certain signalling proteins may be required before a significant decrease in levels of phosphorylation is observed. Treatment with LY294002 results in inhibition of Akt phosphorylation from 1 hour (data not shown), so it may also be that I3C is required to be metabolised (or undergoes acid condensation) before it appears in a form that exerts these inhibitory effects. Alternatively, it may take longer to get into the cells, or elicit its effects via a less direct manner. Longer-term I3C treatments were unable to sustain a continued downregulation of Akt phosphorylation (*figure 4.5*). Indeed, following a 24 hour I3C treatment, there was evidence of an increase in phosphorylation levels, possibly due to a negative feedback mechanism following this initial decrease. However, it is possible that a short-term inhibition would be sufficient to initiate a variety of downstream consequences which may lead to the induction of an irreversible cascade, such as apoptosis.

The activity of the Akt kinase was reduced to a similar extent when compared to levels of phosphorylation following an I3C treatment (*figure 4.6*). This is in agreement with reports in the literature (Datta *et al.*, 2000) that inhibition of phosphorylation of either the ser473 or thr308 site is sufficient to inhibit enzyme activity. When I3C was added directly to the

kinase assay, it did not significantly inhibit the activity of the enzyme (measured by the extent of GSK-3 phosphorylation). This lack of direct inhibition is indicative of I3C being an inhibitor of events upstream of Akt phosphorylation. It is unlikely that I3C elicits its effects through mechanisms such as induction of phosphatase inhibitors, as following an okadaic acid treatment (phosphatase 2a inhibitor) in conjunction with I3C, Akt phosphorylation in the MDA MB468 cells remained inhibited to a similar extent as for I3C alone.

So far, inhibition of Akt phosphorylation has been discussed as an amalgamation of all three Akt isoforms. There is increasing evidence in the literature that the different isoforms are involved to varying extents in different cellular processes, and that Akt 3 is overexpressed in cell lines that have lost their estrogen dependence (Nakatani *et al.*, 2000). It was therefore hypothesized that Akt 3 may be overexpressed in the MDA MB468 cells, and that the decrease in phosphorylation induced by I3C may be due to inhibition of this specific isoform. However, whilst there was a decrease in phospho-Akt 3 in both cell lines following an I3C treatment, it was not sufficient to explain the decrease observed in the MDA MB468 cells for phospho-Akt(1,2,3) (*figure 4.7*). Although total Akt3 levels were not directly compared between ER-ve and ER+ve cell lines, these results would suggest that Akt3 was not the predominant isoform in either of these ER-ve cell lines, although due to poor antibody quality, a definite assertion would be difficult. The Akt 1 isoform has a greater role in growth and differentiation than Akt 2 (Datta *et al.,* 1999) and is generally the most abundant isoform within the cell. It was then proposed that this isoform may be responsible for the decreased phosphorylation observed in response to I3C treatment in the MDA MB468 cells. Akt 1 phosphorylation was significantly decreased following a 5 hour I3C treatment in the MDA MB468 cells, although not to the same extent as total phosphorylated Akt (1,2,3), with significance being reached only by 500μM I3C. Akt2 phosphorylation was not significantly decreased in either cell line following an I3C treatment, so cannot account for the discrepancy observed between the decrease in Akt(1,2,3) phosphorylation compared to the decreases seen for Akt3 and Akt 1 phosphorylation alone. This suggests that Akt2 is a minor component of the total Akt levels, however, it is possible that some discrepancy may have resulted from an increased variability seen with the immunoprecipitation techniques required to measure the

phosphorylation status of the isoforms, when compared to direct western blotting for p-Akt(1,2,3).

It has recently been shown that there is a significant inverse relationship between Akt phosphorylation and ER expression, although whether there is a direct causal relationship is as yet unknown (Shi *et al.,* 2003). The MDA MB468 cells exhibited the highest basal levels of Akt phosphorylation in keeping with their origin as an aggressive estrogen-independent breast tumour cell line. Both the MCF7 and T47D cell lines are ER+ve tumour-derived cell lines, representing less aggressive carcinomas than the MDA MB468 cells, and so it was proposed that they may have lower phosphorylated Akt levels. The 'normal derived' HBL 100 cells were an exception in that they possessed similar levels of phosphorylated Akt to the tumour-derived T47D cell line, which may reflect upon the estrogen insensitivity of HBL 100 cells or their SV40 transformation. Interestingly, there is increasing evidence to suggest a role for Akt in estrogen-mediated signalling, as it can be stimulated by estrogen in an estrogen receptor-independent fashion (Tsai *et al.,* 2001). Phosphorylation of ERα by Akt2 has been shown to increase the transcriptional activity of the estrogen receptor (Sun *et al.*, 2001), and this hormone-independent induction of the ER by Akt has been proposed as a mechanism for development of tamoxifen resistance in ER+ breast tumours (Campbell *et al.*, 2001).

It is proposed that the MDA MB468 cells may have a greater reliance upon the PI3K pathway for their survival than the HBL 100 cells, for which further evidence is provided in *figure 4.8*. This suggests constitutive activation of Akt in the MDA MB468 cells, phosphorylation of which remains undepleted in the absence of serum. This constitutive activation will be discussed in greater detail in conjunction with PTEN expression.

In chapter 3 (*figure 3.6)*, it was shown that apoptosis was not significantly induced until an 8 hour, 500μM I3C treatment in the MDA MB468 cells. It is now known that inhibition of Akt phosphorylation is occurring at a lower dose and earlier time point than this, in this cell line. This led to the hypothesis that the I3C-induced decrease in Akt phosphorylation was at least in part responsible for the I3C-induced apoptosis. Indeed, it was shown (*figure 3.4.3)* that inhibition of the PI3K pathway by LY294002 was sufficient to induce apoptosis in this cell line, but had no effect upon the HBL 100 cells even though it inhibited Akt

phosphorylation to a similar degree. It was unlikely, however, that all of the I3C-induced apoptosis was entirely due to inhibition of Akt phosphorylation. Apoptosis levels following an I3C treatment were far greater than those following an LY294002 treatment, despite the greater decrease in Akt phosphorylation caused by LY294002 (even following a 1000μM I3C treatment, phosphorylated Akt levels are not completely depleted in the MDA MB468 cells). However, a complete depletion of phosphorylated Akt was not necessary for induction of apoptosis in the MDA MB468 cells by I3C. It was unfortunate that constitutive activation of Akt using the HA-Akt1 and kinase dead constructs did not yield informative results with regards to the necessity of inhibition of Akt phosphorylation (induced by I3C) for the induction of apoptosis in the MDA MB468 cell line. However, this problem was addressed to a certain extent, using the PI3K inhibitor, LY294002. LY294002 (50μM) treatment of the HBL 100 and MDA MB468 cells (*figure 4.15*) resulted in a complete inhibition of Akt phosphorylation in both cell lines. Maximal apoptosis following a 24 hour treatment with I3C occured at 500μM, a concentration that inhibited Akt phosphorylation by approximately 60%. In order to ascertain whether I3C was inducing apoptosis by mechanisms other than inhibition of Akt phosphorylation, a concentration of LY294002 was determined (10μM) that resulted in similar levels of Akt inhibition as 500μM I3C in the MDA MB468 cell line. Treatment of these cells with 10μM LY294002 for 24 hours, resulted in approximately 18 times less apoptosis than for I3C, although significant apoptosis occurred following a 48 or 72 hour LY294002 treatment.

PTEN levels (*figure 4.10*) in the four breast cell lines were consistent with the levels of Akt phosphorylation. The MDA MB468 cells did not appear to express PTEN, which may explain their constitutive Akt activity, as this regulatory process was not available for reduction of phosphoinositide generation. I3C treatments did not induce PTEN expression in either the MDA MB468 cells or the HBL 100 cells (*figure 4.11*), in contrast to the data by Meng *et al.,* (2000) shown for the T47D cells, although data presented in this thesis would suggest that the MDA MB468 cells are actually PTEN (protein) null and therefore would be uninducible in this respect.

The *in vitro* kinase screen developed by Cohen *et al.,* (Davies *et al.*, 2000) is a useful tool for predicting potential kinases of interest with regards to the inhibitory effects of I3C (*table 4.1*). Interestingly, whilst PI3K itself was inhibited by 72%, other kinases

downstream in this pathway including PDK1, Akt1, GSK-3β and p70S6K were to a large extent unaffected. Direct inhibition of PI3K by I3C using protein precipitated from the HBL 100 and MDA MB468 cells was confirmed using a radioactive kinase assay, detecting D-3 phosphorylated phosphatidylinositol (*figure 4.12*). This provided further evidence that I3C was capable of inhibiting PI3K (immunoprecipitated) directly.

Interestingly, the HBL 100 cells possessed higher PI3 kinase activity than the MDA MB468 cells, which would suggest that were it not for the presence of PTEN, high levels of phosphorylated Akt might also be expected in the HBL 100 cell line. When cells were treated for 5 hours with I3C or LY294002 and the protein immunoprecipitated, no decrease in PI3K activity was observed in either cell line (*figure 4.12*). LY294002 is a well-characterised inhibitor of PI3K (Vlahos *et al.*, 1994, Davies *et al.,* 2000), and from the decreases in Akt phosphorylation observed in both cell lines upon treatment with this compound, PI3K inhibition must be occurring. This would suggest a problem with the assay itself that was preventing observation of the expected PI3K inhibition, and unfortunately means that interaction of I3C with PI3K following whole cell treatment, was difficult to characterise. However, new, more easily quantifiable technologies are now becoming available for determination of PI3K activity which may soon outmode the current radioactive kinase assay.

To examine the effects of I3C on the PI3K in more detail, basal levels of the p85α subunit were determined for each cell line (*figure 4.13*). It has previously been shown that tumour cells exhibit higher p85 levels than do normal surrounding tissues (Gershtein *et al.*, 1999), which might have been responsible for the high phosphorylated Akt levels seen in the MDA MB468 cells (due to enhanced binding of the PI3K to phosphorylated receptors and p110 activation). However, there was little difference in total levels of p85α between all 4 cell lines. Following a 5 hour I3C treatment, tyrosine-phosphorylated proteins were then immunoprecipitated from cell lysates using the antibodies to the p85α and p110β subunits. Theoretically, this should allow determination of levels of these subunits associated with a tyrosine phosphorylated receptor, and therefore be indicative of PI3K activity. Unfortunately, this method does not allow differentiation between the subunits pulled down due to association with a receptor and those subunits which may themselves be phosphorylated on a tyrosine residue. However, there was no decrease in phosphorylation

observed in either cell line, suggesting that I3C treatment does not decrease binding of the subunits to a phosphorylated receptor, or direct phosphorylation of the subunits themselves.

The accumulating evidence for the MDA MB468 cells suggested that Akt was constitutively active in this cell line, and that inhibition of Akt activity by I3C may be one of the direct mechanisms for the induction of apoptosis. During the course of this project, the effect of I3C upon Akt phosphorylation in PC-3 cells has been investigated (Chinni and Sarkar, 2002), with I3C not only able to inhibit Akt phosphorylation, but also EGF-induced Akt activation in this cell line. Further studies within this thesis have examined growth factor-mediated stimulation of Akt, and in particular, EGF-induced signalling through the EGFR.

A 5 hour I3C pre-treatment had little effect on EGF-induced phosphorylation of the EGFR in either cell line (and was therefore unlikely to directly inhibit receptor-ligand interaction), which was also reflected at the level of ERK phosphorylation in the MDA MB468 cells. However, in the HBL 100 cells, I3C significantly inhibited EGF-stimulated ERK phosphorylation from 100μM, and decreased phosphorylation back to basal levels following a 1mM I3C or 50μM LY294002 treatment. This would suggest an effect of I3C that is independent from EGFR-mediated effects on ERK in this cell line.

In the HBL 100 cells, EGF induced Akt phosphorylation by approximately 12 fold. This increase in Akt activity remained unaffected following an I3C pre-treatment, but was prevented by an LY294002 pre-treatment. EGF-induced phosphorylation of the EGFR also appeared to be prevented by an LY294002 pre-treatment, as was ERK phosphorylation, which may suggest that inhibition of PI3K could mediate EGFR signalling in this cell line. However, in the MDA MB468 cells, EGF was only able to stimulate Akt phosphorylation by approximately 2 fold, despite a 12 fold induction in EGFR phosphorylation. It may be that because Akt is constitutively active in this cell line, EGF was unable to greatly increase levels of Akt phosphorylation above basal levels. A 5 hour 1mM I3C pre-treatment was sufficient to prevent EGF stimulation of Akt phosphorylation above basal levels, as was a 50μM LY294002 treatment. These results would suggest, however, that I3C was eliciting its effects on Akt phosphorylation independently from the EGFR in the

MDA MB468 cells, as EGF-induced Akt phosphorylation was prevented by I3C, but EGF-induced EGFR phosphorylation was not.

There appears to be a fundamental difference between the HBL 100 and MDA MB468 cell lines, reflected in their response to I3C. The hypothesis that this response was directly related to the constitutive activation of the signalling molecule Akt as a consequence of uncontrolled phosphoinositide signalling due to the absence of PTEN, was further tested using two prostate cell lines.

The relationship observed between phosphorylated Akt and PTEN levels was confirmed in the DU145 and LNCaP prostate cells, in that high levels of phosphorylated Akt correlated with low PTEN levels and *vice versa* (*figure 4.23*). The LNCaP cells possessed very high levels of phosphorylated Akt and low PTEN compared to the DU145 cells, and it was in the former cell line that I3C was able to inhibit Akt phosphorylation. I3C caused a significant induction of apoptosis in the LNCaP cells (*figure 4.24*), as did the blocking of Akt phosphorylation by LY294002, which was comparable to the effects of I3C and LY294002 in the MDA MB468 cells. As in the case for the MDA MB468 cells, complete inhibition of Akt phosphorylation and induction of apoptosis by LY294002 in the LNCaP cells again did not equate fully with the extent of Akt inhibition and apoptosis induced by I3C.

I3C appears to be a promising agent with respect to inhibition of the PI3K cascade and its ability to cause apoptosis, particularly in cells of an aggressive tumourigenic nature that lack (or express low levels of) the tumour suppressor PTEN. It is, however, clear that I3C can also induce apoptosis independently of PI3K inhibition, which will be investigated to some extent in the following chapters.

CHAPTER 5

EFFECT OF I3C ON DOWNSTREAM TARGETS OF AKT

INTRODUCTION

As previously described, Akt can be thought of as a major effector protein within the PI3K pathway, and is able to interact with and impinge upon many other signalling processes. In this chapter, many of the targets of Akt shown in *figure 1.7* have been further investigated in an attempt to distinguish functional consequences of the I3C-induced inhibition of Akt phosphorylation.

Constitutive activation of NF-κB has so far been observed in many cancers, including Hodgkins lymphoma, melanomas, leukaemia and more recently, in breast cancer (Newton *et al.,* 1999), with several oncoproteins able to activate NF-κB including Her2/neu (Galang *et al.*, 1996). In addition to this, the 100kDa precursor of NF-κB2 (which forms the p52 subunit) is overexpressed in a high percentage of breast cancers (Dejardin *et al.,* 1995), with active p65 found in many breast cancer cell lines and inversely correlated to estrogen receptor expression (Nakshatri *et al.*, 1997). Tumour cells in which NF-κB is constitutively overexpressed are usually highly resistant to conventional anticancer drugs, and inhibition of NF-κB can greatly increase sensitivity to treatment (Karin *et al.,* 2002).

Breast cancer cell lines commonly exhibit raised NF-κB levels, consisting of the constitutively active p50:p65 heterodimer and p50 homodimer, although this expression pattern may not be truly indicative of breast cancers, as the p50:p65 dimer increases proportionately with time in culture in MDA MB231, MCF7 and T47D cells (Cogswell *et al.,* 2000). It appears likely that overexpression or increased activity of NF-κB may suppress apoptosis in breast cancer (Mayo *et al.,* 1997, Cogswell *et al.*, 2000) as its inhibition promotes cell death in breast cancer cell lines (Sovak *et al.*, 1997).

Due to the relationship between NF-κB and the apoptotic process, combined with the established links between NF-κB and PI3K signalling, the NF-κB pathway was focussed upon to ascertain whether inhibition of Akt phosphorylation by I3C may be consequential within NF-κB signalling in the MDA MB468 cell line.

There is recent evidence to suggest a role for Wnt signalling and de-regulated β-catenin expression in breast tumourigenesis although it is likely to play differing roles between

breast and colon carcinomas (Wong *et al.*, 2002). Interference with GSK-3 signalling through chemopreventive agents may provide a novel mechanism by which these agents are able to perturb the carcinogenic process. Akt signalling through other molecules such as the forkhead transcription factors, HSP70, and p70S6K may additionally play a role in determination of cell survival and also warranted further investigation.

RESULTS

5.1 Effects of I3C on NF-κB

5.1.1 Determination of NF-κB status in the breast cell lines

Nuclear levels of the NF-κB components p65, p50 and Rel B were determined in the HBL 100 and MDA MB468 cell lines under normal growth conditions, following serum starvation, and after serum starvation followed by serum stimulation *(figure 5.1).* The differing treatment regimes did not affect basal levels of nuclear p65 or p50 in either cell line, suggesting that the cells possessed active NF-κB in the nucleus without stimulation, although there appeared to be a slight decrease in Rel B levels in both cell lines following serum starvation. Nuclear p65 levels under resting basal conditions were approximately 2 fold higher in the HBL 100 cells when compared to the MDA MB468 cells.

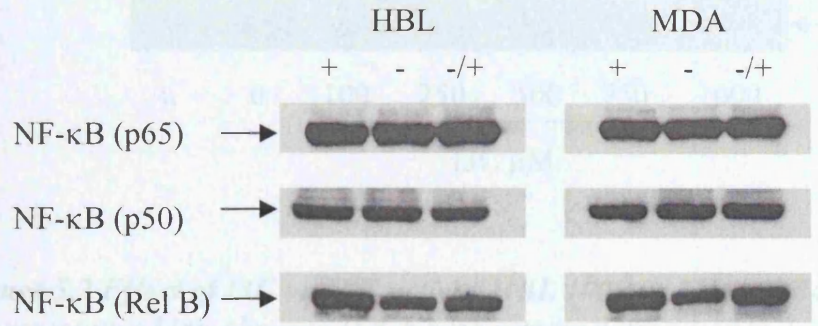


Figure 5.1 Relative levels of NF-κB proteins in HBL 100 and MDA MB468 cells. *Representative western blots of NF-κB protein constituents in HBL 100 and MDA MB468 cells following (+) growth under normal serum conditions, (-) growth in the absence of serum for 24 hours or (+/-) growth in the absence of serum for 19 hours followed by 5 hours in normal 10% serum growth medium (Performed by Mrs. C.E. Houghton). Each blot was performed on at least 3 separate occasions with both the HBL 100 and MDA MB468 samples run on the same gel .*

5.1.2 Effect of I3C on IKK activity and nuclear NF-κB levels.

It has been proposed that Akt phosphorylation is closely linked to NF-κB nuclear translocation due to the ability of Akt to directly phosphorylate and activate IKK (Ozes *et al.,* 1999, Romashkova *et al.,* 1999). *Figure 5.2* shows that I3C did not decrease IKK activity in an *in vitro* kinase assay as determined by IκB phosphorylation, nor did it consistently decrease IKK total protein levels.

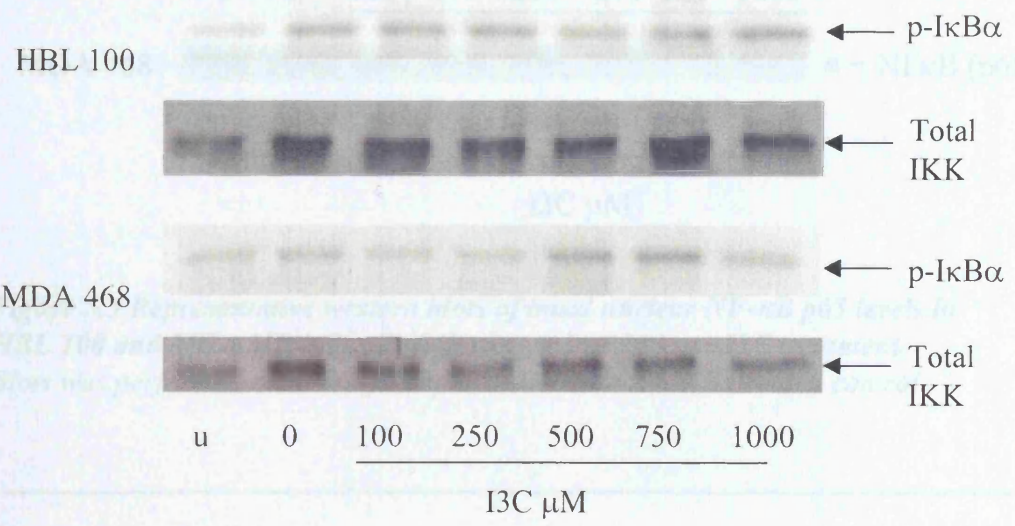


Figure 5.2 Effect of I3C on IKK activity HBL 100 and MDA MB468 cells. *Representative blots showing IKK kinase activity determined by IκBα phosphorylation in vitro (as described in 2.8.2) and total IKK protein levels in HBL 100 and MDA MB468 cells following a 5 hour I3C treatment. Each blot was performed upon at least 3 separate occasions. u = untreated control.*

Evidence for this lack of involvement of IKK was further substantiated upon investigation of nuclear p65 levels following a 5 hour I3C treatment. *Figure 5.3* demonstrates that I3C did not alter p65 nuclear levels following a 5 or 24 hour treatment, suggesting that there was no effect on translocation, in agreement with an inability to inhibit IKK activity.

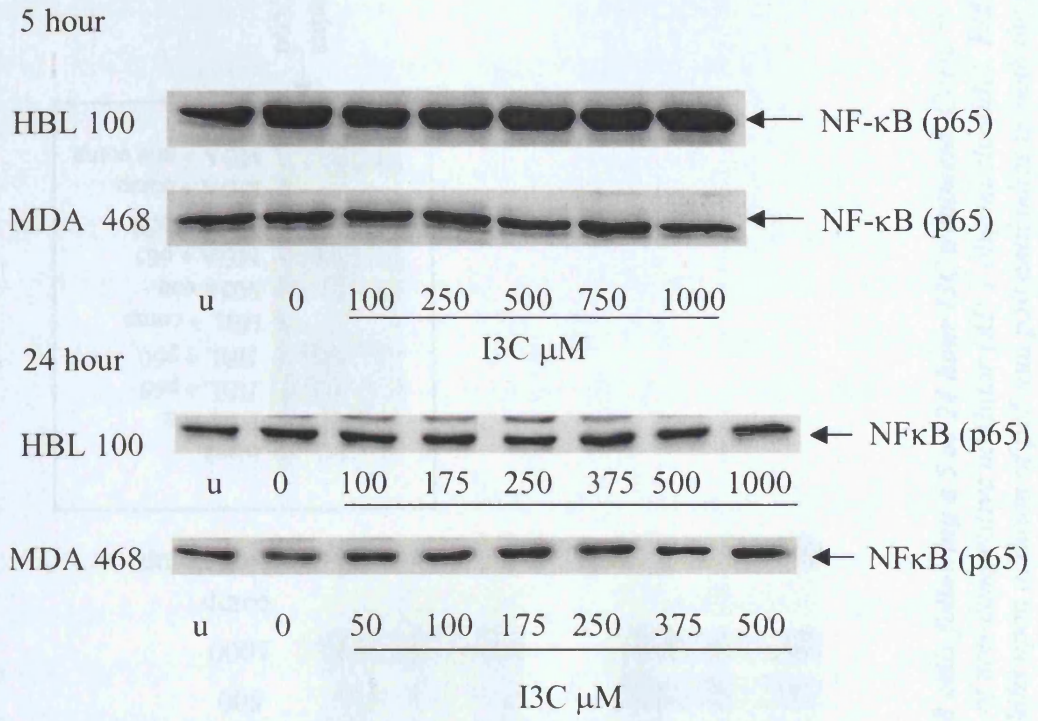


Figure 5.3 Representative western blots of basal nuclear NF-κB p65 levels in HBL 100 and MDA MB468 cells following a 5 or 24 hour I3C treatment.
Blots was performed on at least 3 separate occasions. u = untreated control.

5.1.3 Effect of I3C on NF-κB DNA binding and transcriptional activity

There is evidence to suggest that Akt may affect NF-κB activity via an IKK-independent mechanism, by targeting the TAD1 domain and so enhancing DNA binding independently of nuclear levels (Madrid *et al.*, 2001, 2002). Even though I3C was unable to inhibit p65 nuclear translocation, it was hypothesised that it may exert an effect directly upon the ability of NF-κB to bind to its consensus sequence in promoters of various genes in the nucleus. The effect of I3C upon NF-κB DNA binding was determined by an electrophorectic mobility shift assay following 5 and 24 hour treatment times *(figure 5.4)*. The banding pattern observed appeared specific for these cells, and could be competitively inhibited by the addition of a 100 fold excess of unlabelled NF-κB oligonucleotide. The addition of a 100 fold excess of an unrelated oligonuceotide sequence did not inhibit NF-κB DNA binding.

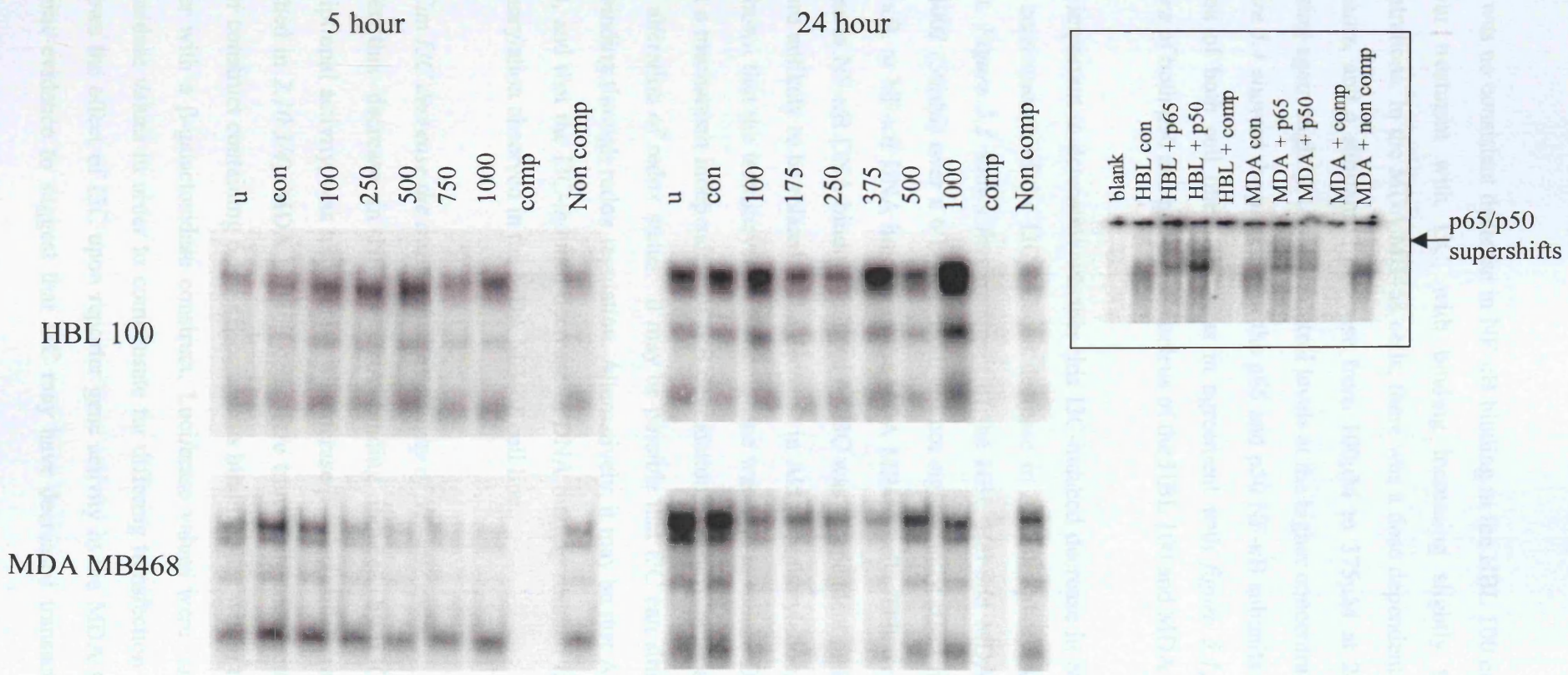


Figure 5.4 EMSA showing NF-κB DNA binding in HBL 100 and MDA MB468 cells following a 5 or 24 hour I3C treatment. *Comp = addition of competitive inhibitor (NF-κB oligonucleotide). Non comp = addition of non-competitive inhibitor (AP-1 oligonucleotide). EMSAs were performed upon at least 3 separate occasions. Inserted panel shows supershifts upon addition of p65 and p50 antibodies in both the HBL 100 and MDA MB468 cells.*

There was no consistent decrease in NF-κB binding in the HBL 100 cells following a 5 or 24 hour treatment with I3C, with binding increasing slightly towards the higher concentrations. In the MDA MB468 cells, there was a dose dependent decrease in binding at 5 hours, and a substantial decrease from 100μM to 375μM at 24 hours with levels increasing again slightly, towards control levels at the higher concentrations. The supershift in *figure 5.4* showed the presence of the p65 and p50 NF-κB subunits in the DNA binding complex of both cell lines. This was in agreement with *figure 5.1*, which showed the presence of both p50 and p65 in the nucleus of the HBL 100 and MDA MB468 cells.

It was important to determine whether this I3C-induced decrease in NF-κB DNA binding was a consequence of the I3C-induced decrease in Akt phosphorylation, or independent from it. *Figure 5.5* shows that treatment of the HBL 100 and MDA MB468 cells with LY294002 (50μM) over a 6 hour period, did not significantly affect nuclear translocation of NF-κB, or NF-κB DNA binding in the MDA MB468 cells. This would suggest that the decrease in NF-κB DNA binding induced by I3C was independent of PI3K inhibition, and therefore unlikely to be related to the decrease in Akt phosphorylation. Heiss *et al.* (2001) have shown that the isothiocyanate Sulforaphane was able to reduce DNA binding of NF-κB via a mechanism independent of IκB degradation, and have proposed that this may be due to alteration of redox status. It may be possible that I3C can similarly affect NF-κB DNA binding through redox regulation. Alternatively, it may be that Akt is downstream of NF-κB, and that the I3C-induced decrease in DNA binding resulted in the decreased Akt phosphorylation observed in the MDA MB468 cell line.

5.1.4 Can I3C decrease the transactivation activity of NF-κB?

Whether this decrease in NF-κB DNA binding induced by I3C would affect its transcriptional activity, was tested using a luciferase/β-galactosidase reporter assay system (described in *2.10.3/4*). MDA MB468 cells were transiently transfected with a luciferase reporter construct containing 6 NF-κB consensus binding sites within the promoter region, together with a β-galactosidase construct. Luciferase values were normalised against β-galactosidase values in order to compensate for differing transfection efficiencies. *Figure 5.6* shows the effect of I3C upon reporter gene activity in the MDA MB468 cells. There was some evidence to suggest that I3C may have decreased transactivation by NF-κB,

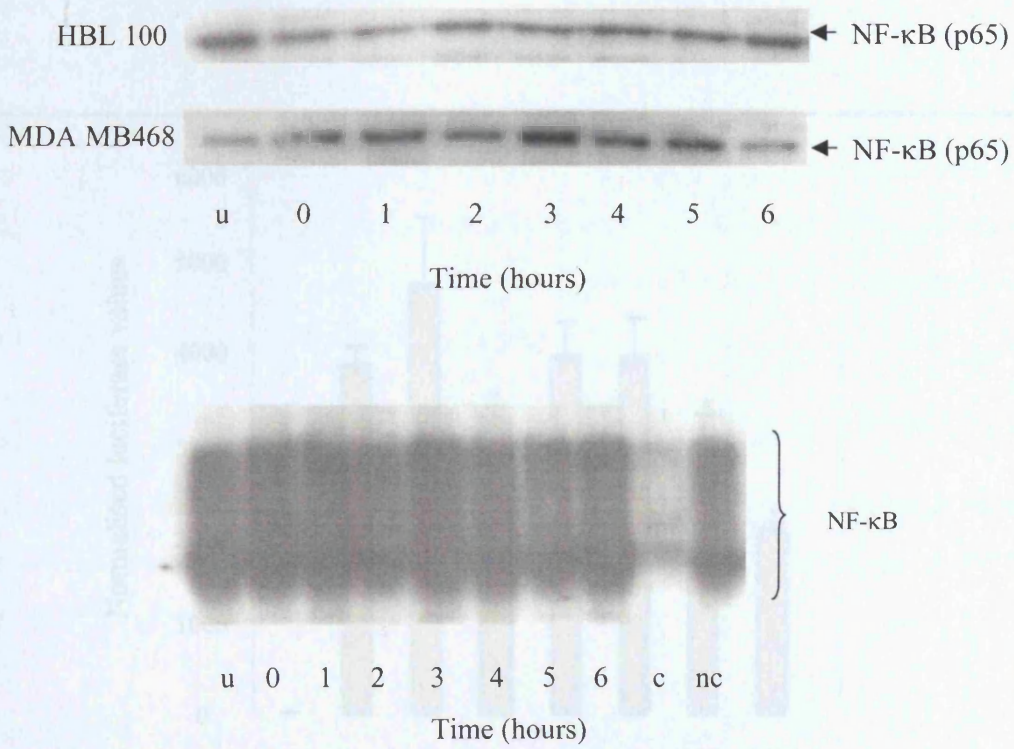


Figure 5.5 Effect of an LY294002 (50μM) time course on NF-κB nuclear levels and DNA binding.
Top panel shows representative western blots for HBL 100 and MDA MB468 cells following a 6 hour treatment with 50 μM LY294002. Blots were probed for nuclear levels of NF-κB p65 and performed on 2 separate occasions.
Lower panel shows NF-κB DNA binding in MDA MB468 cells, following a 6 hour treatment with 50μM LY294002. c = competitor (NF-κB oligonucleotide), nc = non competitor (AP-1 oligonuleotide).

although there was no dose response effect observed below 500μM. This may have been in part, due to difficulties within the methodology.

The method used to transfect the cells, required an overnight incubation with the transfection/DNA mix. The effect of I3C on NF-κB transcriptional activity ideally needed to be explored at a treatment time of 5 hours or earlier to investigate immediate consequences of inhibition of DNA binding in the MDA MB468 cells. Consequently, this could result in I3C-induced changes to transactivation by NF-κB being masked by the high reporter activity already present following an overnight transfection. A 24 hour I3C

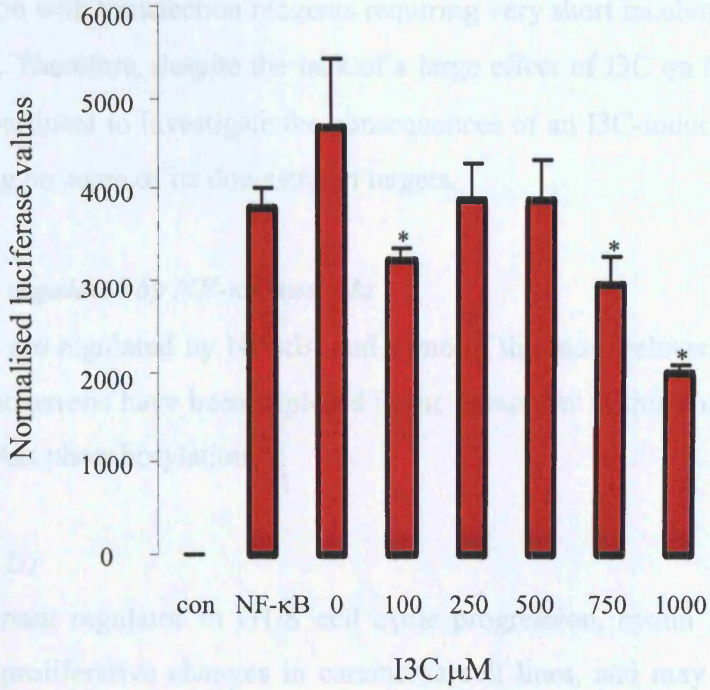


Figure 5.6 Effect of I3C on NF-κB transcriptional activity in the MDA MB468 cell line. *Chart showing the effects of a 5hr I3C treatment upon NFκB reporter activity when transfected into the MDA MB468 cells, con = TK luciferase plasmid without the 6NF-κB insert. All other results represent the reporter activity following an overnight transient transfection with p6NFκB (n=3, ± SEM, p ≤0.05). All luciferase values have been normalised to β-galactosidase to compensate for varying transfection efficiency. * represents a significant difference from the DMSO control as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

treatment was also undertaken in order that the transfection procedure could be carried out within this treatment time rather than prior to it, to determine whether this would result in greater inhibition of reporter activity. However, the combined toxicity of this longer-term I3C treatment, together with toxicity observed from the transfection reagent, resulted in the loss of too many cells for consistent results to be obtained. Similarly, an I3C pre-treatment in conjunction with transfection reagents requiring very short incubation times, also proved overly toxic. Therefore, despite the lack of a large effect of I3C on NF-κB transactivation, it was still pertinent to investigate the consequences of an I3C-induced decrease of NF-κB DNA binding on some of its downstream targets.

5.2 Proteins regulated by NF-κB and Akt

Many genes are regulated by NF-κB, and some of the more relevant ones with respect to breast carcinogenesis have been explored in the remainder of this chapter, as have proteins affected by Akt phosphorylation.

5.2.1 Cyclin D1

As an important regulator in G1/S cell cycle progression, cyclin D1 levels are closely linked with proliferative changes in carcinoma cell lines, and may provide an important marker for breast cancer (Hulit *et al.*, 2002). Cyclin D1 is not only directly regulated by NF-κB, but is also indirectly regulated by Akt via GSK-3/β-catenin signalling through the TCF/Lef transcription factors. Nuclear cyclin D1 levels were investigated following a 5 and 24 hour I3C treatment *(figures 5.7* and *5.8).* There was no change in cyclin D1 levels in either cell line following a 5 hour I3C treatment. Following a 24 hour treatment there was a significant decrease in cyclin D1 levels of approximately 50% from 100μM I3C in the HBL 100 cells only, with almost complete depletion observed at 500μM.

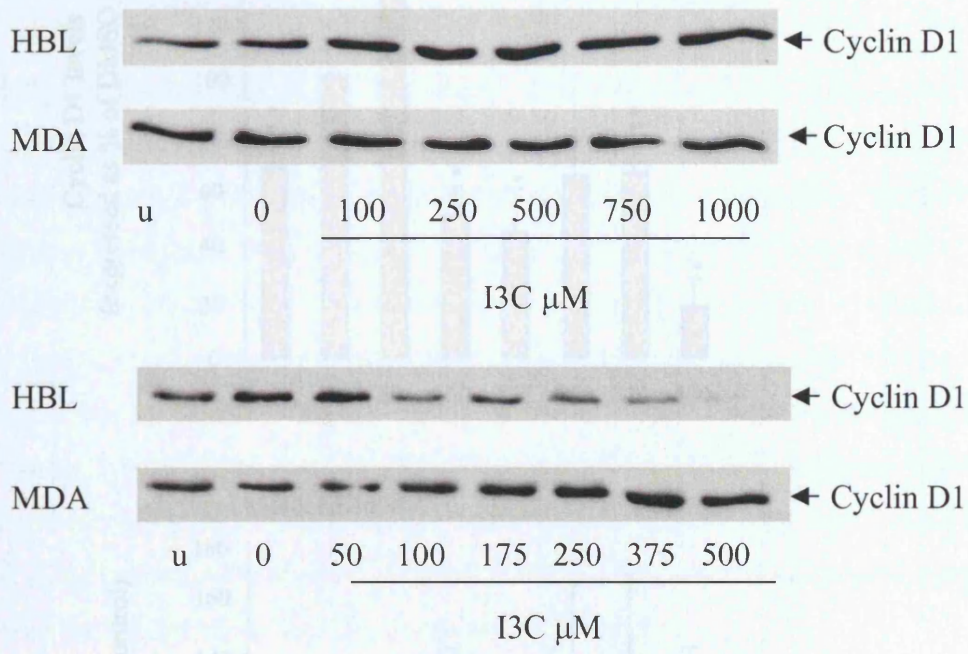


Figure 5.7 Representative western blots showing nuclear cyclin D1 levels following a 5 or 24 hour I3C treatment in the HBL 100 and MDA MB468 cells. *Blots were performed upon at least 3 separate occasions.*

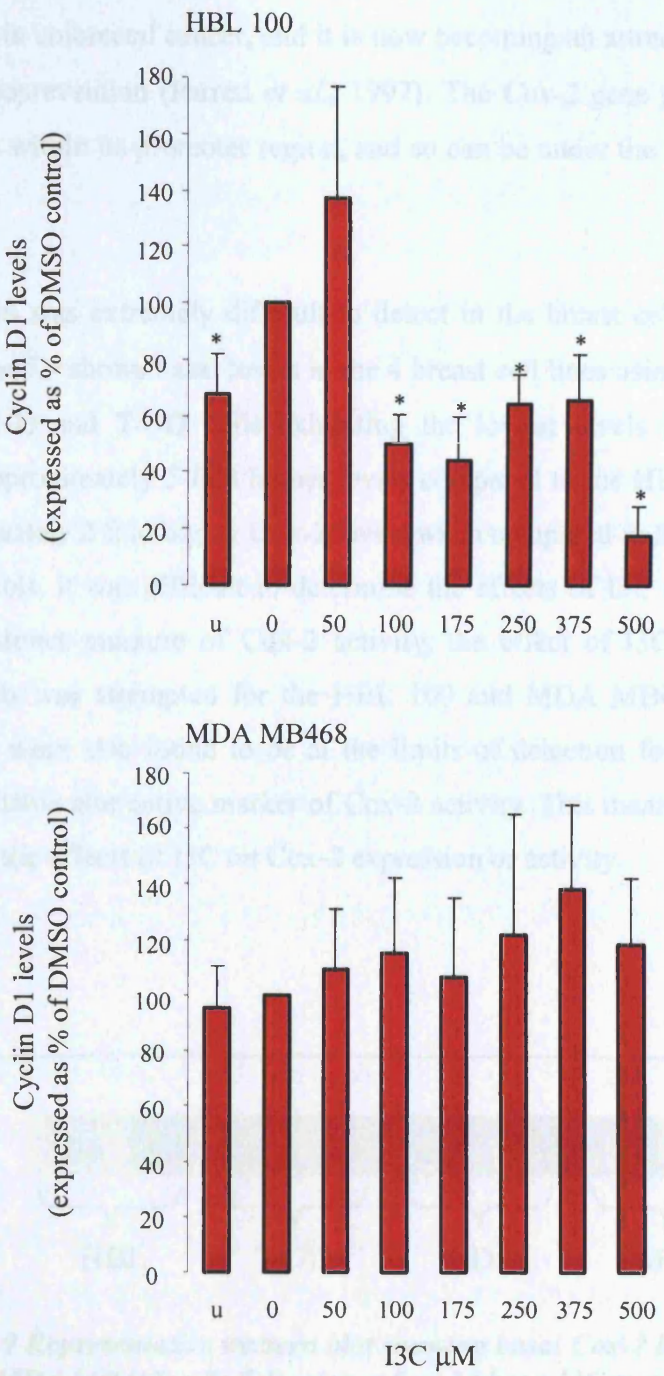


Figure 5.8 Effect of a 24 hour I3C treatment on nuclear cyclin D1 levels in the HBL 100 and MDA MB468 cell line. ** represents a significant difference from the DMSO (n=3, ± SEM, p ≤0.05) control as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test*

5.2.2 COX-2

Cox-2 overexpression and its association with high nuclear levels of NF-κB have been well documented in colorectal cancer, and it is now becoming an attractive biomarker for breast cancer chemoprevention (Parrett *et al.,* 1997). The *Cox-2* gene possesses 2 NF-κB DNA binding sites within its promoter region, and so can be under the regulatory control of NF-κB.

Cox-2 protein was extremely difficult to detect in the breast cell lines, due to low basal levels. *Figure 5.9* shows basal levels in the 4 breast cell lines using 200μg protein per lane, with HBL 100 and T47D cells exhibiting the lowest levels and MDA MB468 cells exhibiting approximately 5 fold higher levels compared to the HBL 100 cells. MCF7 cells had approximately 2 fold higher Cox-2 levels when compared to the HBL 100 cells. Due to such low levels, it was difficult to determine the effects of I3C upon Cox-2 in these cell lines. As a direct measure of Cox-2 activity, the effect of I3C upon prostaglandin E2 (PGE2) levels was attempted for the HBL 100 and MDA MB468 cell lines. However, PGE2 levels were also found to be at the limits of detection for this assay, and did not provide a suitable alternative marker of Cox-2 activity. This meant that it was not possible to determine the effects of I3C on Cox-2 expression or activity.

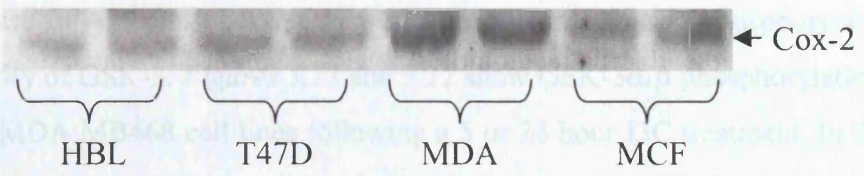


Figure 5.9 Representative western blot showing basal Cox-2 levels in the HBL 100 and MDA MB468 cells following a 5 or 24 hour I3C treatment. *Blots were performed on 2 separate occasions. Duplicates represent different samples, loading 200μg protein per lane (exposure time = 1 hour).*

5.2.3 Inhibitors of apoptosis

The transcriptional activity of NF-κB also plays an important role in the regulation of the X-linked inhibitor of apoptosis (XIAP), inhibition of which has been shown to result in an increase in apoptosis (Deveraux *et al.*, 1997). *Figure 5.10(A)* shows the XIAP levels in the HBL 100 and MDA MB468 cells following a 5 hour I3C treatment. Both cell lines possessed similarly high XIAP levels, which did not decrease with increasing doses of I3C. In section *3.4,* it was shown that apoptosis cannot be significantly detected in the MDA MB468 cell line until approximately 6 hours following a 500μM I3C treatment. In order to confirm whether I3C affected XIAP at other time points, both cell lines were treated for times up to 10 hours with 500μM I3C. No decrease in XIAP protein levels was observed at any time point. Interestingly, XIAP is also a substrate for caspase 3 and is often cleaved and inactivated during apoptosis. The lack of any decrease in XIAP levels in this experiment suggests not only that there is no effect in response to I3C treatment, but also that there was no cleavage of this protein by caspase activity under these conditions. Using 500μM I3C, small amounts of apoptosis were induced in the MDA MB468 cells from 2 hours (*figure 3.7*), suggesting that the caspases were active. However, it may be that there was greater caspase activity in the apoptotic cells that are floating, in which case the lack of cleavage of XIAP may be explained, as adherent cells only were used in this assay.

5.2.4 GSK-3

GSK-3 is phosphorylated and inactivated by Akt, so it might be expected that an I3C-induced decrease in Akt phosphorylation would result in decreased phosphorylation and increased activity of GSK-3. *Figures 5.11* and *5.12* show GSK-3α/β phosphorylation in the HBL 100 and MDA MB468 cell lines following a 5 or 24 hour I3C treatment. In the HBL 100 cells there was a significant dose dependent decrease in GSK-3α phosphorylation induced following an LY294002 treatment only. From *figure 5.12*, GSK-3β levels in the HBL 100 cells appeared more susceptible to the effects of I3C and were significantly inhibited from 500μM I3C and following an LY294002 treatment. In contrast, the MDA MB468 cells appeared more sensitive to the inhibition of GSK-3α phosphorylation by I3C than did the HBL 100 cells. Again, phosphorylation levels of both GSK-3α and β were significantly inhibited upon an LY294002 treatment in this cell line. Following a 24 hour

I3C treatment, there appeared be an increase in GSK-3α levels when compared to the DMSO control in both cell lines.

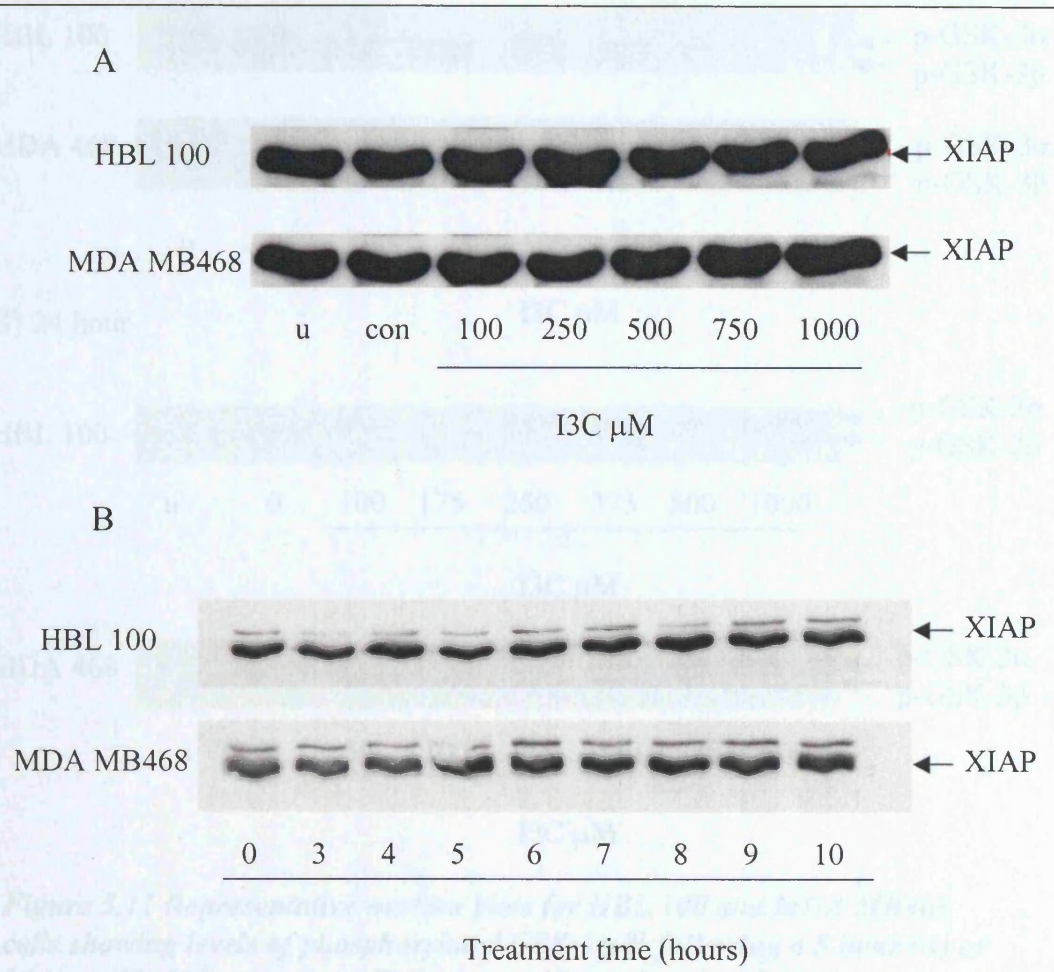


Figure 5.10 Effects of I3C on XIAP levels in HBL 100 and MDA MB468 cell lines. *Panel A shows representative western blots of XIAP levels in HBL 100 and MDA MB468 cells following a 5 hour I3C treatment. Panel B shows representative western blots for HBL 100 and MDA MB468 cells following a 500μM I3C treatment for times up to 10 hours. Blots were performed on 3 separate occasions.*

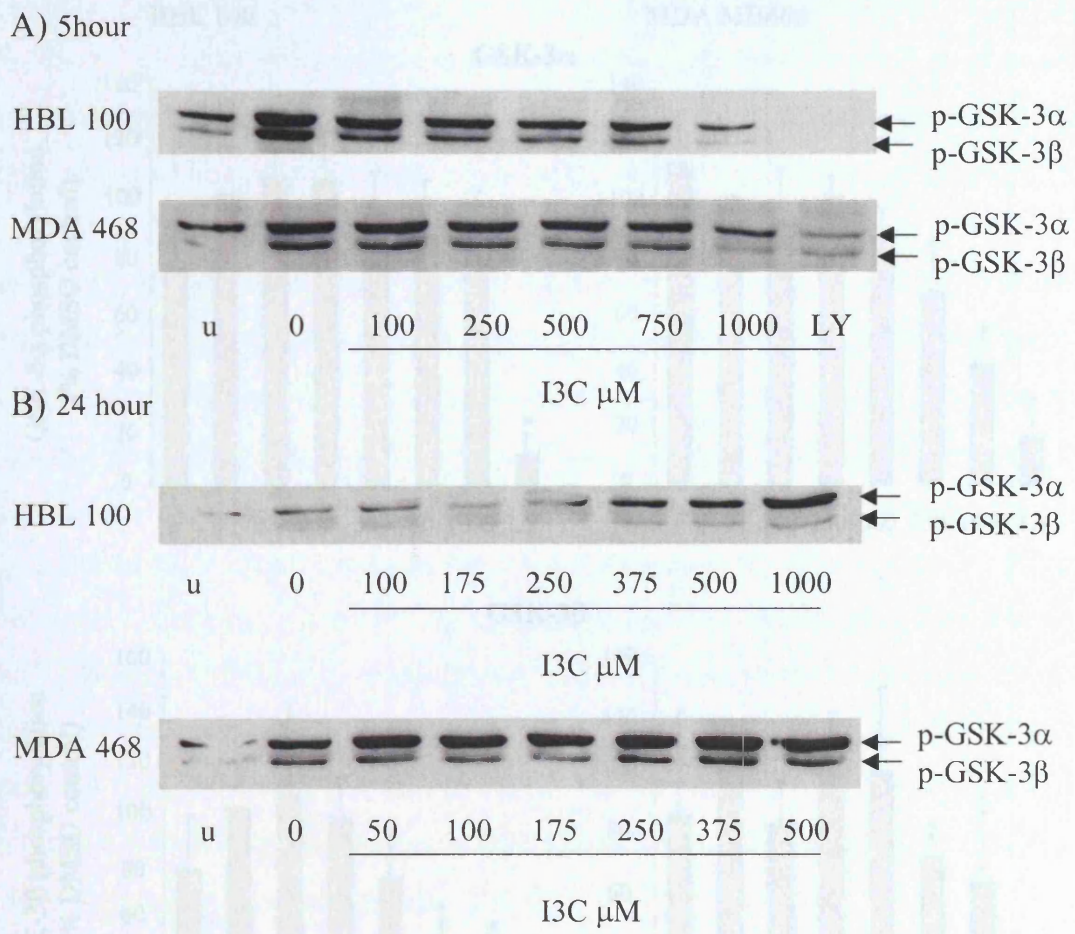


Figure 5.11 Representative western blots for HBL 100 and MDA MB468 cells showing levels of phosphorylated GSK-3α/β following a 5 hour (A) or 24 hour (B) I3C treatment. *Blots were performed upon at least 3 separate occasions.*

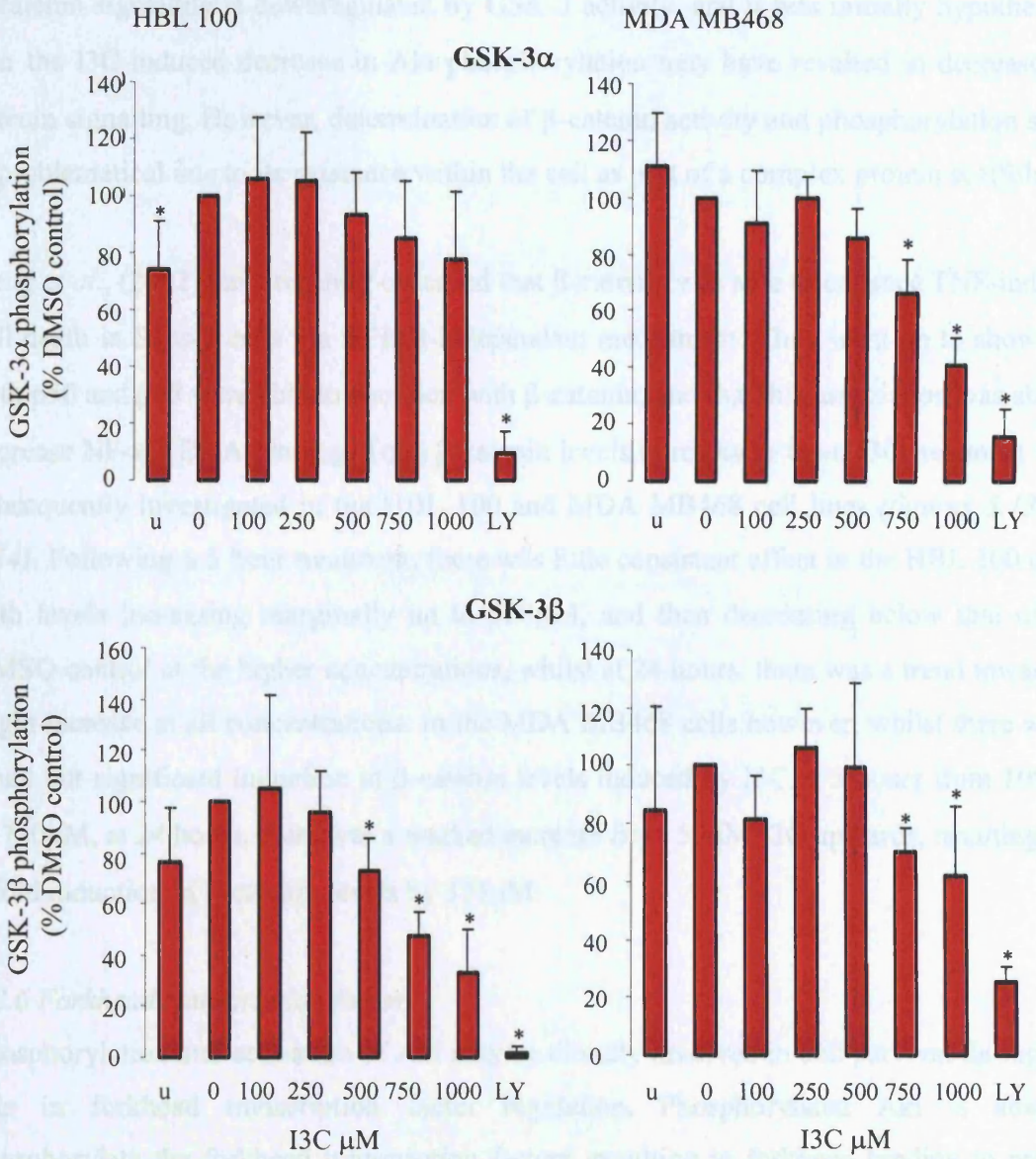


Figure 5.12 Effects of a 5 hour I3C treatment on GSK-3α/β levels in the HBL 100 and MDA MB468 cells. ** represents a significant difference from the DMSO control (n=3, ± SEM, p ≤0.05) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test*

5.2.5 β–Catenin

β-catenin signalling is mediated through its interaction with the TCF family of transcription factors and has been proposed as a pro-survival factor in many cancers (Morin *et al.,* 1999). β-catenin signalling is downregulated by GSK-3 activity, and it was initially hypothesised that the I3C-induced decrease in Akt phosphorylation may have resulted in decreased β-catenin signalling. However, determination of β-catenin activity and phosphorylation status is problematical due to its existence within the cell as part of a complex protein scaffold.

Deng *et al.*, (2002) have recently observed that β-catenin was able to enhance TNF-induced cell death in Saos-2 cells via an IκB-independent mechanism. They went on to show that both p50 and p65 were able to complex with β-catenin, and that this association was able to decrease NF-κB DNA binding. Total β-catenin levels in response to an I3C treatment were subsequently investigated in the HBL 100 and MDA MB468 cell lines *(figures 5.13* and *5.14)*. Following a 5 hour treatment, there was little consistant effect in the HBL 100 cells, with levels increasing marginally up to 500μM, and then decreasing below that of the DMSO control at the higher concentrations, whilst at 24 hours, there was a trend towards a slight increase at all concentrations. In the MDA MB468 cells however, whilst there was a small but significant induction in β-catenin levels induced by I3C at 5 hours from 100μM to 750μM, at 24 hours, there was a marked increase from 50μM I3C upwards, resulting in a 2 fold induction in β-catenin levels by 375μM.

5.2.6 Forkhead transcription factors

Phosphorylation and activation of Akt may be directly involved in cell survival through its role in forkhead transcription factor regulation. Phosphorylated Akt is able to phosphorylate the forkhead transcription factors, resulting in forkhead binding to protein 14-3-3, and subsequent targeting for ubiquitination and degradation. This ultimately results in decreased transcriptional upregulation of apoptosis-associated genes such as FasL (*section 1.6.3.2). Figure 5.15* shows that in this system, a decrease in Akt phosphorylation was not sufficient to consistently decrease phosphorylation of the forkhead transcription factors FKHR and FKHRL1 in the MDA MB468 cells, nor was there an Akt-independent effect in the HBL 100 cells using whole cell extracts.

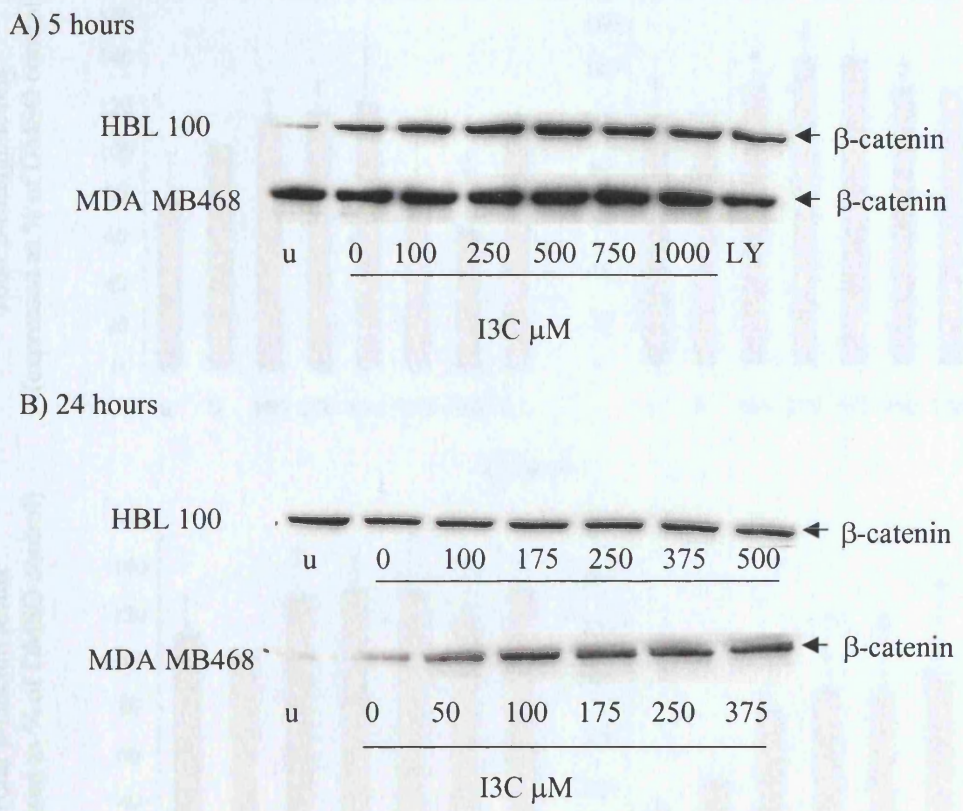


Figure 5.13 The effect of a 5 or 24 hour I3C treatment on total β-catenin levels in the HBL 100 and MDA MB468 cells. *Blots were performed on at least 3 separate occasions.*

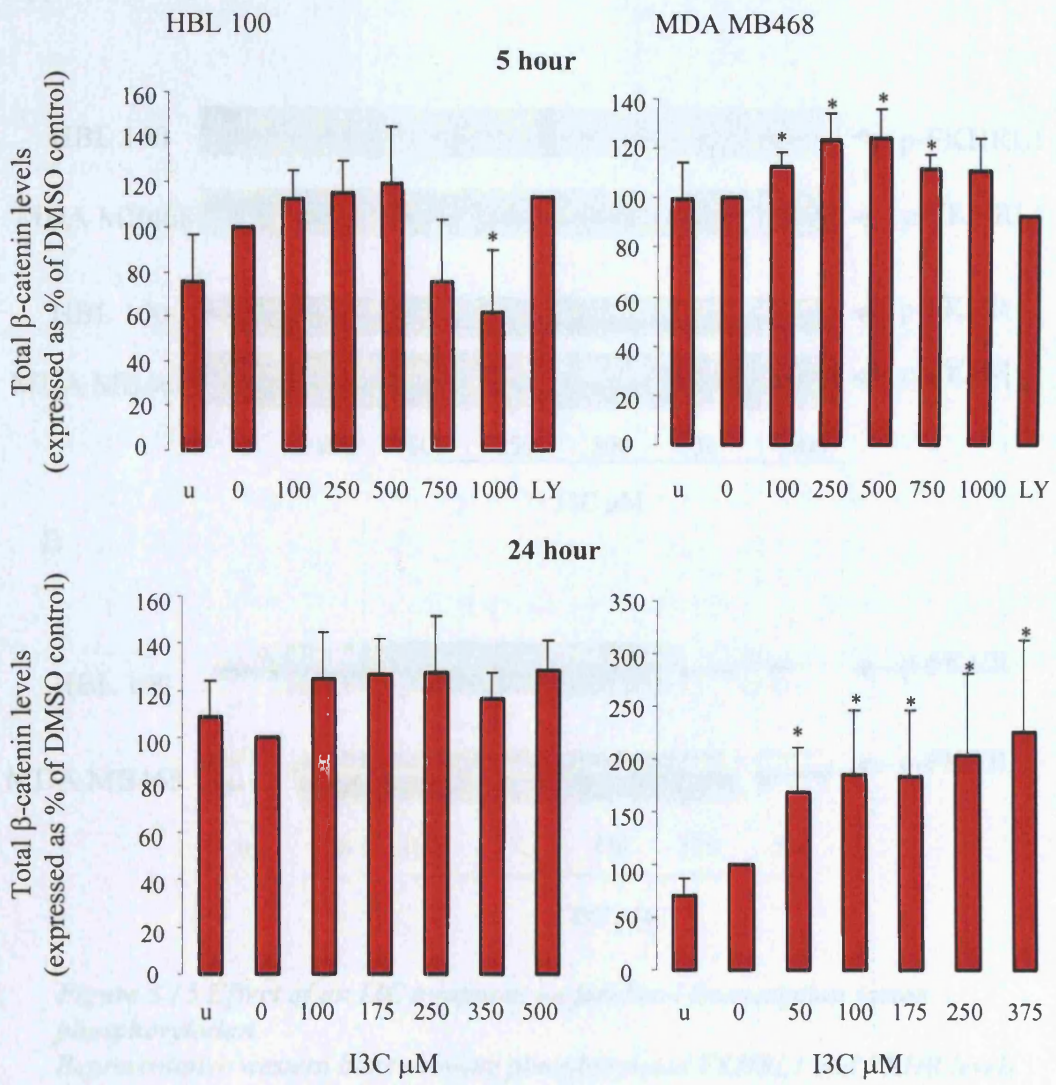


Figure 5.14 The effect of a 5 or 24 hour I3C treatment on β-catenin levels in the HBL 100 and MDA MB468 cell line. ** represents a significant difference from the DMSO control (n=3, ± SEM, p ≤0.05) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

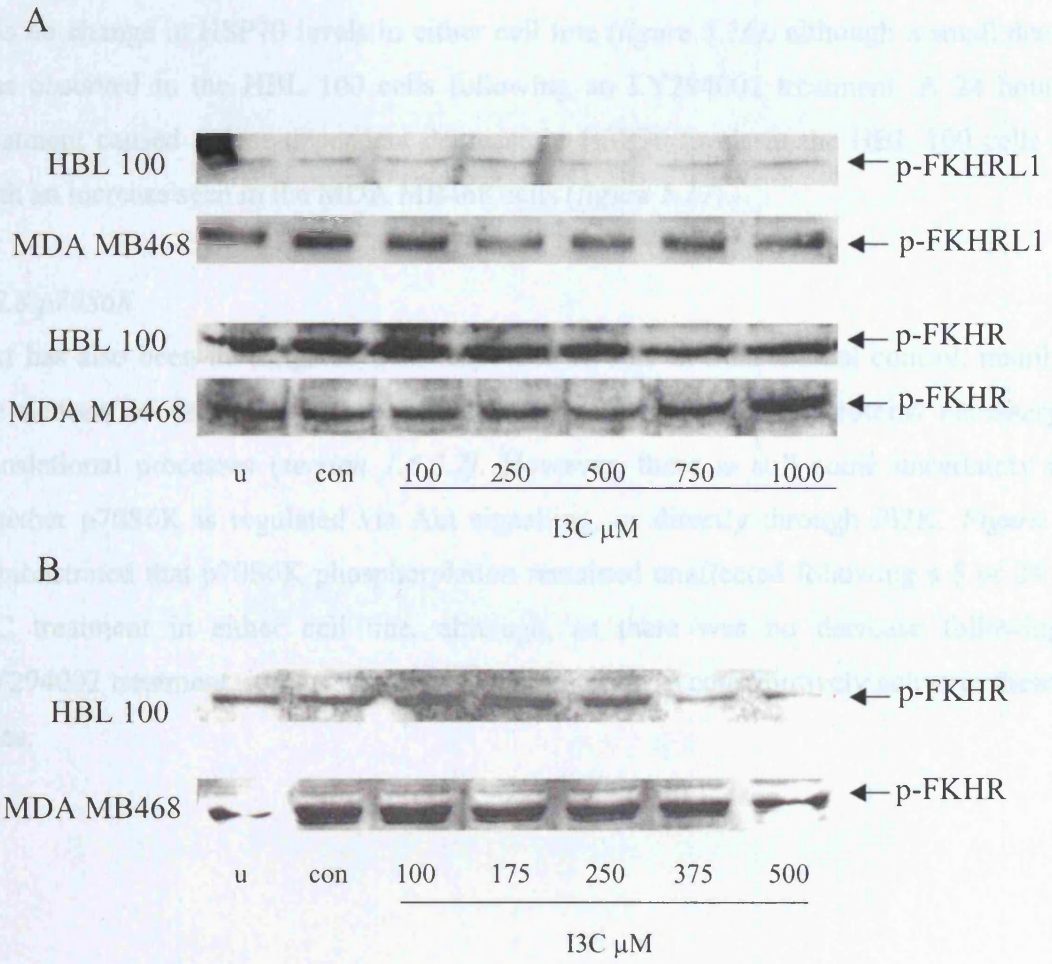


Figure 5.15 Effect of an I3C treatment on forkhead transcription factor phosphorylation.
Representative western blots showing phosphorylated FKHRL1 and FKHR levels in (A) HBL 100 and MDA MB468 cells following a 5 hour I3C treatment, and (B) phosphorylated FKHR levels in HBL 100 and MDA MB468 cells following a 24 hour treatment using whole cell lysates. Blots were performed upon at least 3 separate occasions.

5.2.7 HSP70

Heat shock proteins are anti-apoptotic molecules whose expression is upregulated by phosphorylated Akt, so promoting cell survival. Following a 5 hour I3C treatment, there was no change in HSP70 levels in either cell line (*figure 5.16),* although a small decrease was observed in the HBL 100 cells following an LY294002 treatment. A 24 hour I3C treatment caused a dose-dependent decrease in HSP70 levels in the HBL 100 cells only, with an increase seen in the MDA MB468 cells (*figure 5.17*).

5.2.8 p70S6K

Akt has also been investigated with respect to its role in translational control, mainly via the actions of the p70S6kinase, which regulates translation of proteins necessary for translational processes (*section 1.6.3.2).* However, there is still some uncertainty as to whether p70S6K is regulated via Akt signalling, or directly through PI3K. *Figure 5.18* demonstrated that p70S6K phosphorylation remained unaffected following a 5 or 24 hour I3C treatment in either cell line, although, as there was no decrease following an LY294002 treatment, it may suggest that this kinase was constitiutively active in these cell lines.

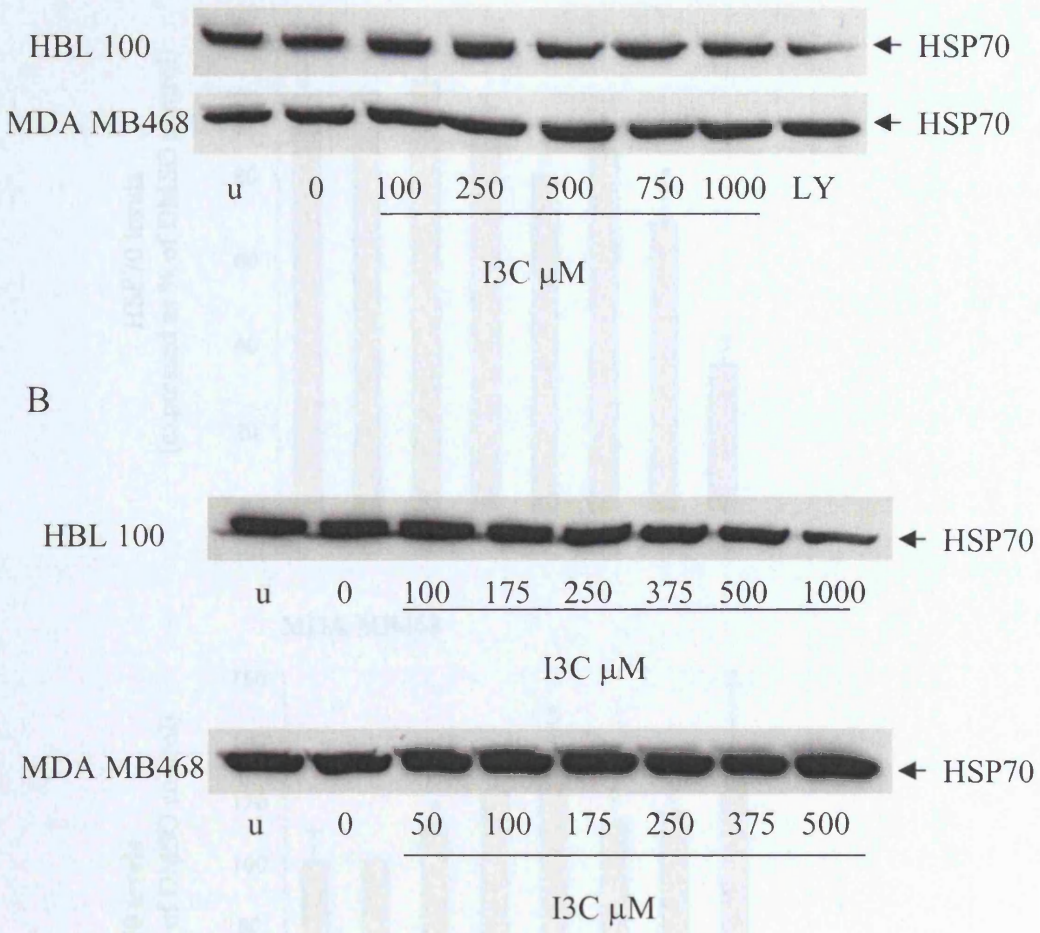


Figure 5.16 The effect of an I3C treatment on HSP70 levels.
Representative western blots showing HSP70 levels in HBL 100 and MDA468 cells following a 5 (A) or 24(B) hour I3C treatment. Blots were performed upon at least 3 separate occasions.

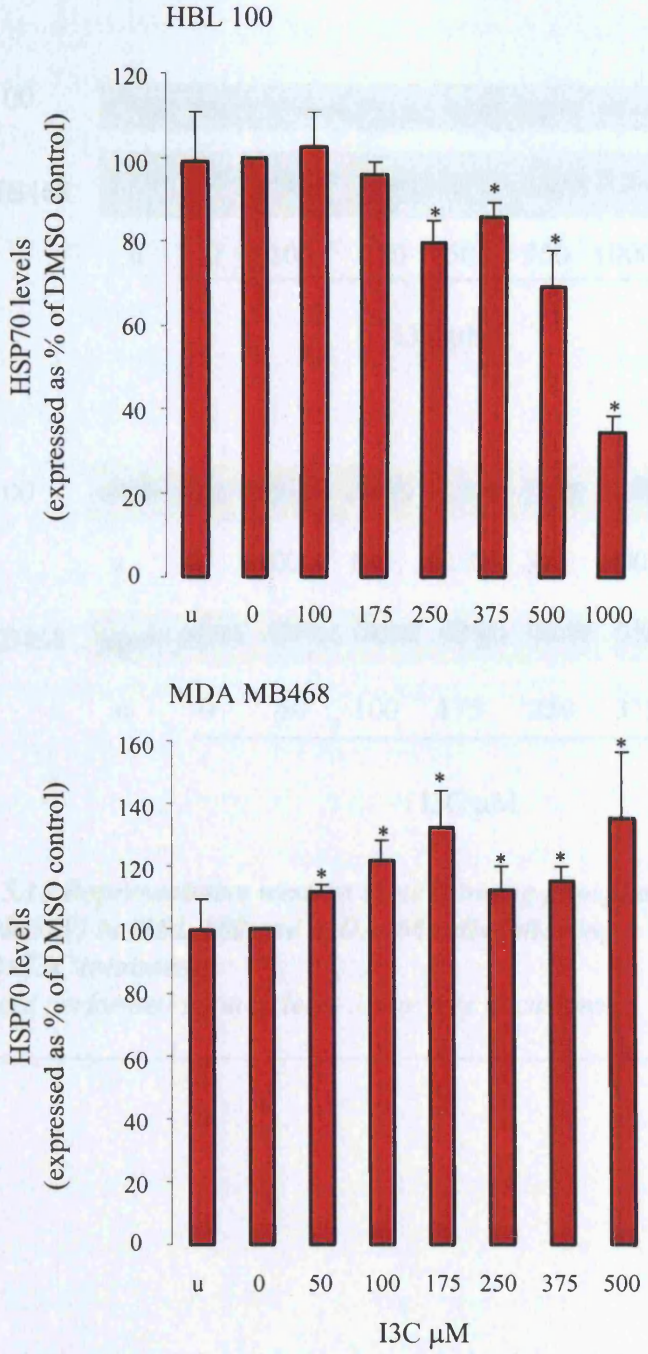


Figure 5.17 Effect of a 24 hour I3C treatment on HSP70 levels in the HBL 100 and MDA MB468 cells lines.

** represents a significant difference from the DMSO control (n=3, ± SEM, p ≤0.05) as determined by a Balanced ANOVA followed by Fisher's least significant difference post hoc test.*

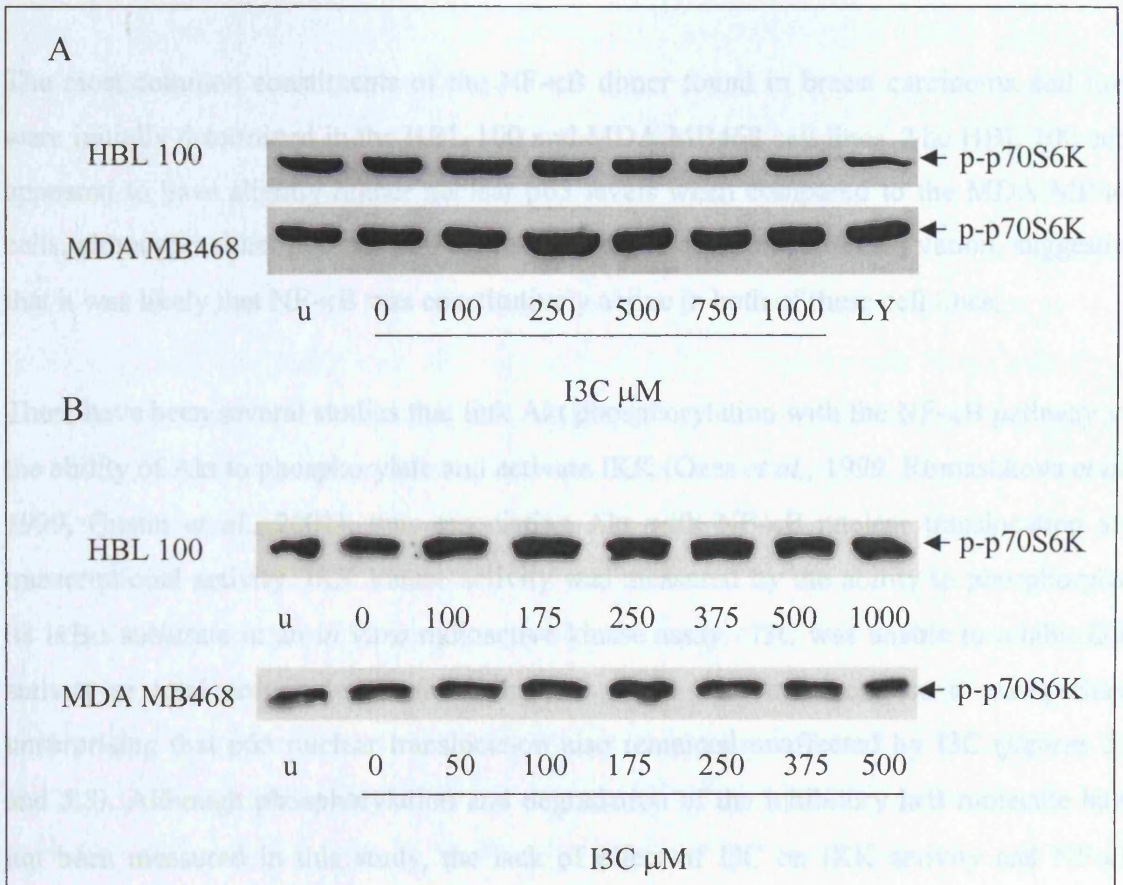


Figure 5.18 Representative western blots showing phosphorylated p70S6K levels (thr389) in HBL 100 and MDA468 cells following a 5 hour (A) or 24 hour (B) I3C treatment.
Blots were performed upon at least 3 separate occasions.

DISCUSSION

The most common constituents of the NF-κB dimer found in breast carcinoma cell lines were initially determined in the HBL 100 and MDA MB468 cell lines. The HBL 100 cells appeared to have slightly higher nuclear p65 levels when compared to the MDA MB468 cells, although neither p65 nor p50 levels were depleted upon serum starvation, suggesting that it was likely that NF-κB was constitutively active in both of these cell lines.

There have been several studies that link Akt phosphorylation with the NF-κB pathway via the ability of Akt to phosphorylate and activate IKK (Ozes *et al.,* 1999, Romashkova *et al.*, 1999, Gustin *et al.*, 2001), thus associating Akt with NF-κB nuclear translocation and transcriptional activity. IKK kinase activity was measured by the ability to phosphorylate its IκBα substrate in an *in vitro* radioactive kinase assay. I3C was unable to inhibit IKK activity or total protein levels following a 5 or 24 hour treatment, so it was perhaps unsurprising that p65 nuclear translocation also remained unaffected by I3C (*figures 5.2* and *5.3)*. Although phosphorylation and degradation of the inhibitory IκB molecule have not been measured in this study, the lack of effect of I3C on IKK activity and NF-κB translocation would suggest that it would have been unlikely to have shown any effect. It can be concluded from these data that the I3C-induced inhibition of Akt phosphorylation did not result in a decrease in NF-κB nuclear translocation or inhibition of the NIK/ IKK signalling pathway, and that therefore this latter pathway was not involved in the growth-inhibitory effects of this agent.

There have been several reports that Akt phosphorylation can affect NF-κB signalling in an IKK-independent manner (Madrid *et al.*, 2000, 2001), so the effect of I3C on NF-κB DNA binding was therefore investigated. I3C decreased NF-κB DNA binding in the MDA MB468 cells only. The specificity of this effect, suggested that this may be a direct consequence leading to the I3C-induced inhibition of Akt phosphorylation and induction of apoptosis also seen in this cell line. In order to test whether decreased Akt phosphorylation was important in the regulation of NF-κB signalling in the MDA MB468 cell line, cells were treated with the PI3K inhibitor LY294002 (*figure 5.5*). Complete inhibition of Akt phosphorylation was not sufficient to prevent NF-κB from being present in the nucleus, nor

was it able to prevent NF-κB DNA binding. This suggested that the I3C-induced decrease in Akt phosphorylation was not responsible for eliciting the decrease in DNA binding observed in *figure 5.4.* However, it may be that the decrease in NF-κB DNA binding was responsible for the decrease in Akt phosphorylation observed by Meng *et al.,* (2002), or that the effects of I3C on NF-κB may be entirely independent from the Akt pathway, but still contribute to apoptosis.

Functional consequences of this decreased binding were investigated using reporter gene technology in which a luciferase construct containing 6NF-κB consensus binding sites, was transfected into the MDA MB468 cells. Transfection of this reporter gene overnight, and then subsequent treatment with I3C for 5 hours *(figure 5.6)* did not result in a decrease in luciferase that matched the extent of the decrease in DNA binding observed in *figure 5.4.* The overnight incubation to ensure maximal transfection efficiency in this assay, could have resulted in a high luciferase background due to the activity of the reporter construct throughout the incubation period once individual cells had become transfected. A pre-treatment with I3C prior to transfection could eliminate high background levels of reporter activity. However, greater toxicity of I3C was observed at longer time points, which was enhanced in the presence of the transfection reagent. This unfortunately meant that the results for transactivation by NF-κB were not clear-cut.

Many genes possess NF-κB consensus sequences within their promoter regions, some of which have particular relevance to breast cancer, including cyclin D1, COX2 and XIAP, which have been investigated in this chapter.

Increased cell cycling is often a consequence of the carcinogenic process, and in breast cancer, cyclin D1 levels are frequently raised (Hulit *et al.,* 2002). In the HBL 100 and MDA MB468 cells however, cyclin D1 levels did not appear to correlate with the effects observed upon the NF-κB pathway following treatment with I3C. In the HBL 100 cells, there was a clear decrease in nuclear cyclin D1 levels following a 24 hour I3C treatment, which was in contrast to the lack of effect observed upon NF-κB DNA binding. There was no clear cell cycle arrest induced by an I3C treatment in this cell line, but it is possible that there was a slight slowing of the cell cycle which may have occurred due to this decrease in cyclin D1. It was apparent that this decrease in cyclin D1 occurred independently from NF-

κB DNA binding and Akt phosphorylation status, as there were no observable changes in these two parameters in the HBL 100 cells. In the MDA MB468 cell line, cyclin D1 levels were slightly raised, despite the I3C-induced decrease in Akt phosphorylation and NF-κB DNA binding. There are many other factors however, involved in the regulation of cyclins, and cyclin D1 in particular is heavily controlled by growth factor stimulation. Transcription of the cyclin D1 gene can thus result from growth factor stimulation of the MAPK pathway leading to production of transciption factors such as AP-1 and ets which are able to bind to and activate the cyclin D1 promoter region (Balmanno *et al.,* 1999). Cyclin D1 expression is also regulated through wnt signalling and the estrogen receptor, with reduced cyclin D1 levels observed upon estrogen deprivation in estrogen dependent cells (reviewed by Barnes and Gillet, 1998).

Cox-2 levels were difficult to determine in the breast cell lines, requiring very large amounts of protein to be loaded onto gels for western blotting. The MDA MB468 cells exhibited the highest basal Cox-2 levels of all the breast cell lines. High Cox-2 levels are often observed in cell lines with aggressive, quickly proliferating phenotypes, with concomitant raised PGE2 levels providing a possible prognostic marker for breast cancer (Parrett *et al.,* 1997). The ability of chemopreventive agents to reduce PGE2 levels in cellular models of colorectal cancer has previously been documented (Ireson *et al.*, 2001), as has LY294002 inhibition of LPS-induced Cox-2 activity and PGE2 production in macrophages, due to destabilisation of Cox-2 mRNA (Monick *et al.*, 2002).

Due to the difficulty in assessing Cox-2 levels, it was considered that PGE2 levels may provide a more convenient measure of Cox-2 activity. However PGE2 levels were so low as to be at the limits of detection for the assay, and could not provide reproducible results, suggesting that Cox-2 would not provide a valuable prognostic marker for the chemopreventive activity of I3C in this system.

The inhibitors of apoptosis are frequently overexpressed in cells that are resistant to apoptosis (McEleny *et al.*, 2002), and their downregulation through inhibition of the NF-κB pathway has been suggested as a potential chemotherapeutic mechanism (Biswas *et al.*, 2003). High levels of XIAP were observed in both the HBL 100 and MDA MB468 cell line, and levels remained unaffected following various I3C treatments (*figure 5.10*). The

IAPs have been shown to be induced by NF-κB in multiple cell lines, with XIAP able to activate NF-κB by increased nuclear translocation of the p65 subunit via the actions of MAP3K (Hofer-Warbinek *et al.,* 2000, LaCassae *et al.,* 1998). That I3C did not affect IAP levels and/or cleavage is surprising, as upon release of active caspases from IAPs, the caspases can themselves then cleave the IAP molecule and so potentiate the apoptotic process. These results would therefore suggest that although present in relatively high amounts, the induction of apoptosis by I3C in the MDA MB468 cells was independent from the potential for IAPs to inhibit active caspases. However, cleaved IAP may also be present in floating cells, which were not examined in this study. The I3C-induced decrease in NF-κB DNA binding ability in the MDA MB468 cells may not affect IAP protein levels at the relatively short (10 hour) time points investigated. However, if a decrease was to occur at longer time points, as with the Bcl-2 data (*section 3.5*), it would indicate that these changes are not a direct cause of the I3C-induced apoptosis, but rather a consequence of it.

GSK-3 is regulated by direct phosphorylation by Akt leading to its inactivation (*section 1.6.3.2*). Inhibition of GSK-3 phosphorylation by I3C occurred in both the HBL 100 and MDA MB468 cell lines, although only to a significant extent at the very highest concentrations (750-1000μM). A 5 hour LY294002 treatment almost completely abolished GSK-3 phosphorylation in both cell lines, which is consistent with the fact that GSK-3 is directly phosphorylated by active Akt. That Akt phosphorylation was not decreased by I3C in the HBL 100 cells, means that this pathway cannot be involved in the I3C-induced decrease in GSK-3 phosphorylation in this cell line. It may be that I3C is interfering with Wnt signalling to decrease GSK-3 phosphorylation in the HBL 100s, which may also explain the NF-κB independent decrease in cyclin D1 observed. The decrease in GSK-3 phosphorylation observed in the MDA MB468 cells followed a similar trend to that induced in Akt phosphorylation following an I3C treatment, although there was no significant decrease in levels of proteins regulated through GSK-3 such as cyclin D1 and HSP70 in this cell line.

β-catenin is regulated by GSK-3 via enhanced degradation (when GSK-3 is active), or increased translocation to the nucleus (when GSK-3 is inactive). Whilst levels of the phosphorylated protein were not determined, an I3C treatment resulted in a marginal increase in total β-catenin protein levels at 5 and 24 hours in the HBL 100 cells, and a

significant increase at both time points in the MDA MB468 cell line (which appeared inconsistent from GSK-3 results). From the recent study by Deng *et al.*, (2002), it was possible that this increase in total β-catenin levels contributed to the I3C-induced decrease in NF-κB DNA binding in this cell line. This could provide an alternate mechanism for induction of apoptosis by I3C, which is independent from Akt phosphorylation.

There was no consistent effect upon forkhead transcription factor phosphorylation by I3C in either the HBL 100 or MDA MB468 cells. This was unsurprising in the HBL 100 cells, as the forkheads can be directly regulated by Akt, which similarly remained unaffected following an I3C treatment. Jackson *et al.,* (2000) showed that PI3K-dependent phosphorylation of FKHR was sufficient to decrease nuclear FKHR levels in the MDA-MB231 cells. Therefore it might be expected that the I3C-induced decrease in Akt activity in MDA MB468 cells would result in increased FKHR nuclear levels (represented by decreased levels of phosphorylation) in this cell line. However, in the MDA MB468 cells, the lack of any I3C-induced effect upon the forkheads may be in part due to their lack of PTEN. Nakamura *et al.,* (2000) observed that FKHR localisation and transcriptional activity is aberrant in PTEN null cells, and so the forkhead transcriptional activity remains relatively inactive. They have also shown that this may in turn result in aberrant p27 expression and so be linked to de-regulation of the cell cycle in these cells. This would therefore suggest that these results showing a lack of effect of I3C on forkhead phosphorylation, are consistent with the MDA MB468 cell genotype and phenotype.

There is evidence to suggest the involvement of PI3K signalling in p70S6K regulation (Gonzalez-Garcia *et al.,* 2002) and that it may be a downstream target of Akt. p70S6K levels remain unaffected following a 5 or 24 hour I3C treatment in either cell line. A 5 hour treatment with LY294002 had little effect upon phosphorylated p70S6K levels in either cell line, which would suggest that p70S6K is not under PI3K regulation in this system. It has been observed in cardiac myocytes that raf/MEK/ERK signalling via PKC plays a major role in S6K activation (Iijima *et al.,* 2002), which may also be the case for these breast cells. However, little evidence has been accrued to date as to whether p70S6K is overexpressed in breast carcinomas.

In this chapter the investigation of further potential signalling targets of I3C has been described. The NF-κB pathway was modulated by I3C in the MDA MB468 cells only, and this appeared to be an Akt-independent effect, although it has yet to be determined whether the decrease in Akt phosphorylation was independent from NF-κB. Other well-documented targets of Akt were also investigated, but few substantial effects that would further our understanding of the chemopreventive properties of I3C, were observed. However, it may be that an additive effect of small changes to phosphorylation or protein levels of these molecules would be sufficient to contribute to the induction of apoptosis. There are potentially many targets of I3C still to be investigated, and in the next chapter, the use of microarray analysis in an attempt to further identify these targets and elucidate more prospective chemopreventive mechanisms of action for this agent, is described.

CHAPTER 6
MICROARRAY ANALYSIS OF I3C INDUCED GENE CHANGES

INTRODUCTION

Gene expression microarrays are an exciting new technology which allow the analysis of global mRNA expression levels, profiling thousands of genes simultaneously. Microarray offers a powerful new tool in cancer research allowing the exploration of potential new therapeutic agents on multiple targets, and detection/characterisation of gene expression patterns associated with disease state which would previously have taken many man hours to investigate *(figure 6.1)*.

In order to create an array chip, cDNA molecules representing the system of interest are first amplified and then spotted onto a glass slide in a unique array format (Shalon, 1998). DNA probes are made from cell samples by extracting mRNA from a control and test cell sample, and the cDNA probes generated are then differentially fluorescently labelled prior to mixing. The use of fluorescent dyes in the arrays allows the gene expression in the two samples to be directly compared, as they compete for binding to the array chip (Rew, 2001). The Cy-3 and Cy-5 dyes used in this study possess different emission wavelengths, so that the ratio of the fluorescence intensity on each spot equals the ratio of expression of each gene in the two samples *(figure 6.2)*. This method is the basic unit from which new technologies are emerging allowing more specific identification of drug-target interactions. Open reading frames of genes in expression vectors can now be printed onto glass slides, transfection reagent added, and the cDNA covered with a layer of cells allowing evaluation of phenotypic changes resulting from the overexpression of specific genes in cells (Mousses *et al.*, 2001). Similarly, it may be that antisense suppression of specific genes could be carried out in an analogous manner, allowing a more physiological representation of gene function.

Tools that are now becoming available to increase the relevance and ease of interpretation of RNA microarray data include the advent of protein microarrays, which will ultimately provide far more functional data essential for understanding biological systems. Protein arrays have the potential to speed up target validation, as often mRNA levels and corresponding protein levels have little correlation. This will hopefully have applications to include target identification and characterisation plus biomarker identification and monitoring in clinical studies (Huels *et al.,* 2002).

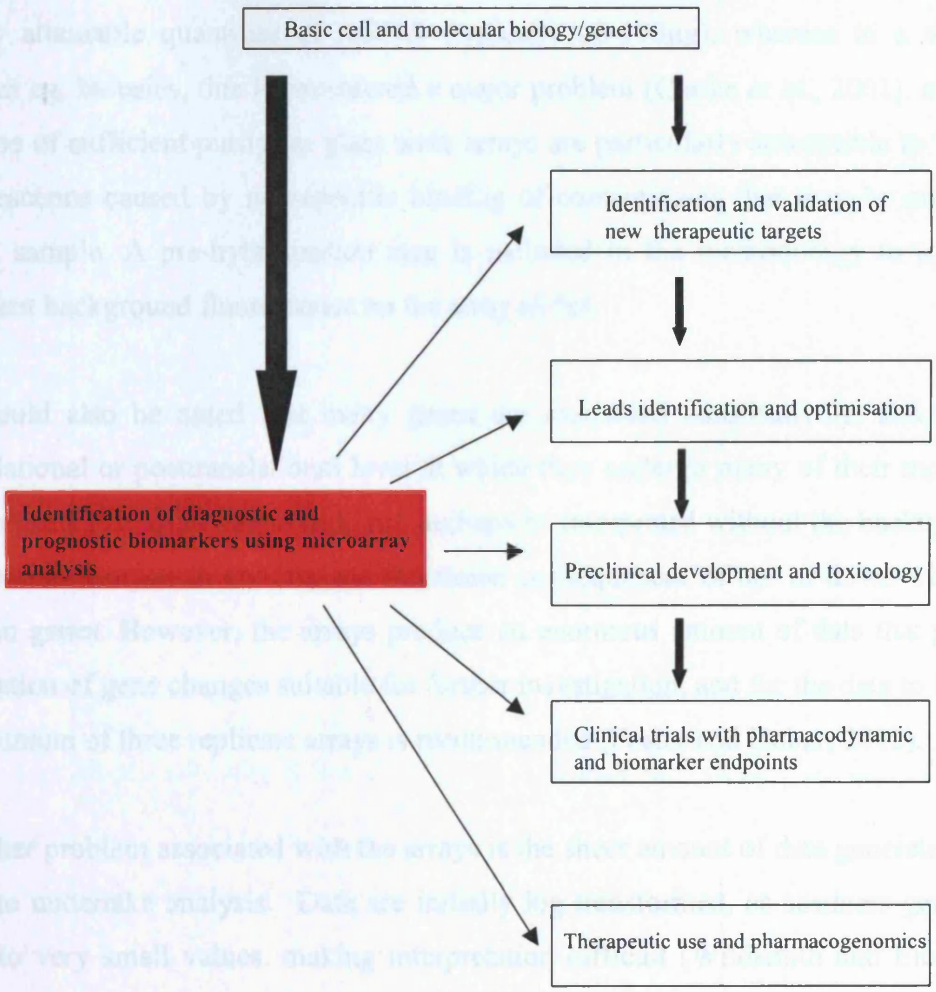


Figure 6.1 Phases in the discovery and development of therapeutic agents and of diagnostic, prognostic and other biomarkers. *Microarray expression profiling can be used to help advance all stages of this process. Adapted from Clarke et al., Biochemical Pharmacology. 2001.*

Microarray trials and tribulations

One of the problems associated with the array is the amount and quality of mRNA required. In the system described here, the use of cells for test and control samples allowed easily attainable quantities of mRNA (typically 20-100μg), whereas in a tissue based system eg, biopsies, this is considered a major problem (Clarke *et al.*, 2001). mRNA must also be of sufficient purity, as glass slide arrays are particularly susceptible to background fluorescence caused by non-specific binding of contaminants that may be present in the RNA sample. A pre-hybridisation step is included in the methodology to try and limit inherent background fluorescence on the array slides.

It should also be noted that many genes are expressed constitutively, and it is at the translational or posttranslational level at which they undergo many of their modifications. This means that array data should not perhaps be interpreted without the backup of protein expression analysis to confirm the functional consequences of up- or down- regulation of certain genes. However, the arrays produce an enormous amount of data that provides an indication of gene changes suitable for further investigation, and for the data to be of value, a minimum of three replicate arrays is recommended (Foster and Huber, 2002).

Another problem associated with the arrays is the sheer amount of data generated, and how best to undertake analysis. Data are initially log transformed, as scanners generally bias data to very small values, making interpretation difficult (Wildsmith and Elcock, 2000). Normalisation of data between arrays must also be carried out, although within each array, there is an internal normalisation due to the simultaneous hybridisation of both labelled probes. In this array system, normalisation is also carried out at the level of the scanner, by obtaining a similar output from the fluorophores, in order to account for differing fluorescence if one probe has hybridised better than the other.

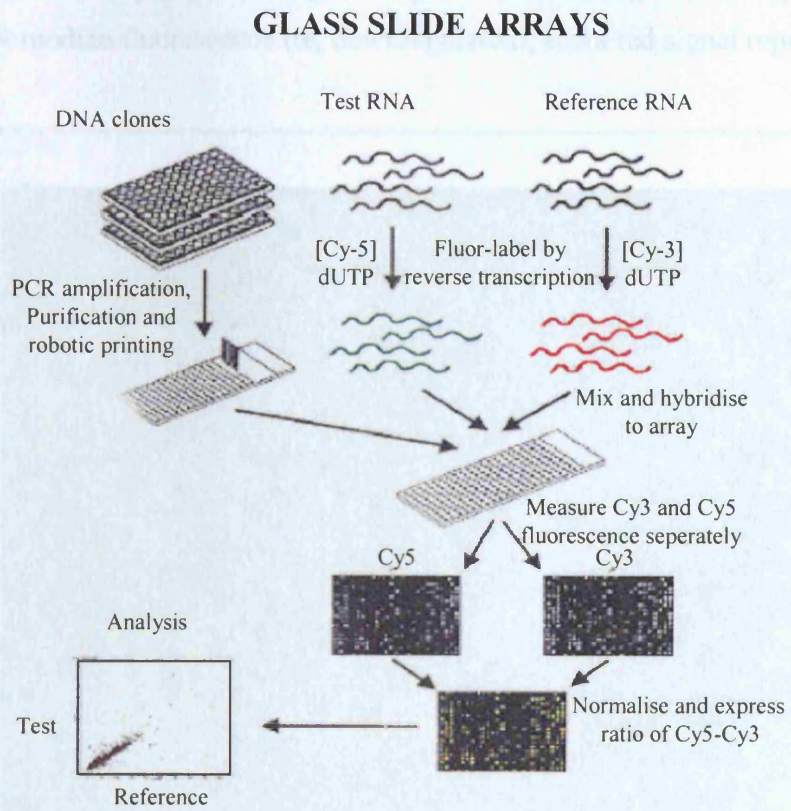


***Figure 6.2 Stages within the microarray experiment**. Adapted from Hedge et al., 2000.*

In the data presented in the results section, only genes that have been up- or down-regulated by twofold or greater have been included as significant (DeRisi *et al.,* 1996), and only genes that are pertinent to the effects of I3C with regards to its anti-proliferative effects have been discussed in greater detail. However, this may be misleading as it is possible that even very small gene changes may have functional consequences at the level of protein expression, so that any changes no matter how small should not necessarily be dismissed as insignificant.

The array data was originally clustered hierarchically via the Cluster and Treeview programmes (referenced in Rhodes *et al.,* 2002) to enable visual examination of gene groups of similar expression patterns, which may be co-regulated. Examples of these

patterns are shown in *figure 6.3.* A green signal represents a gene, the expression of which is below the median fluorescence (ie, downregulated), and a red signal represents one with

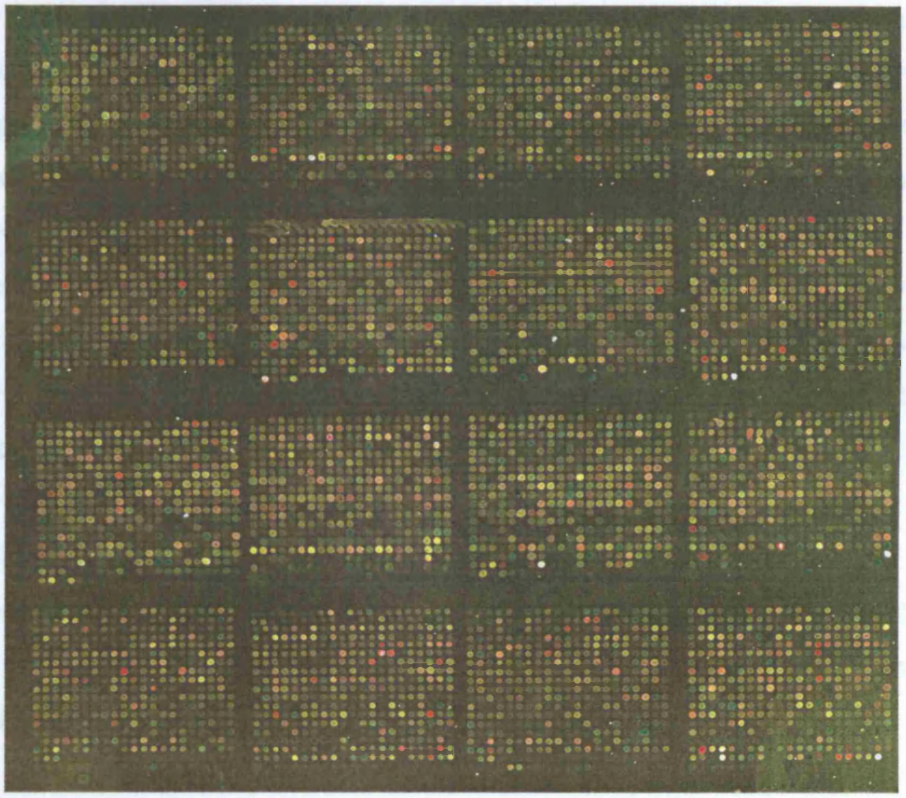


Figure 6.3 Representative array slide showing gene changes in the MDA MB468 cells following a 5 hour I3C treatment.

above median fluorescence (ie, upregulated). The intensity of the colour is proportional to the degree of difference from the median (ie, the degree of up- or downregulation). Investigation into gene function and relevance to the carcinogenic process was undertaken using web-based searches such as GeneCard and SwissProt, permiting clustering of genes into pertinent groups to allow a global analysis of gene changes.

Use of arrays in breast cancer research

Overexpression of certain genes that contribute to tumour development or confer drug resistance are common to breast cancers, such as overexpression of *ERBB2, MDM2* and *CDK4* (*refer to section 1.2*). Amplifications such as these are of particular interest, as they

are of use as clinical markers of tumour origin, stage and grade. A problem with microarray characterisation of human tumour specimens, particularly with breast tumours, is that the specimens are invariably representative of differing cell types. This may require microdissection of tumours to separate populations rich in cancer cells to try and remove other cells such as those of stromal or lymphocytic lineage that may mask real gene changes in the tumour cells (Cooper, 2001). Interestingly, gene profiling has revealed distinct expression patterns for ER-ve and ER+ve breast cell lines. ER+ve tumours were characterised by the expression of genes specific to breast luminal cells, whereas ER-ve tumours had differing expression patterns based on myoepithelial cells or *ERBB2* overexpression (Perou *et al.*, 2000). Microarray analysis may potentially identify a 'poor prognosis' signature by recognition of genes involved in cell cycle and metastasis, allowing breast cancer therapy and management to be better tailored to individual needs with regards to cancer stage and grade (Zajchowski *et al.,* 2001, Van 't Veer *et al.,* 2002).

In this chapter a comparison of the HBL 100 cells with the tumourigenic MDA MB468 cells has been made, and the effects of an I3C treatment at 5 or 24 hours upon the gene expression in both of these cell lines investigated in an attempt to identify novel gene targets for I3C.

RESULTS

In this chapter, results are only presented for genes for which the mean change in expression (generated from 3 arrays) was in excess of 2 fold. Genes were then grouped according to function via the genecard programme (genes may appear in more than one category), in order to assess whether there were similar changes to expression patterns in pertinent clusters. Due to the large amount of data obtained, even when including only 2 fold changes, it is perhaps easier to discuss gene profiles of groups before examining individual gene changes with specific relevance within the context of this thesis.
Full names for all gene abbreviations are included in the appendix.

6.1 Differential gene expression between HBL 100 (control) and MDA MB468 (test) cells

The MDA MB468 cells possess a highly tumourigenic phenotype, and it would perhaps be expected that they would have an upregulation of genes associated with cell survival, and downregulation of genes associated with apoptosis when compared to the normal-derived HBL 100 cells. However, interpretation of data from comparisons between 2 cell lines derived from differing individuals is inherently problematical, due the intrinsic nature of gene variability between individuals, and that the HBL 100 cells have been SV40 immortalised. Results shown in table 6.1 (a-j) depict fold differences in gene expression in MDA MB468 cells compared to HBL 100 cells, with upregulation shown in red and downregulation in green.

a) Apoptosis

With the exception of caspase 1, caspases appeared to be downregulated in the MDA MB468 cells when compared to the HBL 100 cells. Downregulation of caspase 3 (*CASP3*), as one of the major effector caspases, would suggest that the MDA MB468 cells under resting conditions do not undergo apoptosis as readily as the HBL 100 cells. An over expression of *E2F1* in the MDA MB468 cells compared to the HBL 100 cells would suggest that the MDA MB468 cells are again less prone to apoptosis and also may be experiencing a higher degree of cell cycle deregulation than their 'normal tissue-derived' counterparts. However, in keeping with the fact that not all changes at the mRNA level correlate with protein levels, *EGFR* mRNA levels were lower in the MDA MB468 cells

when compared to the HBL 100 cells, when in fact protein levels of this receptor are approximately ten times higher in the MDA MB468 cells (Squires *et al.,* 2000).

b) Angiogenesis

The gene encoding the angiogenic protein *ANGPT1* (angiopoeitin 1) was upregulated in the MDA MB468 cells when compared to the HBL 100 cells, which is indicative of the higher rates of angiogenesis often observed in tumour growth. However, the HBL 100 cells appeared to posses higher expression levels of the matrix metalloproteinases MMP2 and MMP14. Work to date by Gordon *et al.,* (2003) has suggested that MMP protein levels were higher in the HBL 100 cells compared to the MDA MB468 cells, and that the invasive potential was greater in this cell line.

c) Cell cycle

There were many cell cycle genes upregulated in the MDA MB468 cells compared to the HBL 100 cells. Of particular interest was upregulation of the gene for cyclin G1 (*CCNG1*) in the MDA MB468 cells which is usually found to be active in the G2/M phase of the cell cycle, causing growth arrest in response to DNA damage. However, consistently high levels are found in breast cancer cells thoughout all stages of the cell cycle (genecard, Erlandsson *et al.*, 2003). Upregulation of proliferating cell nuclear antigen (PCNA) in the MDA MB468 cells would also be expected in a tumour cell line, due to its ability to increase the rate of DNA replication and so enhance progression through the cell cycle. Protein phosphatase genes (*PPP2CB, PPP2R2B, PPP2R5E*) are actively involved in the modulation of cell signalling molecules such as PKB, p70S6K, CK2 and MAPKK, so that the upregulation observed in the MDA MB468 cells could be pertinent in regulation of pathways involved in proliferation, apoptosis and cell cycle progression.

d) Cell growth

Perhaps the most interesting difference between the HBL 100 and MDA MB468 cells in this category was the upregulation of the gene for the PI3K catalytic p110β subunit in the MDA MB468 cells (*PIK3CB*). There is increasing evidence for over expression of PI3K in tumour cell lines (Shayesteh *et al.*, 1999), although section *4.3* suggests that at a protein level, there is little difference between the cell lines, and that it is in fact the HBL 100 cells

that have higher basal PI3K activity (although this may be due to co-expression of differing isoforms).

e) DNA damage

The gene product for cyclin dependent kinase inhibitor 1A (*CDKN1A*) is otherwise known as p21 and acts to block cell cycle progression particularly in response to DNA damage. Repression of cell cycle inhibitors in tumour cell lines (as shown in the MDA MB468 cells versus the HBL 100 cells) is common, and it has been shown that high levels of phosphorylated Akt may further decrease their inhibitory ability (Graff *et al,*. 2000, Zhou *et al.,* 2002). The downregulation of the *MDM2* (mouse double minute 2) gene in MDA MB468 cells compared to the HBL 100 cells, was perhaps indicative of the genotype for this cell line as MDM2 is a p53 binding protein. In non-tumourigenic cells, MDM2 functions to bind to and inhibit p53, in order to mediate p53 driven apoptosis and cell cycle arrest. However, the MDA MB468 cells are p53 mutant, rendering *MDM2* non-functional in this respect and perhaps leading to its downregulation/low levels of expression.

f) Drug metabolism and resistance

The *ABCB1* gene encodes the multidrug resistance protein 1 (MDR1) otherwise known as p-glycoprotein, which is the drug efflux pump responsible for decreased intracellular drug accumulation that is often overexpressed in tumour cell lines (Das *et al.,* 2001). That the tumourigenic MDA MB468 cells expressed lower levels than the HBL 100 cells is surprising, although again mRNA levels may not reflect levels of a functional protein.

g) Metastasis

Metastasis-associated matrix metalloproteinase genes were generally expressed at lower levels in the MDA MB468 cells compared to the HBL 100 cells, with the exception of *MMP7*. It has already been discussed that *in vitro,* the HBL 100 cells appear to have high MMP protein levels and are phenotypically more invasive than the MDA MB468 cells.

h) Oncogenes

CCND1 is the gene encoding cyclin D1, and appeared to be expressed at high levels in the MDA MB468 cells compared to the HBL 100 cells, which again may be expected in cells with a tumourigenic phenotype and deregulated cell cycle control. However, at the level of

the protein (section *5.2.1*) there appeared to be little difference in expression between the two cell lines, although this was not compared directly on the same gel.

i) Protein kinases

Mitogen activated protein kinase 9 (*MAPK9* – otherwise known as *JNK2*) was expressed at higher levels in the MDA MB468 cells when compared to the HBL 100 cells (although this was not the case for protein levels (Squires *et al.*, 2003)), and codes for an essential protein kinase that acts in response to pro-inflammatory cytokines. Ultimately, phosphorylation of MAPK9 at a protein level would lead to phosphorylation and activation of transcription factors such as ATF2 and c-jun.

The gene product of *RIPK1* (receptor (TNFRSF1) interacting serine-threonine kinase1) is closely associated with the induction of apoptosis via its ability to associate with the death domains of FAS and TRADD, and is required for TNFR1 induction of NF-κB. Its expression at lower levels in the MDA MB468 cell line, may again suggest that this cell line is less able to undergo apoptosis under resting conditions.

j) Transcription factors

The *FOS* gene is responsible for production of c-fos, which is an essential transcription factor in the stress-induced signalling pathways, and its expression has been found to be significantly correlated to pathological stage in breast cancer (Morad et al., 1998). This gene appeared to be expressed at approximately 2 fold lower levels in the MDA MB468 cells compared to the HBL 100 cells (however, protein expression was higher in the MDA MB468 cells compared to the HBL 100 cells (Squires et al., 2003)). It would be predicted that expression would be highest in the more aggressive, tumourigenic MDA MB468 cells, although again, gene expression is not necessarily correlated to protein expression or activity.

Table 6.2 shows the differences between the HBL 100 and MDA MB468 cell lines in gene expression of molecules investigated within this study, compared with the level of protein expression in these two cell lines. Whilst not all proteins have been directly compared between cell lines, the table illustrates that gene expression patterns did not always correlate with protein expression.

Table 6.1 (a-j) Tables show fold change in gene expression in MDA MB468 cells compared to HBL 100 cells.

Figures in green represent a downregulation in MDA MB468 cells when compared to the HBL 100 cells, and those in red represent an upregulation.

a)

Apoptosis	Fold Change
AXL	2.43
BCL2L1	2.35
CASP1	2.77
CASP3	2.13
CASP4	2.03
CASP6	2.57
CDKN1A	3.47
E2F1	2.83
EGFR	2.09
ELK1	2.85
MCL1	2.75
TYRO3	3.25
UBL1	2.17

b)

Angiogenesis	Fold Change
ANGPT1	2.18
EPHB4	2.48
MMP 14	2.96
MMP 2	2.43
NOS3	2.25

c)

Cell cycle	Fold Change
ASNS	2.24
ATM	2.36
CBX3	3.30
CCNC	2.25
CCND3	2.16
CCNG1	3.79
CDC10	2.23
CDC16	2.18
CDK10	2.00
CDK5R2	3.28
CDK9	2.24
CDKN1A	3.47
CDKN2A	3.06
CDKN3	2.22
CUL2	2.00
DUT	2.56
E2F1	2.83
KIA0014	2.17
KIAA0075	2.44
MAPK3	1.99
MAPK6	2.75
MAPK9	2.65
MAPK9	2.47
MCM6	2.13
MDM2	2.67
PCNA	2.66
PPP2CB	2.41
PPP2R2B	2.39
PPP2R5E	2.47
RB1	2.02
RBBP8	2.13
RBMS1	2.04
STK4	3.25
TAF2N	7.15
TK1	2.61
UBE2V2	2.37
WRN	3.45
XRCC5	2.58

d)

Cell growth	Fold Change
GSPT1	2.28
ING1	2.50
PIK3CB	2.31
SPARC	7.63

e)

DNA damage	Fold Change
ATM	2.36
CDKN1A	3.47
MDM2	2.67
XRCC5	2.58

f)

Drug metabolism	Fold Change
ALDOB	2.03
ABCB1	4.09
CYP27	2.21
DDC	3.52
EGFR	2.09
ICAM1	2.06

g)

Metastasis	Fold Change
DDC	3.52
LAMR1	2.19
MMP 1	2.78
MMP 13	2.67
MMP 14	2.96
MMP 2	2.43
MMP 7	6.29
MMP24	2.06
MMP9	2.17
MTA1	2.77

h)

Oncogenes	Fold Change
ARHB	2.48
CCND1	2.08
CDKN1A	3.47
EGFR	2.09
ELK1	2.85
ELK4	2.00
ETS2	2.22
GOLGA5	2.85
MDM2	2.67
MET	2.26
MMP 2	2.43
RAB35	2.33
WNT5A	2.10
YES1	2.62

i)

Protein kinases	Fold Change
AXL	2.43
CCND1	2.08
CCND3	2.16
CDK10	2.00
CDK9	2.24
EPHB4	2.48
MAPK6	2.75
MAPK9	2.65
MAPK9	2.16
MET	2.26
NTRK2	2.27
RIPK1	2.21
STK4	3.25
TYRO3	3.25

j)

Transcription factors	Fold Change
E2F1	2.83
FOS	2.09
FOS	2.56
MEIS1	3.68
NAB1	3.46
NFATC1	2.29
PKNOX1	3.54
POU2F1	2.24

PROTEIN	PROTEIN EXPRESSION	GENE EXPRESSION
Akt1	**2 fold higher in HBL**	**No difference**
Akt2		No difference
Akt3		Not on array
Bax	Not directly compared	No difference
β-catenin	No difference	Not on array
Bcl-2	Not directly compared	No difference
Cox-2	5 fold higher in MDA	Not on array
Cyclin D1	Not directly compared	No difference
EGFR	**10 fold higher in MDA**	**2 fold lower in MDA**
ERK	Not directly compared	Not on array
FKHR	Not directly compared	No difference
GSK-3	Not directly compared	No difference
hIAP	Not directly compared	No difference
HSP70	Not directly compared	Not on array
p110	Not directly compared	2 fold higher in MDA
p50	**No difference**	**No difference**
p65	**2 fold higher in HBL**	**No difference**
p70S6K	Not directly compared	Not on array
p85	**No difference**	**No difference**
PTEN	**Not expressed in MDA**	**No difference**
RELB	**No difference**	**No difference**

Table 6.2 Comparison of gene expression profiles from the array with levels of proteins studied in this thesis between the HBL 100 and MDA MB468 cells. *'No difference' pertains to the fact that protein/genes were expressed at a similar level between cell lines.*

Overall, it would appear from the gene changes shown, that the MDA MB468 cells would be less likely to undergo apoptosis, but to exhibit increased cell cycling, which is in keeping with their origin as a highly tumourigenic cell line. However, the higher expression

of angiogenic and metastasis-associated genes in the HBL 100 cells was somewhat unexpected, although this may have resulted from their SV40 immortalisation. As mentioned previously, it is difficult to have a true comparison between cell lines originating from differing individuals due to the inherently large amounts of genetic variability expected.

6.2 The effect of I3C on gene expression in the HBL 100 cells

HBL 100 cells were treated for 5 or 24 hours with 50μM I3C, and changes to gene expression levels were compared to control cells treated for the same length of time with an equivalent percentage of DMSO. *Table 6.3 (a-i)* depicts gene changes (fold difference) in I3C-treated compared to DMSO control cells.

In these cells, I3C appeared to downregulate many genes 2 fold or more, following a 5 hour treatment, with upregulation in differing genes observed generally only following a 24 hour treatment (although there were far fewer gene changes observed at this later time point). Genes were again clustered into groups significant within the carcinogenic process, with downregulated expression observed in many genes that are often overexpressed within this disease state. The large number of genes in which changes were induced suggests that not all of these changes are functional at the level of the protein, although decreased expression of the cyclin proteins, for example, was indicative of the slowing of the cell cycle observed in *section 3.3,* and the I3C-induced decrease of cyclin D1 protein levels observed in *section 5.2.1.* As the HBL 100 cells were originally derived from a non-tumour cell line, it was difficult to assess the relevance of some of the I3C-induced alterations to gene expression with respect to chemopreventive effects in breast cancer. However, array data may suggest that SV40 immortalisation of the HBL 100 cells has engendered characteristics typical of tumour cell lines

a) Angiogenesis

I3C induced a decrease in expression of angiogenic proteins following a 5 hour treatment, but had no effect following a 24 hour treatment. MMP14 expression was approximately 3 fold higher in the HBL 100 cells compared to the MDA MB468 cells, which was reduced by approximately 3.5 fold upon a 5 hour I3C treatment. This may suggest that I3C is able

to inhibit the invasive potential of the HBL 100 cells, potentially providing a mechanism for its chemopreventive action.

b) Apoptosis

Downregulation of many of the caspases (including 3 and 7) occurred following a 5 hour I3C treatment. This may explain the resistance to apoptosis observed in this cell line even following very high doses of I3C. Even though the anti-apoptotic *BCL2* was also downregulated, it is unlikely that this event alone could overcome the decreased activity of the caspases.

c) Cell adhesion

The protein product of *PECAM1* (platelet/endothelial cell adhesion molecule) is expressed at endothelial intercellular junctions, and is involved in transendothelial migration, which interestingly, may be under the control of PI3K (Poggi *et al.,* 2002) and is associated with angiogenic initiation. *PECAM1* downregulation by I3C, may therefore result in decreased angiogenesis and invasion potential in this cell line.

d) Cell cycle

Following a 5 hour I3C treament, numerous cell cycle genes were downregulated by 2 fold or more in the HBL 100 cells. Following a 24 hour I3C treatment, no genes were downregulated by as much as 2 fold, suggesting that I3C had a transitory, short-lived effect. Cell cycle genes that were upregulated following a 24 hour treatment included *E2F5* and the transcription factors *Dp1/2 (TFDP1/2).* E2F5 binds to E2F sites that are present in the promoters of many genes involved in cell proliferation, with TFDP1 and 2 also able to stimulate E2F dependent transcription. It would be interesting to see whether further long-term I3C treatments also induced stimulation of E2F-regulatory gene, as no increase in cell cycling was observed following a 48 hour I3C treatment.

e) Drug metabolism and resistance

Following a 5 hour I3C treatment, gene expression of the ATP-binding cassette protein *ABCB1* (*MDR1* encoding p-glycoprotein) was reduced by approximately 4 fold. This may suggest that I3C could decrease drug efflux from these cells through inhibition of *ABCB1* levels. Interestingly, *ABCB1* expression levels were approximately 4 fold higher in the HBL 100 cells when compared to the MDA MB468 cells.

f) Metastasis

Gene expression of matrix metalloproteinases 16, 17 and 26 was decreased following a 5 hour I3C treatment, suggesting that there may be potential for I3C as an anti-metastatic agent.

g) Oncogenes

The *ELK1* gene encodes the Elk1 protein, which as a member of the Ets oncogene family functions to stimulate transcription as does the gene product of *JUND*, which is able to bind AP-1 consensus sequences within promoter regions. *BRAF* (v-raf murine sarcoma viral oncogene homologue B1) and *PLD1* (phospholipse D1) are both involved in signal transduction from cell membrane to nucleus and so can promote the oncogenic process through deregulated cell signalling. Downregulation of these genes by I3C may further suggest its potential as an anti-oncogenic agent.

h) Protein kinases

Following a 5 hour I3C treatment, *AKT2* gene expression was downregulated approximately 2.4 fold, which was unlikely to occur at the level of protein at the same time point, as total Akt (1, 2, 3) protein levels did not decrease following treatments with up to 1mM I3C (section *4.1*). At 5 hours, there appeared to be a consistent downregulation of protein kinase genes associated with tumourigenesis such as *JAK1, MAPK9* and *MAPK12,* suggesting that I3C may have the ability to interfere with a wide variety of signalling events.

i) Transcription factors

The gene product of *NFKB1* is associated with the processing of the NF-κB p50 subunit. A 2 fold downregulation in gene expression was observed following a 5 hour I3C treatment, whilst after 24 hours, a 2 fold upregulation was observed. p50 protein levels in response to I3C have not been investigated, although I3C had no effect upon p65 gene expression and protein levels, which appeared to be constitutive in this cell line (section *5.1.3*).

Table 6.3 (a-i) Effects of a 5 or 24 hour I3C treatment (50μM) on gene expression in the HBL 100 cells. *Values in green represent a downregulation of genes, and those in red, an upregulation. Values represent a mean of changes in expression from 3 arrays.*

a)

Angiogenesis			
5hr	Fold Change	24hr	Fold Change
LMO2	3.95		
MMP 14	3.43		
MMP9	2.57		
NOS3	2.14		

b)

Apoptosis			
5hr	Fold Change	24hr	Fold Change
BCL2	2.30	CASP10	2.36
CASP1	2.30	HRK	2.55
CASP1	3.30		
CASP10	5.95		
CASP3	2.49		
CASP4	2.60		
CASP5	4.06		
CASP6	2.55		
CASP7	3.00		
CASP8	2.01		
CASP9	2.64		
DEDD	2.85		
DFFA	2.49		
MAP2K6	2.11		
TNFRSF10D	2.22		

c)

Cell adhesion			
5hr	Fold Change	24hr	Fold Change
PECAM1	3.68		

d)

Cell cycle			
5hr	Fold Change	24hr	Fold Change
BCL2	2.30	E2F5	2.15
CCND2	2.05	TFDP1	2.47
CCND3	2.34	TFDP2	2.24
CCNE1	1.99		
CDC25A	2.02		
CDC25B	2.02		
CDC25C	2.35		
CDC2L	2.23		
KIAA0075	2.65		
LMNA	2.57		
MAPK9	3.37		
PCTK1	2.34		
PCTK3	3.18		
PPP1R1A	2.25		
RBL2	2.25		
STK4	2.04		
TOP2A	2.14		
Wee1	2.38		

e)

Drug Metabolism			
5hr	Fold Change	24hr	Fold Change
ABCB1	4.11		
TOP2A	2.14		
TPMT	2.50		

f)

Metastasis			
5hr	Fold Change	24hr	Fold Change
MMP16	2.20		
MMP17	2.34		
MMP26	1.96		

g)

Oncogenes			
5hr	Fold Change	24hr	Fold Change
BRAF	6.60		
CCND2	2.05		
ELK1	2.79		
ERBB3	2.32		
JUND	2.49		
KRAS2	2.21		
MYCN	2.14		
PLD1	2.17		

h)

Protein Kinases			
5hr	Fold Change	24hr	Fold Change
AKT2	2.41	FGR	2.09
BRAF	6.60		
CAMK4	2.04		
CCND2	2.05		
CCNE1	1.99		
ELK1	2.79		
ERBB3	2.32		
ERBB3	2.32		
FGR	4.79		
JAK1	4.22		
MAP2K5	2.52		
MAP2K6	2.11		
MAPK12	4.11		
MAPK9	3.37		
RIPK1	3.27		
STK4	2.04		

Transcription factors			
5hr	Fold Change	24hr	Fold Change
JUN	1.99	NFKB1	2.03
JUND	2.49	TFDP1	2.47
MAPK10	1.97		
MYCN	2.14		
NFKB1	2.17		

6.3 The effect of I3C on gene expression in the MDA MB468 cells.

There were many alterations to gene expression levels in response to I3C treatment in this cell line at both 5 and 24 hours, although again, genes up or downregulated at 5 hours were not consistently up or downregulated at 24 hours. I3C may be affecting genes differentially according to treatment time, or it could be that genes altered following a 24 hour treatment, may be altered in response to I3C metabolites or acid condensation products. This may partly explain the lack of consistent alterations to the same genes at the two time points.

a) Angiogenesis

The angiopoeitin 1 gene (*ANGPT1*) plays an essential role in blood vessel stabilisation and maturation, and was expressed at approximately 2 fold higher levels in the MDA MB468 cells compared to the HBL 100 cells (*Table 6.1b)*. Following a 5 hour I3C treatment, this gene was downregulated approximately 3.5 fold, as was the matrix metalloproteinase *MMP14*. *MMP14* appeared consistently downregulated following a 5 hour I3C treatment in both MDA MB468 and HBL 100 cells, and may provide an interesting candidate for further study.

b) Apoptosis

Expression of many pro-apoptotic genes again appear to be downregulated following an I3C treatment, particularly at 5 hours. Genes that were consistently downregulated at both 5 and 24 hours include *BCL2* and *TRADD* (TNFRSF1A-associated via death domain), whose gene products both possess anti-apoptotic activity. Interestingly, I3C did not decrease Bcl-2 protein levels until a 48 hour treatment (*section 3.5*). Other anti-apoptotic genes that are

downregulated by I3C include *TRAF6* encoding TNF receptor associated factor 6, which is involved in NF-κB activation.

c) Cell adhesion

The cell adhesion gene *PECAM1* was the only cell adhesion gene downregulated in both the HBL 100 and MDA MB468 cells following a 5 hour I3C treatment. Following a 24 hour treatment, *CMAR* (cell matrix adhesion regulator) was downregulated by approximately 2 fold, although this gene may actually act to suppress tumour invasion via influencing cell adhesion to collagen (Durbin *et al.,* 1997).

d) Cell cycle

Following a 5 hour I3C treatment, genes for cyclins B1 (accumulates during G2 phase of the cell cycle), D2, D3 (essential for control of G1/S) and G2 (peaks in S phase – possible negative cell cycle regulator) were downregulated similarly to the HBL 100 cells. This suggests that I3C was able to slow or inhibit cell cycle progression, which may be indicated by the S phase accumulation of cells shown by flow cytometry, although no specific phase arrest was observed (section *3.3*). Downregulation of *CDC25A* (cell division cycle 25A) might have occurred in conjunction with *CCNB1* downregulation, as enzyme activity for the protein product of *CDC25A* is stimulated by cyclin B1. cdc25c protein is similarly essential for cell cycle progression due to its ability to activate cdc2, with reduced expression of *CDC25C* possibly resulting in the downregulation of *PIN1* (protein (petidyl-prolyl cis/trans isomerase) NIMA-interacting 1) which has an important role in regulation of mitosis. The downregulation of these two genes provided an interesting target for further investigation. However, when the HBL 100 and MDA MB468 cells were treated for 5 hours with I3C, no consistent decrease in PIN-1 protein levels was observed (data not shown).

In this cell line, only *BCL2* and *CDC25A* were consistently downregulated at both treatment times. Gene expression of the cell cycle inhibitors *CDKN1A* (p21) and *CDKN2A* was lower in the MDA MB468 cells than the HBL 100 cells, and following a 24 hour I3C treatment, levels were further repressed in the MDA MB468 cell line.

e) Cell growth

Few changes were observed in other growth regulatory genes, other than a downregulation of *TGFB1* (transforming growth factor, beta 1) following a 5 hour I3C treatment.

f) DNA damage

The *GADD45A* gene product (growth arrest and DNA damage inducible, alpha) stimulates DNA excision repair. The downregulation of *GADD45A* in conjunction with the downregulation of *CDKN1A* following a 24 hour treatment, may result in the entry of damaged cells into the cell cycle, suggesting a negative effect of I3C.

g) Drug metabolism and resistance

The ATP-binding cassette genes (*ABCB1, ABCC2* and *ABCC6)* were downregulated following a 5 hour I3C treatment. Decreased expression of the *ABCB1* (p-glycoprotein) gene, as with the HBL 100 cells, may result in a decreased drug efflux from cells, and so enhance efficacy of drug treatments. A decreased expression of the ABCC6 gene may similarly result in an enhanced drug efficacy, due to a decrease in active transport of drugs from cellular organelles.

h) Metastasis

Gene expression of the matrix metalloproteinase family appeared to be greatly influenced by a 5 hour I3C treatment in this cell line, although only MMPs 16, 17 and 26 were similarly downregulated in the HBL 100 cells. However, the genes that were downregulated following a 5 hour treatment were not decreased following a 24 hour treatment.

i) Oncogenes

A decrease in *EGFR* gene expression was observed following a 5 hour I3C treatment, although there was little evidence for this occurrence at the protein level. Downregulation of NF-κB genes (*NFKB2* and *RELB*) were also observed at this time point. Whilst protein levels for the gene products of *NFKB2* (p100 precursor for p52) and *RELB* have not been examined in response to an I3C treatment, there was no effect of I3C upon NF-κB nuclear translocation suggesting that even if protein levels were decreased, it would be of little functional consequence. An interesting effect following a 24 hour I3C treatment, is the 3.8

fold decrease in *ERBB2* (otherwise known as HER2/NEU) gene expression, which is frequently overexpressed in breast cancers and may provide a suitable candidate for further investigation.

j) Protein kinases

The serine/threonine kinase ILK (integrin linked kinase) gene was downregulated following a 5 hour I3C treatment, and whilst one of its functions is in integrin-mediated signal transduction, it is also one of the putative candidates for phosphoinositide-dependent kinase 2 (PDK2). PDK2 is necessary for the phosphorylation of Akt on its serine 473 residue, in order for the molecule to become fully active, and it may be that a downregulation of ILK (if it is does act as PDK2) may result in decreased Akt phosphorylation (refer to *section 1.6*). Recent preliminary data suggests that whilst I3C does not affect total ILK protein levels following a 5 hour treatment, it may decrease ILK kinase activity (data not shown).

k) Transcription factors

Downregulation of the transcription factor gene *JUND* following a 24 hour I3C treatment was again indicative of the ability of I3C to interfere with cell signalling pathways, and if translated to the level of the protein, could ultimately result in decreased transcription of genes containing an AP-1 binding site.

Table 6.4 (a-k) Effect of I3C (50μM) on gene expression in MDA MB468 cells following a 5 or 24 hour treatment. *Values in green represent a downregulation of gene expression, and those in red, an upregulation. Values represent the mean of changes in gene expression from 3 arrays.*

a)

Angiogenesis			
5hr	Fold Change	24hr	Fold Change
ANGPT1	3.44		
BAI3	2.48		
MMP 14	3.83		

b)

Apoptosis			
5hr	Fold Change	24hr	Fold Change
BCL2	2.38	BCL2	2.15
CASP10	6.38	BCL2L1	2.08
CASP4	3.82	CASP3	1.97
CASP6	4.30	CASP6	2.05
DFFA	2.29	CDKN1A	2.25
TRADD	3.45	CSE1L	3.55
TNFRSF10D	3.03	DEDD	2.70
TRAF6	2.48	MCL1	2.32
TRAILR4	2.15	TNFAIP3	1.96
TYRO3	2.11	TRADD	3.52
		TRAF4	2.30

c)

Cell adhesion			
5hr	Fold Change	24hr	Fold Change
PECAM1	2.90	CMAR	2.21
		ITGB3	2.52

d)

Cell cycle			
5hr	Fold Change	24hr	Fold Change
BCL2	2.38	BCL2	2.15
CCNB1	3.10	BCL2L1	2.08
CCND2	1.96	CCNH	2.05
CCND3	2.13	CDC25A	2.16
CCNG2	2.29	CDKN1A	2.25
CDC25A	3.93	CDKN2A	2.08
CDC25C	2.58	CSE1L	3.55
CDK8	2.15	NCOR2	2.25
MAPK10	2.01	PCTK1	2.51
MAPK8	2.37	PPP2R1A	2.96
NOL1	2.73	PPP2R5B	2.38
PIN	2.11	PPP4C	3.78
		PPP5C	5.13
		STK4	2.04

e)

Cell growth			
5hr	Fold Change	24hr	Fold Change
TGFB1	2.77		

f)

DNA damage			
5hr	Fold Change	24hr	Fold Change
GADD45A	2.02	CDKN1A	2.25
		MDM2	2.14

g)

Drug metabolism			
5hr	Fold Change	24hr	Fold Change
ABCB1	2.82	CYP11B1	2.45
ABCC2	2.02	CYP2D6	2.21
ABCC6	4.74		
CYP1A1	4.37		
CYP1A1/IL2RB	15.79		
CYP1B1	1.71		
CYP4B1	2.07		
DPYD	3.33		
EGFR	2.10		

h)

Metastasis			
5hr	Fold Change	24hr	Fold Change
MMP 1	3.95	ETV4	2.39
MMP 13	2.80	MMP15	2.33
MMP 14	3.83		
MMP10	2.55		
MMP16	2.00		
MMP23B	2.97		
MMP8	3.61		

i)

Oncogenes			
5hr	Fold Change	24hr	Fold Change
EGFR	2.10	CDKN1A	2.25
NFKB2	2.90	EGFR	2.19
PLD1	2.91	ERBB2	3.83
RELB	5.07	JUND	3.39
YES1	2.04	MDM2	2.14
		NCOA4	3.39

j)

Protein kinases			
5hr	Fold Change	24hr	Fold Change
CCND3	2.13	MAP2K2	2.36
ILK	2.37	STK4	2.04
MAP2K6	1.98	TGFBR3	2.00
MAP4K1	3.44		
MAPK10	2.01		
MAPK8	2.37		
NTRK3	3.48		
TESK1	2.00		

k)

Transcription factors			
5hr	Fold Change	24hr	Fold Change
MAPK10	2.01	JUND	3.39
MAPK8	2.37		
NCOA1	2.54		
NFKB2	2.90		

DISCUSSION

The results section has given a brief overview of some of the changes to gene expression induced by I3C, and discussed those that may be relevant within the context of breast cancer and chemoprevention. Whilst the microarray has provided many potential targets for investigation, it would prove impossible to further explore all of these targets, even though germaine to this project. There are four potential gene targets, which would be useful to further our understanding of the effects of I3C in these breast cells. Two of these targets have already been investigated with respect to I3C in breast cell systems, so providing corroboration for this data, and two targets provide potentially novel candidates.

Cytochrome P4501A1

I3C has been well documented to cause an increase in phase I and II metabolising enzymes (Manson *et al.,* 1998, Katchamart and Williams, 2001, Larsen-Su and Williams, 2001), and is typical of many chemopreventive agents in that it can enhance detoxification and elimination of carcinogens. Estrogen can be metabolised via two major pathways to form 16α hydroxy estrone (which is highly estrogenic) or 2-hydroxy estrone (which is anti estrogenic). As already discussed in section *1.3.3,* the cytochrome p450s and in particular CYP1A1 play an essential role in estrogen metabolism, with CYP1A1 induction leading to an increase in metabolism via the 2-hydroxy non-genotoxic pathway. The induction of CYP1A1 by I3C in ER+ve cells such as the MCF7 and T47Ds has previously been shown at the level of the protein and associated changes to estrogen metabolism (Ashok *et al.,* 2001). However, the study by Ashok did not show any effect upon the MDA MB468 cells, whereas gene expression was upregulated by 15 fold in the present study (*table 6.4g*) probably due to induction of the aryl hydrocarbon receptor (Vang *et al.,* 1999).

ABCB1 (p-glycoprotein, MDR1)

Success of chemotherapy in the treatment of breast cancer may often be hindered by the development of drug resistance. An MDR1 phenotype has been reported in breast cancers (Das *et al.,* 2001), with a good prognosis associated with an MDR1-negative phenotype in breast cancer patients (Filipits *et al.,* 1999). A contributory role for MDR1 has therefore been suggested in multidrug resistance in conjunction with alterations to other genes, such as those involved in drug metabolism. I3C and its acid condensation products have been

shown to increase the cellular accumulation of doxorubicin, which is a substrate for the p-glycoprotein drug efflux pump, and also to sensitise resistant cells to chemotherapeutic agents (Christensen and LeBlanc, 1996), whilst recently, I3C has been shown to reverse the overexpression of p-glycoprotein in vinca-alkaloid-induced drug resistance (Arora and Shukla, 2003). Cover *et al.,* (1999) showed that I3C was able to enhance the efficacy of tamoxifen-induced cell cycle arrest in the MCF7 cells, and whilst they did not propose a mechanism for this cooperation, it may be due to the ability of I3C to decrease drug efflux from the cell and so enhance accumulation. There have been data however, to suggest that the MDA MB468 cells are relatively sensitive to doxorubicin toxicity despite their aggressive phenotype. MDA MB468 cells have been shown to express MDR1 at similar levels to that seen in the HBL 100 cells (Turton *et al.,* 2001), although data here suggests a four fold lower expression in the MDA MB468 cells compared to the HBL 100 cells. However, the downregulation of MDR1 and upregulation of CYP1A1 gene expression in the MDA MB468 cells in our study, suggests that I3C may exhibit potential for co-administration in combination therapy in order that chemotherapeutic agent uptake may be enhanced, whilst at the same time, toxicity may be reduced.

Interestingly, the MDR1 gene possesses a consensus NF-κB DNA binding site and NF-κB can transactivate MDR1 in HCT15 colon cancer cells (Bentires-Alj *et al.,* 2003). The downregulation of NF-κB DNA binding by I3C observed in *section 5.1*, may be consequential for this downregulation of the MDR1 gene. There is also recent evidence to suggest that membrane ruffling (which is one of the earliest membrane changes associated with cellular invasion) caused by MDR1-mediated drug transport, is mediated through activation of PI3K (Yang *et al.,* 2002). This may again suggest a potential role for chemoprevention by I3C, as it was not only able to decrease PI3K activity when added directly into an *in vitro* kinase assay in the MDA MB468 cells, but also decreased MDR1-gene expression.

ERBB2

The *c-erbB2* gene shares a high degree of homology with the *EGFR*, and is frequently found to be overexpressed at the gene and/or protein level in breast cancer. *ERBB2* gene amplification has been correlated with a poor clinical outcome and is generally associated with ERα-ve breast cancers (Revillion *et al.*, 1998) and tamoxifen resistance (De Placido *et*

al., 1998, Stearns *et al.,* 1998, Tsutsui *et al.,* 2002). Turpin *et al.,* (2002a,b) found *ERBB2* overexpression to frequently be associated with *TP53* (gene encoding p53) mutations in highly aggressive inflammatory breast cancers, with this gene also being mutated in the MDA MB468 cells. Interestingly, erbB2 is able to mediate *PIN1* expression via E2F, which is then able to enhance erbB2-mediated transformation (Ryo *et al.,* 2002). However, in the MDA MB 468 cells, *ERBB2* gene expression was not downregulated until 24 hours, whilst *PIN1* was downregulated at 5 hours. PIN1 protein levels remained constant following a 5 hour I3C treatment (data not shown), suggesting that the downregulation of both of these genes by I3C was probably not mechanistically connected in this system. Although there was no difference in the degree of *ERBB2* gene expression between the HBL 100 and MDA MB468 cells, as both cells are ERα-ve, this was not entirely unexpected. The decrease in *ERBB2* gene expression induced by I3C in the MDA MB468 cells, however, may prove interesting as it could be supportive of a reduction in their tumourigenicity.

Matrix metalloproteinases (MMPs)

In order for tumour cells to invade surrounding tissues and metastasise, proteolytic degradation of the extracellular matrix must first occur. MMPs function to degrade extracellular matrix molecules, and can be upregulated via a variety of cytokines including EGF, PDGF, IL-1 and TNFα (Benaud *et al.*, 1998). MMPs have been documented to be overexpressed in tumour tissues compared to surrounding normal tissues (reviewed by Toi *et al.*, 1998), with certain MMPs being upregulated specifically in breast cancer. MMP2 is expressed in the early stages of breast cancer, and purported to be a marker of malignant phenotype (Murray, 2001). Upregulation of MMPs 1 and 9 has also been observed in pre-invasive and invasive breast carcinomas, with their transcriptional control under the regulation of the Ets-1 transcription factor (Barrett *et al.,* 2002, Behrens *et al.*, 2001). Deregulation of the Akt pathway has also been established as a major invasion-promoting factor in cancer cells, with Kim *et al.,* (2001) observing an increase in MMP9 (which possess an NF-κB DNA binding site in its promoter region) mediated by an Akt-induced activation of NF-κB transcriptional activity.

I3C decreased levels of *MMP1* gene expression in the MDA MB468 cells. However, *MMP1* was expressed at higher levels in the HBL 100 cells than the MDA MB468 cells, which may explain the increased invasiveness of this cell line (Gordon *et al.,* 2003). I3C

has been found to decrease invasion and migration of T47D breast cells in culture through upregulation of the cell-cell adhesion molecule E-cadherin (Meng *et al.*, 2000), but there is currently no evidence in the literature for an I3C-induced decrease in invasive potential due to decreased MMP expression.

CHAPTER 7
GENERAL DISCUSSION

The potential for I3C as a chemopreventive agent has been well documented in the literature over the past ten years, but it is only recently that its mechanisms of action at a cellular level have been investigated. The advantages of using naturally derived agents such as I3C, are that they have a prolonged history of use within the human population so much is already known about their safety, and that they are generally cheap to produce, unlike many current anti-cancer drugs. However, the need for better understanding of the mechanisms of action of chemopreventive agents has been highlighted by studies such as the CARET (β-carotene and retinol efficacy trial) and ATCB (alpha-tocopherol, beta-carotene cancer prevention study) trials. In these studies, there was no cancer preventive effect of β-carotene or retinol in a healthy population, but individuals who smoked, and continued smoking throughout the trial, developed a higher risk for lung cancer, resulting in early cessation of the trials (Smigel, 1996, De Smet, 1996, Patrick, 2000). However, these studies investigated β-carotene with respect to its behaviour as an antioxidant, and did not take into account the potential for non-antioxidant-mediated effects. It is now thought likely that the majority of β-carotene's effects *in vivo* were due to its effects on biochemical pathways, rather than antioxidant properties (Pryor *et al.,* 2000). These trials demonstrate not only the importance of investigation into all of the properties of an agent, but also show how difficult it is to isolate a single component of a healthy diet as the beneficial element.

To date, there have been no long-term studies in humans undertaken using I3C. However, short-term studies in both healthy and high-risk individuals have shown success with regards to favourable alteration to biomarkers (Wong *et al.,* 1997, Michnovicz., 1998) and inhibiting progression of pre-neoplastic conditions (Rosen *et al.*, 1998, Bell *et al.*, 2000). It has been observed in some animal models that whilst I3C may be effective in prevention of estrogen-dependent cancers, there might be detrimental effects with regards to promotion of other cancers (reviewed by Dashwood, 1997). Manson *et al.,* (1998) showed I3C to prevent aflatoxin-induced liver carcinoma in a long-term feeding study when administered both pre- and post-initiation. This was due to the ability of I3C to induce phase I and II drug metabolising enzymes when administered pre-initiation, and possibly through the induction of suppressing mechanisms such as inhibition of total tyrosine kinase and ornithine decarboxylase activity, when administered post-initiation. However, others have proposed I3C to be a tumour promoter (Dashwood, 1997), and it is for these reasons that it

is essential to characterise the molecular mechanisms of action and pharmacokinetics of I3C prior to commencement of large prevention studies in humans.

At a cellular level it was interesting to observe that I3C was four-fold more potent at inhibiting proliferation in the MDA MB468 tumour cell line compared with the normal-derived HBL 100 cell line, suggesting that it may be the possession of a tumourigenic phenotype that allows I3C to exert its most striking effects. The MDA MB468 cells lack or have a mutated version of the tumour suppressor genes p53, Rb and PTEN, overexpress EGFR, lack ERα, possess constitutive NF-κB activity and have high levels of Akt phosphorylation. All of these alterations from a 'normal' phenotype will ultimately result in gross deregulation of cell cycle and cell survival properties, so that from these phenotypic characteristics alone, the MDA MB468 cells are representative of an aggressive tumour cell type. It is possible that these alterations could render this cell line more susceptible to the effects of a chemopreventive agent, particularly if there is specific targeting of one of these deregulated events by the agent.

Further investigation showed that neither the HBL 100 nor MDA MB468 cell lines underwent a phase-specific cell cycle arrest in response to I3C, and that the MDA MB468 cells only, underwent apoptosis. The HBL 100 cells, as well as being more resistant to the effects of I3C, were also more able to recover than the MDA MB468 cells once the treatment was removed. This difference in reversibility could be explained by the early commitment to the apoptotic process induced by an I3C treatment in the MDA MB468 cells. The occurrence of apoptosis in the PTEN null MDA MB468 cells only, led to the hypothesis that I3C may be able to exert this effect by alteration to signalling events within a cell survival pathway such as the PI3K pathway.

The MDA MB468 cells were found to possess approximately four-fold higher levels of phosphorylated Akt than the HBL 100 cells. This high level of phosphorylation was not unexpected, due to the lack of the PTEN tumour suppressor in this cell line. Under normal circumstances, PTEN would regulate signalling through the PI3K pathway by decreasing levels of PIP signalling intermediates. That I3C was able to decrease levels of Akt phosphorylation in the MDA MB468, but not in the HBL 100 cells, led us to consider that this may be an important event in the induction of apoptosis in the former cell line. The

hypothesis that this may be related to PTEN status was supported when no decrease in Akt phosphorylation and little induction of apoptosis was observed following an I3C treatment, in the high PTEN-expressing prostate cell line DU145, in contrast to the low PTEN-expressing LNCaP prostate cells, which underwent greater induction of apoptosis. The LNCaP cells possess high Rb levels (Lu *et al.,* 2000), low EGFR levels (Sherwood *et al.*, 1998), wildtype p53 and are androgen dependent (Gupta *et al.*, 2000), only sharing similar characteristics with the MDA MB468 cells with respect to their Akt and PTEN status.

It was expected that this decrease in Akt phosphorylation would potentially result in many changes to expression of Akt-regulated molecules within the signalling cascades involved in apoptosis and cell proliferation. The Bcl-2 family members were investigated in more detail in the HBL 100 and MDA MB468 cells. Bcl-2 levels were decreased in both cell lines following a 48 hour I3C treatment, whilst there was no observable decrease in Bax levels at any time point. Increased expression levels of Bax and an increase in its translocation to the mitochondria, have previously been reported in response to I3C in the MDA MB435 cell line (Rahman *et al.,* 2000). However, whilst it is possible that I3C-induced Bax mitochondrial translocation was a contributing mechanism for induction of apoptosis in the present study, no change in the levels of anti-apoptotic Bcl-2 were observed until time points later than 24 hours.

It was also surprising that no clear cell cycle arrest was observed in either the HBL 100 or MDA MB468 cells, as I3C has previously been shown to induce a G1 cell cycle arrest in MCF7 cells (Cover *et al.*, 1998). This was also unexpected following microarray investigation into I3C-induced changes to gene expression in these cell lines. Many cell cycle regulatory genes were found to be downregulated by I3C, and in the HBL 100 cells, cyclin D1 protein levels were also diminished. In the MDA MB468 cells, it could be proposed that the decrease in Akt phosphorylation should have resulted in cell cycle inhibition due to the association of high levels of phosphorylated Akt with inhibition of the cell cycle inhibitors p21 and p27 (Graff *et al.* 2000). Phosphorylated Akt is able to phosphorylate p21 and so prevent its nuclear translocation and activity as an inhibitor of cell cycle progression. It was proposed that this lack of cell cycle inhibition observed in the MDA MB468 cells may have been due to the rapid induction of apoptosis by high concentrations of I3C, with alterations to cell cycling perhaps occurring following long-term low-dose I3C treatments that would not cause significant apoptosis. However, recent

data have shown that long-term, low-dose I3C treatments serve only to further increase S-phase accumulation in this cell line (experiments performed by Dr. A. Hudson – data not shown). Suprisingly, p21 mRNA levels were decreased in the MDA MB468 cells following a 24 hour I3C treatment. If I3C decreased p21 protein levels, the lack of effect of decreased phospho-Akt on cell cycle inhibition could be explained, as I3C-induced p21 nuclear transport would not occur due to diminished levels of this protein in its presence.

The observed effects of LY294002 on PARP cleavage were not as substantial as those induced by I3C in the MDA MB468 cells, even though LY294002 was able to completely inhibit Akt phosphorylation (*chapter 4*), whilst in the HBL 100 cells, LY294002 treatment did not cause any PARP cleavage (*chapter 3*). This led to the hypothesis that it was not purely the ability of I3C to inhibit Akt phosphorylation that was responsible for the induction of apoptosis in the MDA MB468 cell line, but the proposal that I3C was likely to affect other signalling pathways pertinent to cell survival. It may be that the decrease in Akt phosphorylation at early time points is sufficient to sensitise cells to other potential pro-apoptotic effects of I3C, which are currently under further investigation.

Evidence in the literature suggests that the effect of I3C on NF-κB DNA binding in the MDA MB468 cell line, could be due to the observed decrease in Akt phosphorylation. However, the lack of effect of LY294402 on NF-κB nuclear levels and DNA binding indicates that the I3C-induced decrease in NF-κB DNA binding is independent from Akt in this cell line.

Interestingly, a recent report suggested that NF-κB was able to influence Akt activity (Meng *et al.,* 2002), which may have contributed to the decreased Akt phosphorylation observed in this study, and explained the lack of effect of an LY294002 treatment upon NF-κB DNA binding.

I3C upregulated total β-catenin levels by approximately 2 fold in the MDA MB468 cells. This may provide an explanation for the I3C-mediated decrease in NF-κB DNA binding, as β-catenin is able to bind the NF-κB p50 and p65 subunits and so prevent its interaction with DNA (Deng *et al.,* 2002).

There are many potential targets for NF-κB, and the effect of this I3C-mediated decrease in NF-κB DNA binding has been investigated in but a few. Downregulation of the *ABCB1 (MDR1)* gene in the MDA MB468 cells following a 5 hour I3C treatment is of particular interest, as it possessed a consensus NF-κB DNA binding site within its promoter region (Bentires-Alj *et al.*, 2003), and the MDR1 phenotype has been found to be reversed by I3C in MDR-1-transfected murine melanoma cells (Christensen and LeBlanc, 1996). However, it is likely that inhibition of NF-κB transactivation in its own right, also contributes to the induction of apoptosis in the MDA MB468 cell line. *Figure 7.1* shows a schematic proposal as to how I3C may induce apoptosis in the MDA MB468 cell line.

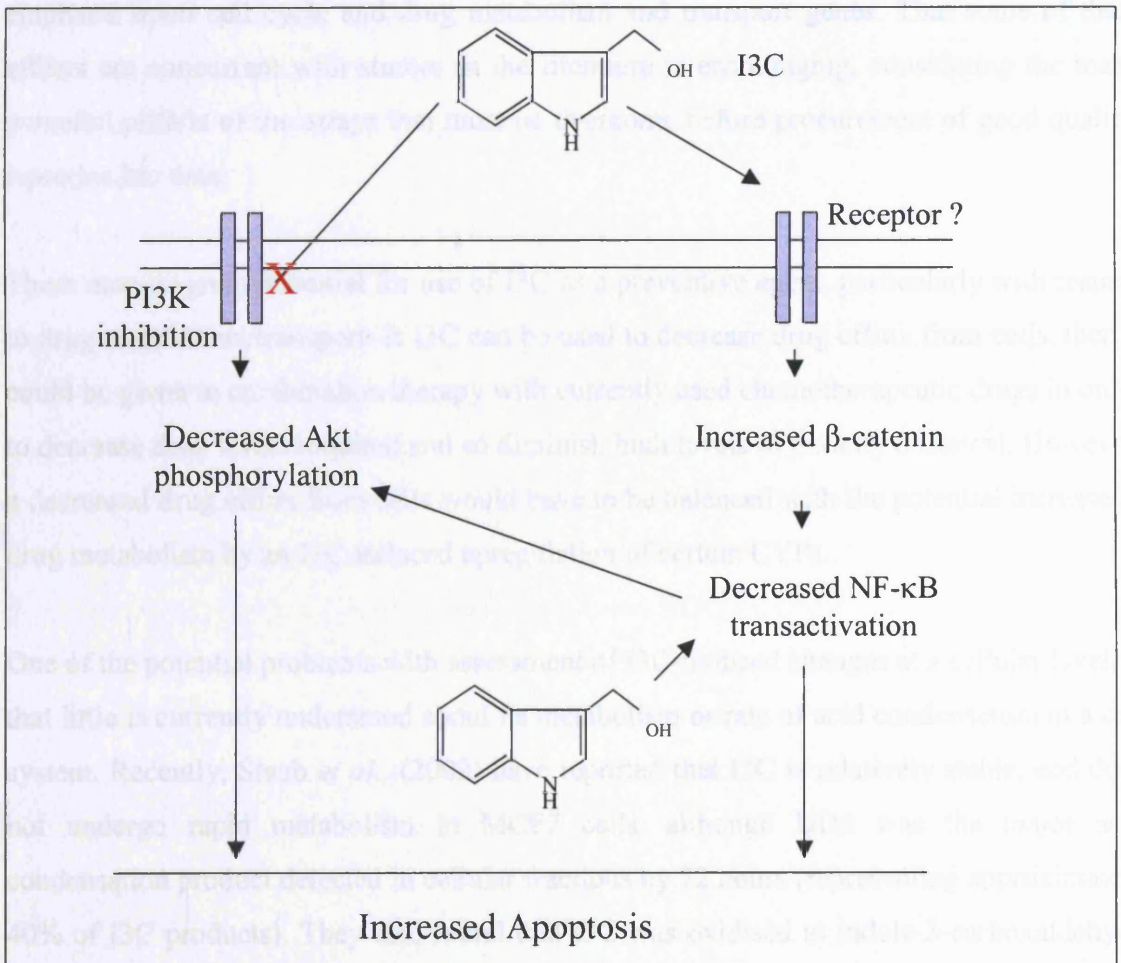


Figure 7.1 Potential mechanisms by which I3C could induce apoptosis through PI3K and NF-κB signalling in the MDA MB468 cells.

That anti-proliferative, pro-apoptotic effects occurred in the MDA MB468 and LNCaP cell lines, suggests a positive role for the chemopreventive effects of I3C in cancers possessing deficiencies in PTEN expression and deregulated PI3K signalling. To date, chemoprevention trials with I3C have utilised the ratio of urinary estrogen metabolites as a biomarker indicative of the efficacy of the agent. Although this gives a good indication that I3C is being systemically absorbed, it allows no prediction as to the efficacy of the agent against hormone-independent tumours. Investigation of the effects of I3C in hormone-independent tumours therefore requires the identification of other suitable molecular biomarkers of efficacy. The use of microarray analysis to identify further potential molecular targets for I3C has resulted in a plethora of potential candidates, with particular emphasis upon cell cycle and drug metabolism and transport genes. That some of these effects are concurrent with studies in the literature is encouraging, considering the many potential pitfalls of the arrays that must be overcome before procurement of good quality, reproducible data.

There may be great potential for use of I3C as a preventive agent, particularly with respect to drug metabolism/transport. If I3C can be used to decrease drug efflux from cells, then it could be given in combination therapy with currently used chemotherapeutic drugs in order to decrease dose levels required and so diminish high levels of toxicity observed. However, a decreased drug efflux from cells would have to be balanced with the potential increase to drug metabolism by an I3C-induced upregulation of certain CYPs.

One of the potential problems with assessment of I3C-induced changes at a cellular level, is that little is currently understood about its metabolism or rate of acid condensation in a cell system. Recently, Staub *et al.,* (2002) have reported that I3C is relatively stable, and does not undergo rapid metabolism in MCF7 cells, although DIM was the major acid condensation product detected in cellular fractions by 72 hours (representing approximately 40% of I3C products). They also found that I3C was oxidised to indole-3-carboxaldehyde and indole-3-carboxylic acid in a cell free system, so it may be possible that this could affect the chemopreventive properties of I3C seen in the studies shown here. *In vivo,* Anderton *et al.,* (unpublished) have shown that following a single oral dose of I3C in mice (250mg/kg), it was rapidly absorbed, and widely distributed throughout the tissues, with complete tissue elimination observed by 2 hours. The elimination of I3C from tissues was

concurrent with increasing DIM and LTr levels, indicating that acid condensation of I3C in the stomach had occurred, with the late tissue accumulation of condensation products probably due to a slower absorption rate. DIM has recently been shown to induce apoptosis and decrease Akt phosphorylation in the MDA MB468 cells (Anderton, Unpublished) and is currently undergoing further investigation within this laboratory. It may be that some of the chemopreventive effects of I3C are indeed elicited upon conversion to metabolites or acid condensation products. It is currently unkown whether in the MDA MB468 cells, I3C undergoes conversion to DIM in a similar manner to that observed in the MCF7 cells in Staub's study. If this is the case, then the early effects observed in response to I3C treatment are likely to be due to the effects of the parent compound, while effects observed at later time points possibly are due to metabolites or products such as indole-3-carboxaldehyde or indole-3-carboxylic acid. However, if metabolism and conversion of I3C are similar to that seen in tissues *in vivo*, then it is likely that these compounds will contribute more to the chemopreventive effects of I3C than the parent compound itself.

Chemopreventive agents should be able to fulfil a role in the restoration of normal cell function with minimal toxicity, which is not observed in current aggressive chemotherapy treatments. This investigation into the effects of I3C at a molecular level has given an insight as to how this agent may be useful in the therapy and/or prevention of aggressive estrogen-independent breast tumours. Biomarkers that may give an indication as to the suitability for use of I3C include PTEN status and levels of Akt phosphorylation, whereas markers of efficacy may include decreased erbB2, matrix metalloproteinases and p-glycoprotein expression and increased cytochrome P450 expression. From the array data, there were many more potential candidates, but these require further investigation. Studies are continuing to determine whether it is I3C or its condensation products that are efficacious with respect to a chemopreventive effect, and ultimately which of these will be taken into the clinic for further evaluation.

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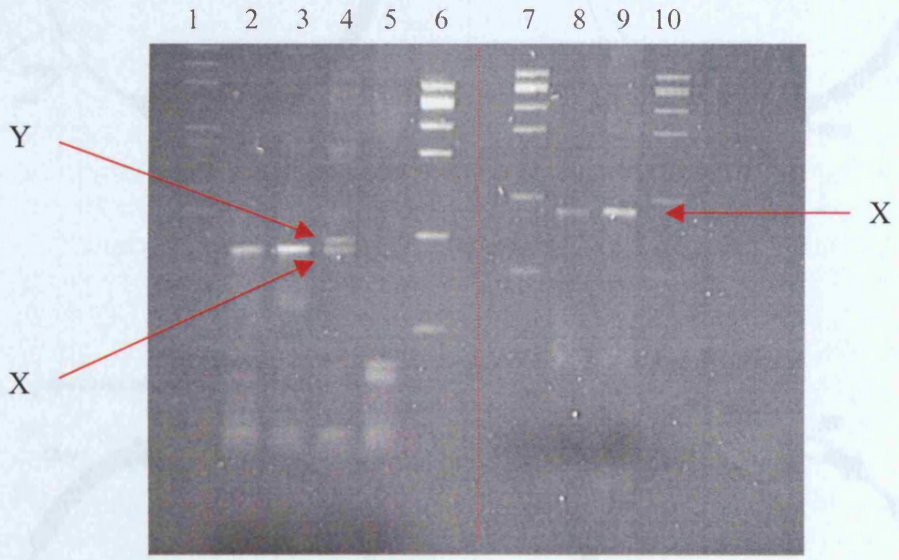
APPENDIX

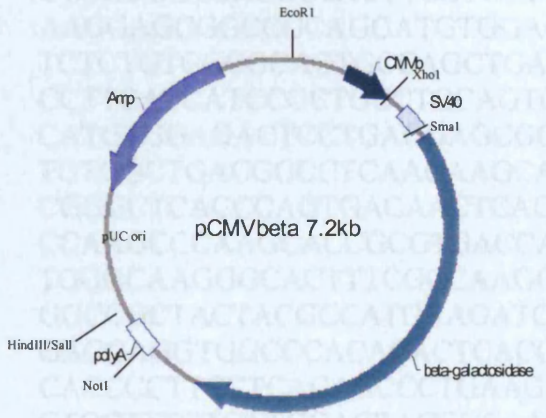
i) Determination of X/Y chromosome status of breast cell lines

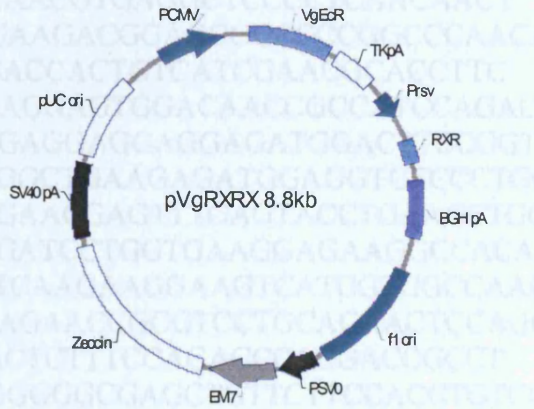
The amelogenin test provides a quantitative, fluorescence-based PCR analysis of the X-Y homologous gene amelogenin (Sullivan *et al.*, 1993). Following PCR amplification, the 106bp (X) and 112bp (Y) products can be analysed by agarose gel electrophoresis in the presence of a known male control, showing the X-Y pattern.

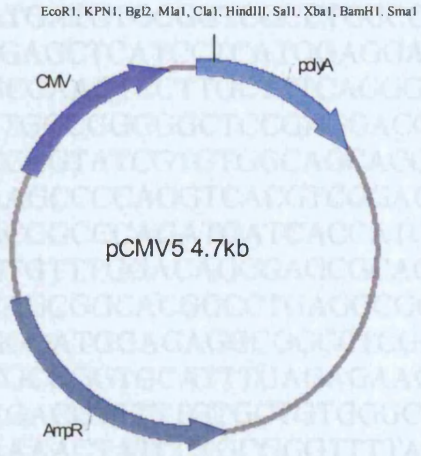


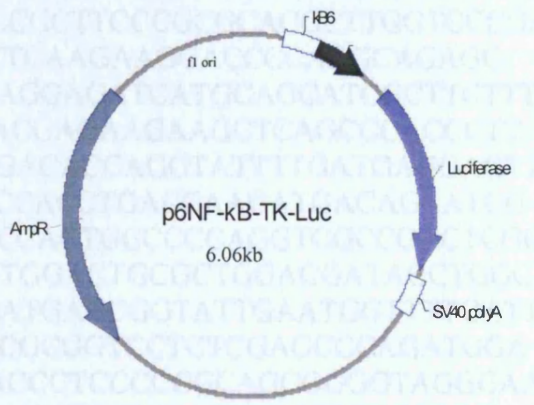
Amelogenin gel showing HBL 100 and MDA 468 cells to be female. *1) marker, 2) MDA 468, 3) HBL 100, 4) known male, 5) negative control, 6) marker, 7) marker, 8) Repeat MDA 468, 9) Repeat HBL 100, 10) marker. Known male shows presence of Y chromosome at 112KDa and X chromosome at 106KDa. HBL 100 and MDA 468 show a 106KDa band only. (Data obtained by Dr. R. C. Barber. Dept. Genetics.)*

ii) Plasmid Maps









pCMV5 Akt1 insert sequence *(differs from NCBI sequence at S478 which is a G. Kinase dead K179 is mutated to A)***.**

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pTK- luc sequence can be found at:

http://www.clontech.com/techinfo/vectors/vectorsT-Z/pdf/pTK-Lucseq.pdf

p6NF-κB sequence

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iii) ***Definition of the Akt consensus binding sequence.***

RXRXXS/T

R = arginine
X = any amino acid
S = serine
T = threonine

iv) Genes and gene products relating to microarray

GENE	**FULL NAME**
ABCB1	ATP binding cassette, sub family B, member 1 (otherwise known as p-glycoprotein)
AKT2	v-akt murine thyoma viral oncogene 2 (protein kinase B beta)
ALDOB	aldolase B, fructose-bisphosphate
ANGPT1	angiopoietin 1 (mediates blood vessel maturation and stability)
ARHB	ras homologue gene family member (links plasma membrane receptors to focal adhesions)
ASNS	asparagine synthetase
ATM	ataxia telandiectasia mutated (involved in signal transduction and protein phosphorylation)
AXL	AXL receptor tyrosine kinase (signal transduction in mesodermal cells)
BAI3	brain-specific angiogenesis inhibitor 3
BCL2	B-cell CLL/lymphoma 2 (anti apoptotic)
BCL2L1	BCL2-like 1 (inhibits caspases)
BRAF	v-raf murine sarcoma viral homologue B1 (conduction of mitogenic signals from cell membrane to nucleus)
CAMK4	calcium/calmodulin dependent protein kinase VI
CASP1	caspase 1
CBX3	chromobox homologue 3 (component of heterochromatin)
CCNB1	cyclin B1
CDC10	cell division cycle 10
CDK10	cyclin dependent kinase 10
CDK5R2	cyclin dependent kinase 5 regulatory subunit 2
CDKN1A	cyclin dependent kinase inhibitor 1A (otherwise known as p21)
CMAR	cell matrix adhesion regulator
CSE1L	CSE1 chromosome segregation 1-like yeast
CUL2	culin 2 (forms a stable complex with the VH2 tumour suppressor)

CYP11B1	cytochrome p450 11B1, 1A1 etc
DDC	dopa decarboxylase
DEDD	death effector domain containing (induces apoptosis)
DFFA	DNA fragmentation factor (inhibitor of caspase activated DNase)
DPYD	dihydropyrimidine dehydrogenase
DUT	dUTP pyrophosphatase
E2F1	E2F transcription factor 1
EGFR	epidermal growth factor receptor
ELK1	member of ETS oncogene family
EPHB4	ephrin receptor 4
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homologue 2
ETS2	v-ets erythroblastosis virus E26 oncogene homologue 2
ETV4	ets variant gene 4
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homologue
FOS	v-fos FBJ murine osteosarcoma viral oncogene homologue
GADD45A	growth arrest and DNA damage-inducible, alpha
GOLGA5	golgi autoantigen, golgin subfamily a, 5
GSPT1	G to S phase transition 1
HRK	harakiri, BCL2 interacting protein
ICAM1	intercellular adhesion molecule 1
ILK	integrin linked kinase
ING1	inhibitor of growth, family member 1
ITGB3	integrin beta 3
JAK1	janus kinase 1
JUN	v-jun sarcoma virus
JUND	jun D proto-oncogene
KRAS2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homologue
LAMR1	laminin receptor 1
LMNA	lamin A/C (provides framework for nuclear envelope)
LMO2	LIM domain only 2 (involved in regulation of red blood cell development)
MAPK3	Mitogen activated protein kinase 3, etc
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)

MCM6	minichromosome maintenance deficient 6 (possibly involved in S phase DNA replication)
MDM2	mouse double minute 2 gene (inhibits p53 mediated cell cycle arrest)
MEIS1	myeloid ecotropic viral integration site 1
MET	met proto-oncogene (hepatocyte growth factor receptor)
MMP 1	matrix metalloproteinase 1, etc
MTA1	metastasis-associated 1
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
NAB1	NGFI-A binding protein1 (transcriptional repressor)
NCOA1	nuclear receptor coactivator 1
NCOR2	nuclear receptor co-repressor 2
NFATC1	nuclear factor of activated T-cells
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NOL1	nucleolar protein 1
NOS3	nitric oxide synthase 3
NTRK2	neurotrophic tyrosine kinase receptor, type 2
PCNA	proliferating cell nuclear antigen
PCTK1	PCTAIRE protein kinase 1
PECAM1	platelet/endothelial cell adhesion molecule
PIK3CB	phosphoinositide-3-kinase, catalytic beta polypeptide
PIN	prolyl isomerase
PKNOX1	PBX/knotted 1 homeobox 1
PLD1	phospholipase D1
POU2F1	POU domain, class 2, transcription factor 1
PPP4C	protein phosphatase 4C
RAB35	member of RAS oncogene family
RB1	retinoblastoma 1
RBBP8	retinoblastoma binding protein 8
RBL2	retinoblastoma-like 2
RBMS1	RNA binding motif, single stranded interacting protein 1
RELB	v-rel reticuloendothelialosis viral oncogene homologue B
RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1
SPARC	secreted protein, acidic, cysteine-rich

STK4	serine/threonine kinase 4
TAF2N	TAF15 RNA, polymerase II TAT box binding protein (TBP)-associated factor
TESK1	testis-specific kinase 1
TFDP1	transcription factor Dp-1
TGFB1	transforming growth factor beta 1
TK1	tyrosine kinase 1
TNFAIP3	tumour necrosis factor, alpha-induced protein 3
TNFRSF10D	TNF receptor super family 10D
TOP2A	topoisomerase (DNA) II alpha
TPMT	thiopurine S-methyltransferase
TRADD	TNFRSF1A-associated via death domain
TRAF4	TNF receptor-associated factor 4
TRAILR4	otherwise known as TNFRSF10D
TYRO3	protein tyrosine kinase
UBE2V2	ubiquitin-conjugating enzyme E2 variant 2
UBL1	ubiquitin-like 1
Wee1	WEE1 + homologue (negative regulator of G2/M transition)
WNT5A	wingless-type MMTV integration site family, member 5A
WRN	werner syndrome (formation of DNA replication focal centres)
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5
YES1	v-yes1 Yamaguchi sarcoma viral oncogene homologue

PUBLICATIONS

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Manson, M. M., Holloway, K. A., **Howells, L. M.**, Hudson. E. A., Plummer, S. M., Squires, M. S., Prigent, S. A. (2000) Modulation of signal transduction pathways by chemopreventive agents. *Biochemical Society Transactions* **28,** 7-12.

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Howells. L. M., Gallacher-Horley, B., Houghton, C. E., Manson, M. M., Hudson, E. A. (2001) Indole-3-carbinol inhibits protein kinase B/Akt and induces apoptosis in the human breast tumour cell line MDA MB468 but not in the nontumourigenic HBL 100 line. *Proceedings of the American Association of Cancer Research #1682.*

Squires, M. S., **Howells, L.,** Hudson, E. A., Dickens, M., Prigent, S. A., Manson, M. M. (2000) Inhibition of cell signalling may contribute to the chemopreventive activity of curcumin and epigallocatechin-3-gallate. *Proceedings of the American Association of Cancer Research #.*

Plummer, S., Wakelin, D., Bouer, M., Shepherd, P., **Howells, L.,** Gescher, A., Chow, S., Manson, M. M. (2000) Inhibition of growth of colon tumour cells correlates with the inhibition of the I kappa B kinase and downregulation of cyclin D1. *British Journal of Cancer* **83**(410 Suppl 1).

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Indole-3-carbinol Inhibits Protein Kinase B/Akt and Induces Apoptosis in the Human Breast Tumor Cell Line MDA MB468 but not in the Nontumorigenic HBL100 Line[1]

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Abstract

We have identified a new target for the chemopreventive dietary agent indole-3-carbinol (I3C) in the antiapoptotic signaling pathway involving phosphatidylinositol 3′-kinase and protein kinase B (PKB)/Akt. I3C inhibited phosphorylation and activation of PKB in the tumor-derived breast cell line MDA MB468, but not in the immortalized breast line HBL100. We propose that this cell type-specific response to I3C contributes to the differential induction of apoptosis and sensitivity to growth inhibition of the two cell lines (approximate IC_{50} = 30 μM for the MDA MB468 line, compared with 120 μM for the HBL100 line). I3C only induced apoptosis in the MDA MB468 cell line, but at higher doses, it increased necrosis in the HBL100 line. The tumor cell line was also markedly less able to recover when I3C was removed from the culture medium. Downstream of PKB, I3C decreased nuclear factor κB DNA binding, independently of an effect on IκB kinase, in the MDA MB468 cell line only. The tumor suppressor PTEN, which prevents phosphorylation and activation of PKB, was expressed in HBL100 cells but was not detected in MDA MB468 cells. In corroboration of the results obtained with the breast cell lines, I3C decreased phospho-PKB levels and induced apoptosis in the prostate cell line LNCaP, which expresses very low levels of PTEN, but did not do so in PTEN-positive DU145 cells. I3C did not affect PTEN levels in any cell line. This is the first study to report a differential mechanistic response of tumor-derived and nontumorigenic cell lines and of PTEN high- and low-expressing cells to I3C and indicates a promising chemopreventive role for I3C against estrogen receptor-α-negative, aggressive-phenotype breast tumors.

Introduction

I3C,[3] a microconstituent derived from cruciferous vegetables such as broccoli and Brussels sprouts, has been shown to exert antitumor and chemopreventive activity against chemically induced tumors at a variety of sites in rodent models (1–4). Two Phase I clinical trials of I3C with promising results have been reported, featuring patients with recurrent respiratory papillomatosis or cervical intraepithelial neoplasia (5, 6). I3C has also been the subject of a breast cancer prevention dose-finding pilot study (7) and has been shown to act cooperatively with tamoxifen *in vitro* (8). There is little in the literature regarding the physiological levels of I3C achievable *in vivo*, but one study reported I3C equivalents of 120 μM in liver after a single oral administration of 50 mg/kg [^{14}C]I3C in rats (9). The mechanism by which I3C exerts its chemoprotective effect has not been fully elucidated, although a number of potential cellular targets have been implicated. We showed previously that I3C not only acted to prevent tumor development when given before the carcinogen in an aflatoxin B_1 hepatocarcinogenesis model but also completely inhibited tumor formation when added to the diet from 6 weeks postinitiation (1). The blocking activity of I3C has been well characterized in terms of modulation of phase I and II drug-metabolizing enzymes, leading to the rapid excretion of the carcinogen and prevention of DNA adduct formation (10–17).

The mechanism by which I3C exerts its tumor-suppressing activity is likely to involve modulation of cell signaling pathways resulting in inhibition of cell proliferation or induction of apoptosis (reviewed in Refs. 18 and 19). In the long-term feeding study mentioned above (1), we showed that after 13 weeks, in rats receiving oral I3C, liver activities of both total tyrosine kinase and ornithine decarboxylase were significantly decreased compared with those in rats not receiving the agent. I3C has also been shown to inhibit total tyrosine kinase activity *in vitro* (20), but little is known about its effect on specific kinases of cell signaling pathways. Cover *et al.* (21) showed that in MCF7 and MDA MB231 breast tumor cell lines, I3C caused G_0-G_1 cell cycle arrest, which was accompanied by a down-regulation of cyclin-dependent kinase 6, decreased retinoblastoma protein phosphorylation, and an increase in $p21^{WAF1}$ and p27. There is also evidence that I3C may exert its chemopreventive activity by induction of apoptosis. The compound caused a small but significant increase in apoptosis in the human breast tumor MDA MB231

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[3] The abbreviations used are: I3C indole-3-carbinol; PKB, protein kinase B; PI3K, phosphatidylinositol 3′-kinase; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; IKK, IκB kinase, PARP, poly(ADP-ribose) polymerase; GSK, glycogen synthase kinase; EMSA, electrophoretic mobility shift assay.

line and in the chemical- and oncogene-initiated 184-B5/BP and 184-B5/HER breast cell lines (22). Recently, Rahman *et al.* (23) showed induction of apoptosis in MDA MB435 cells and concluded that this was due to translocation of the proapoptotic molecule Bax to the mitochondria and an increase in the Bax:Bcl-2 ratio. However, an earlier study by Ge *et al.* (24) reported that I3C induced apoptosis in MCF7 cells via a p53- and Bax-independent pathway, although it should be noted that earlier time points were investigated in the latter study. In a previous study, Ge *et al.* (25) showed that 3,3′-diindolylmethane, an acid condensation product of I3C, induced apoptosis in MCF7 and T47D breast tumor cell lines and in Saos2 human osteosarcoma cells.

Many cell signaling pathways have been implicated in apoptosis, including components of the mitogen-activated protein kinase cascades and PKB (otherwise known as Akt) signaling pathways (reviewed in Ref. 26). Activation of c-Jun NH_2-terminal kinase and p38 kinase is generally associated with proapoptotic signaling (26–29), although there are some studies that implicate c-Jun in protection against stress-induced apoptosis (reviewed in Ref. 30). In contrast, activation of the extracellular signal-regulated kinases 1 and 2 and PKB (which lies downstream of PI3K) is associated with cell survival (26, 29, 31). Undoubtedly, it is the balance between pro- and antiapoptotic signaling under particular circumstances that ultimately determines the fate of a cell.

PKB has been shown to influence apoptosis via multiple downstream targets (32–34). It can phosphorylate and inactivate several proapoptotic proteins, including caspase 9 (35), members of the Forkhead family of transcription factors (Refs. 33 and 36 and the references therein), and Bad (both directly and via kinase $p65^{pak}$; Refs. 37–39). Phosphorylated Bad is sequestered by the cytosolic protein 14-3-3, thus preventing it from binding and inhibiting, the antiapoptotic proteins Bcl-2 and Bcl-xL (40). Bcl-2 itself has also been shown to be up-regulated by PKB via the transcription factor cAMP-responsive element-binding protein CREB (41). Activation of signaling via PKB also increases NF-κB-mediated cell survival (42, 43).

The phosphatase PTEN (PTEN/MMAC1/TEP1) tumor suppressor has also been implicated in the regulation of PKB and therefore in induction of apoptosis. Phosphorylation of PKB is required for full activation of the kinase (34). PTEN has been shown to dephosphorylate PI3K-generated phosphatidylinositol 3,4,5-trisphosphate molecules, the signaling intermediates required for activation of PKB (33, 44, 45).

In this study we compared the effects of I3C between the human breast tumor-derived cell line MDA MB468 and the normal tissue-derived HBL100 cell line. We investigated the induction of apoptosis in these cell lines and the role that inhibition of the PKB signaling pathway and of downstream effectors, including NF-κB, might play in this process. In agreement with our preliminary findings (46), our data indicated that I3C inhibited the phosphorylation and activity of PKB in the tumor cell line only and that this is likely to contribute to the induction of apoptosis and chemopreventive mechanism of action of I3C in these breast tumor cells. To confirm our results, we then extended the study to incorporate two prostate cancer cell lines, LNCaP and DU145.

Materials and Methods

Materials. I3C and okadaic acid were obtained from Sigma-Aldrich Company Ltd. (Poole, United Kingdom), and LY294002 was obtained from Calbiochem-Novabiochem (United Kingdom) Ltd. (Beeston, United Kingdom). The complete protease inhibitor mixture was purchased from Roche Diagnostics Ltd. (Lewes, United Kingdom). The IκBα substrate and anti-NF-κB p65 (C-20), anti-IKKα, and anti-Akt-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PTEN and Akt (detects total PKBα, β, and γ, phosphorylation state independent), the two phosphorylation site-specific anti-phospho-Akt antibodies [anti-phospho-Akt (Ser 473) detects PKBα when phosphorylated at Ser-473 and detects PKBβ and PKBγ when phosphorylated at equivalent sites; and anti-phospho-Akt (Thr 308), which does not cross-react with the Ser-473 phosphorylation site], and the Akt kinase assay kit were from Cell Signaling Technology (Hitchin, United Kingdom). Antibody against Bcl-xL was purchased from BD Transduction Laboratories (Lexington, KY); the anti-Bcl-2 and Bax antibodies were from Dako Ltd. (Ely, United Kingdom), anti-PARP antibody was from Alexis Corp. (United Kingdom) Ltd. (Bingham, United Kingdom), and FITC-conjugated annexin V was from Bender MedSystems (Vienna, Austria). Rabbit antisera to NF-κB subunits p50, p65, and RelB were a kind gift of Dr. Nancy Rice (Molecular Basis of Carcinogenesis Laboratory, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD).

NF-κB and AP-1 consensus oligonucleotides were obtained from Promega (Southampton, United Kingdom). All cell culture media and reagents were purchased from Invitrogen (Paisley, United Kingdom).

Cell Lines and Treatments. The human-derived breast carcinoma cell line MDA MB468 and the immortalized nontumorigenic cell line HBL100[4] were kindly provided by Prof. Rosemary Walker (Department of Pathology, University of Leicester, United Kingdom). Both cell lines are regarded as estrogen receptor-α negative, although they may possess estrogen receptor-β (47). The human-derived prostate carcinoma cell lines DU145 and LNCaP were obtained from American Type Culture Collection (Manassas, VA). The DU145 cell line lacks androgen receptors, whereas the LNCaP cell line is hormone sensitive.

Both breast cell lines were cultured as described previously (48). DU145 cells were cultured in MEM with Earle's Salts containing 2 mM glutamax, supplemented with nonessential amino acids (1×), 1 mM sodium pyruvate, and 10% FCS. The LNCaP cells were cultured in RPMI 1640 supplemented with 2 mM glutamax, 1 mM sodium pyruvate, glucose (1.25 g/500 ml), and 10% FCS. All cell lines were negative when tested for *Mycoplasma* infection. I3C was prepared as a stock solution in DMSO, and cells were treated in such a way that all control and treated cells received equal volumes of DMSO, which did not exceed a final concentration of

[4] Following concerns raised by the American Type Culture Collection that the HBL100 cell line contained a Y chromosome, we had our own stock karyotyped and found it was positive for X only.

0.05%. The purity of the I3C stock in DMSO was verified as approximately 99.9% by HPLC (data not shown). For each experiment, cells were initially seeded in normal growth medium and allowed to adhere for at least 4 h before treatment. The concentrations of I3C used were varied according to the duration of the experiment and the different sensitivities of the cell lines. Lower concentrations of I3C (typically 10–100 μM for the MDA MB468 line and 100–250 μM for the HBL100 cell line) were used in the proliferation and apoptosis studies that were performed over several days and in some Western blots that were performed after 72 h. Higher concentrations of I3C were used in the signaling experiments because these were performed over short time periods (up to 5 h).

Cell Proliferation Studies. Cells were seeded at 5×10^4 onto 6-well plates and cultured in the presence of I3C for times from 72 to 240 h. Approximate IC_{50} values were obtained from a plot of cell number expressed as a percentage of control *versus* I3C concentration. To determine their ability to recover proliferative capacity after treatment, cells (1×10^4 cells on 12-well plates) were cultured in the presence of I3C for 24 h, after which they were either maintained in treated medium or washed and replenished with fresh medium and allowed to recover before harvesting at 168 h. The proliferation rate of cells was calculated as fold increase in cell number after the initial treatment period. For analysis of cell cycle, 5×10^5 cells were seeded onto 9-cm plates and treated with I3C (up to 200 μM) for 48 h. Cells were harvested by trypsinization, fixed overnight in 70% ethanol at 4°C, and then collected by centrifugation, resuspended in PBS containing 0.1 mg/ml RNase and 5 μg/ml propidium iodide, and incubated overnight at 4°C. DNA content was analyzed using a Becton Dickinson FACScan and Cell Quest software. Subsequent data analysis was performed using ModFit LT software (Becton Dickinson United Kingdom Ltd., Cowley, United Kingdom).

Measurement of Phosphatidylserine Externalization. Cells were seeded at 2×10^5, 5×10^5 or 1×10^6 onto 9-cm plates (depending on treatment time) and treated with I3C for times up to 168 h. After treatment, cells obtained by trypsinization were combined with those that had spontaneously detached during the incubation. Phosphatidylserine externalization was determined by annexin V staining. Cells were pelleted and resuspended in 1 ml of annexin buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$]. FITC-conjugated annexin V was added to a final concentration of 100 ng/ml, and cells were incubated for 8 min at room temperature, after which propidium iodide (1.5 μg) was added, and cells were analyzed by flow cytometry using a FACScan flow cytometer and Cell Quest software.

Detection of Total and Phosphorylated PKB, PTEN, Bcl-2, Bcl-xL, and Bax. Cells were seeded at between 1.5 and 3×10^6 onto 9-cm plates, depending on the duration of the experiment, and treated with I3C for 5, 24, 48, or 72 h. In a separate experiment, cells were seeded as described above and cultured for 24 h under normal or serum-depleted (0%) growth conditions or serum-starved for 24 h followed by replenishment of serum (10%) for 4 h. To detect levels of phospho-PKB, PTEN, Bcl-2, Bcl-xL, and Bax, treated cells were lysed in a cell lysis buffer (as detailed in the Akt assay kit) containing 2% (v/v) complete protease inhibitor mixture. Samples were analyzed by SDS-PAGE and immunoblotting, followed by visualization using an enhanced chemiluminescence detection system (Amersham Life Science Ltd., Little Chalfont, United Kingdom). Equal amounts of protein were loaded in each lane. Blots were scanned using a densitometer (Molecular Dynamics, Sunnyvale, CA) and quantified using Image Quant software. In all cases, bands were confirmed as being within the linear range of the film. For determination of total PKB, samples were prepared by washing cells twice in ice-cold PBS and harvesting directly in sample buffer. Samples were then sonicated and boiled for 5 min and analyzed as described above. Loading controls are shown in the figures for blots in which there was a marked change in protein level in response to the treatment. Blots were stripped and reprobed with an anti-tubulin antibody.

Detection of PARP and Its Cleavage Products. Cells were seeded and treated with I3C as described above. Adherent cells were harvested by scraping and combined with those that had detached during the treatment incubation. Cells were then pelleted and subjected to three rounds of freeze thawing before resuspension in 40 μl of sample buffer and sonication. Equal volumes were separated by SDS-PAGE and detected as described above, using an anti-PARP antibody.

Immune Complex Kinase Assay for PKB and IKK. Cells were seeded at $1–2 \times 10^6$ onto 9-cm plates and allowed to adhere. Cultures were treated with I3C for 5 h before harvesting in cell lysis buffer as described above. PKB activity was determined according to the protocol supplied with the Akt kinase assay kit. PKB was immunoprecipitated from 200 μg of cell lysate protein, which gave results within the linear range of the assay. Phosphorylation of the glutathione *S*-transferase-GSK-3 substrate was measured by SDS-PAGE using the phospho-GSK-3α/β antibody, and detection was by chemiluminescence using the Lumiglo detection reagent supplied in the kit. No phosphorylation of the substrate was detected in the absence of PKB.

IKKα was immunoprecipitated from 500 μg of cell lysate protein using an anti-IKKα antibody conjugated to protein A beads and then resuspended in kinase assay buffer as described above. Total IKKα protein levels were detected by SDS-PAGE using the same anti-IKKα antibody. IKKα activity was determined by incubation (30°C, 30 min) with IκBα substrate (1 μg) in the presence of 50 μM ATP (containing 0.074 MBq [γ-^{32}P]ATP). Proteins were separated by SDS-PAGE as described above, and phosphorylated IκBα was detected by PhosphorImager analysis (Molecular Dynamics).

Measurement of Nuclear NF-κB. Cells were seeded and treated as described for the determination of levels of PKB. Nuclear extracts were prepared according to the method of Staal *et al.* (49), and protein content was determined using the Bio-Rad protein assay. Samples were denatured in sample buffer and analyzed by SDS-PAGE as described above, using NF-κB p65, p50, or RelB subunit antibodies.

EMSA for NF-κB DNA Binding. Nuclear extracts were prepared from cells treated with I3C for 5 h, as described above. EMSAs were performed as described previously (50)

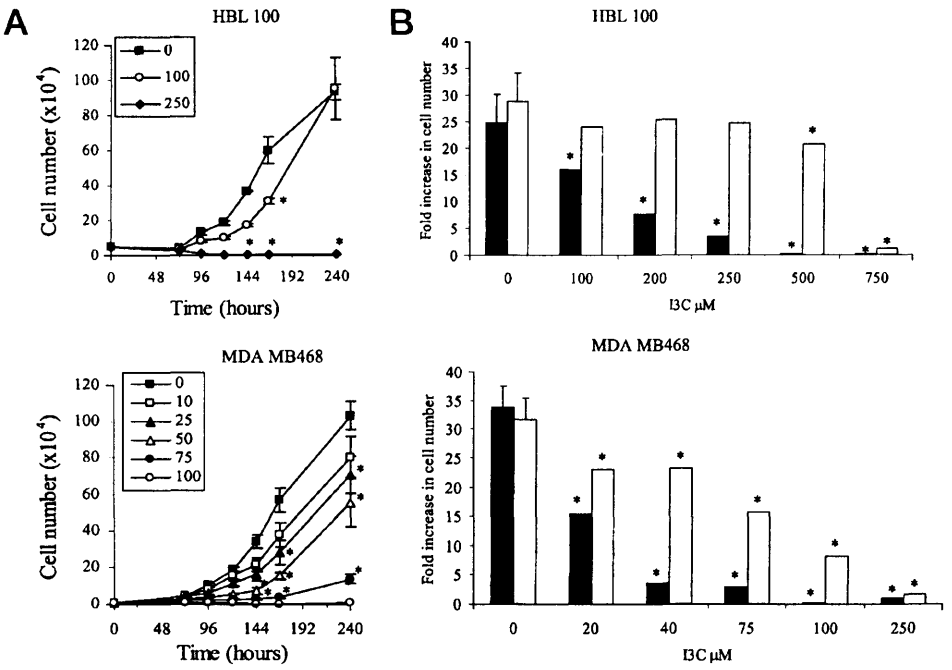

Fig. 1. Effect of I3C on proliferation and recovery of the HBL100 and MDA MB468 cell lines. *A*, growth curves were performed in the presence of increasing concentrations of I3C and counted as detailed in "Materials and Methods." *B*, cells were treated with I3C for 168 h (■) or for 24 h followed by 144 h recovery (□). Data shown are mean cell number ± SE (*A*) or fold increase in cell number ± the SD of the sets (shown on control bars only; *B*). * indicates significant difference from control cell data at that time point ($P < 0.05$), as determined by the ANOVA general linear model followed by Fisher's least significant difference *post hoc* test ($n = 6$).

using a ^{32}P-end labeled NF-κB consensus oligonucleotide (5'-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3') and an excess of either unlabeled NF-κB oligonucleotide or an unrelated AP-1 consensus sequence (5'-CGC-TTG-ATG-AGT-CAG-CCG-GAA-3').

Statistical Analysis. Statistical significance was assessed using ANOVA balanced or general linear models (51) followed by Fischer's least significant difference *post hoc* test (52).

Results

Inhibition of Cell Growth and Recovery after Treatment with I3C. I3C caused a dose-dependent inhibition of cell proliferation in both cell lines (Fig. 1*A*). However, the MDA MB468 cell line was approximately 4-fold more sensitive to the growth-inhibitory effect of I3C than the HBL100 line, with approximate IC_{50} values (at 168 h) of 30 and 120 μM, respectively. The ability of the two cell lines to recover from I3C treatment also differed markedly. The HBL100 cell line was capable of complete recovery (fold increase in cell number was not significantly different from the control) from 24 h of treatment with concentrations up to 250 μM and almost complete recovery from 500 μM (Fig. 1*B*). In contrast, the MDA MB468 cells only partially recovered from 24 h of treatment with 20 μM I3C and were unable to recover from 24 h of treatment with 250 μM (Fig. 1*B*).

The decrease in cell proliferation caused by I3C may be due to either cytostatic or cytotoxic effects or a combination of both. To determine the nature of the growth inhibition, we determined the effect of I3C on cell cycle progression. No apparent cell cycle phase-specific arrest was observed in either cell line (data not shown), but a clear dose-dependent sub-G_1 peak, indicative of apoptotic cells, was visible in the MDA MB468 cell line only, from 100 μM (Fig. 2*A*).

Induction of Apoptosis by I3C. Using annexin V and propidium iodide staining, we determined the percentage of cells undergoing apoptosis or necrosis by flow cytometry in treated cultures. No increase in the proportion of apoptotic cells was observed for the HBL100 cell line after any dose of I3C. However, as the proportion of live cells decreased over time, a significant dose-dependent increase in the percentage of necrotic cells occurred, from 5% in control cells after 24 h up to 37% in cells treated with 250 μM for 144 h (Fig. 2*B*). Treatment of MDA MB468 cells, on the other hand, resulted in a dose- and time-dependent induction of apoptosis (Fig. 2, *B–D*). This was accompanied by a steady increase in the percentage of necrotic cells, which at higher concentrations (500 μM) or longer time points is likely to encompass a proportion of cells undergoing secondary necrosis after apoptosis (Fig. 2). An early increase in apoptosis was detected in the MDA MB468 cells with 500 μM I3C, which reached significance by 8 h (Fig. 2*D*). These results were confirmed by Western analysis of PARP cleavage, in which cleavage products were detected in MDA MB468 cells by 8 h (Fig. 3), whereas in contrast, no such products were readily detected in the HBL100 cell line over the same time

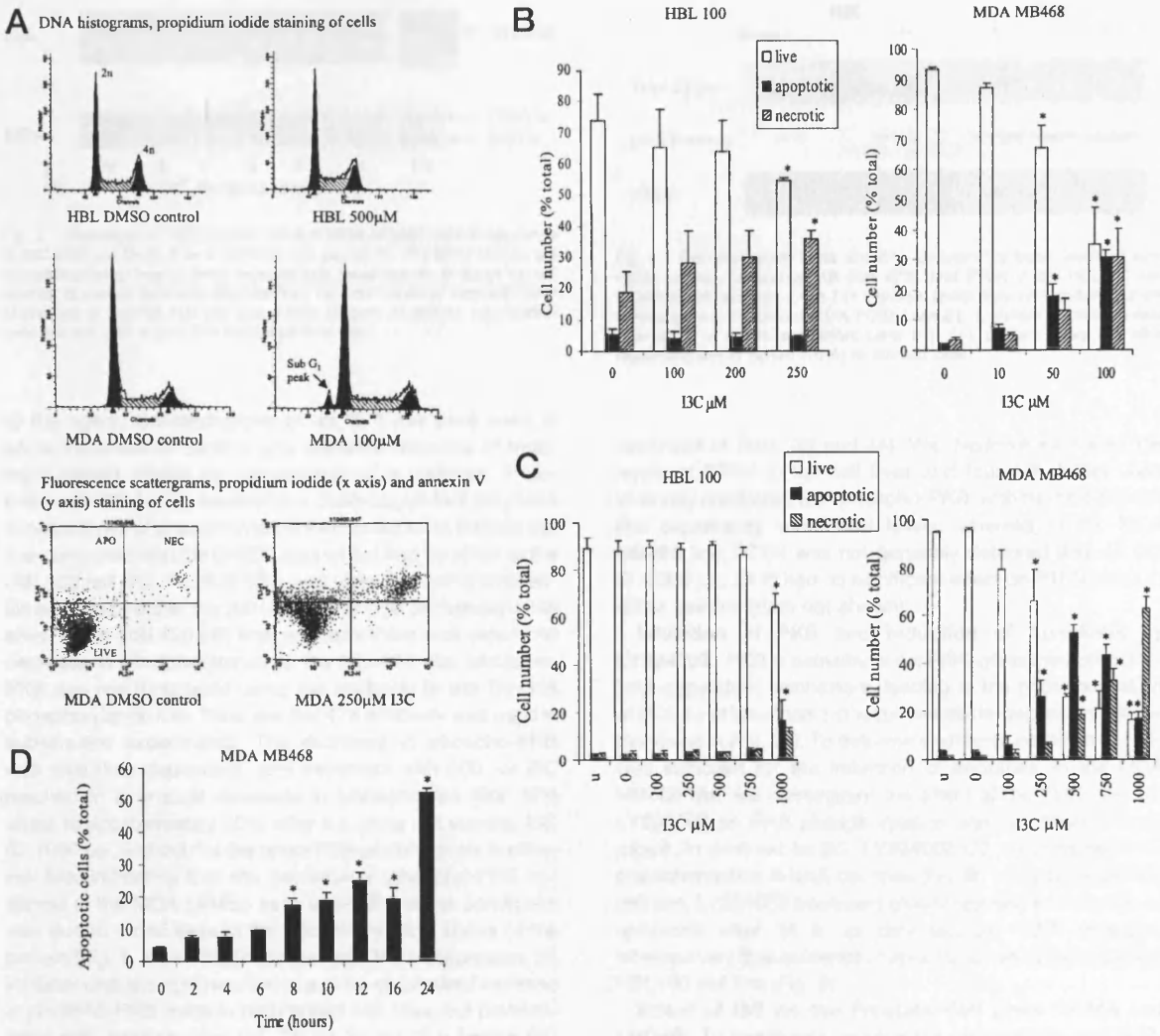

Fig. 2. Effect of I3C on cell cycle progression and induction of apoptosis and necrosis. *A,* sample panels showing flow cytometric analyses of control and treated cells. The *top panels* show DNA content histograms of control and I3C-treated (48 h) HBL100 and MDA MB468 cells determined by propidium iodide staining; the *X* axis shows red fluorescence (propidium iodide), and the *Y* axis represents cell counts. The *bottom panels* show profiles of MDA MB468 cells (24-h treatment) labeled with propidium iodide (shown on the *X* axis) and FITC-conjugated annexin V (*Y* axis); live cells appear in the *bottom left quadrant*, whereas apoptotic (*APO*) and necrotic (*NEC*) cells appear in the *top quadrants* as indicated. The effect of I3C on the proportion of live, apoptotic, or necrotic cells, as determined by annexin V staining in the breast cell lines, is shown after 144 h in *B* and 24 h in *C*. A time course (0–24 h) for the induction of apoptosis by I3C (500 μM) in the MDA MB468 cell line is shown in *D*. Results shown are mean ± SE ($n = 3$). * indicates significant difference from the control (DMSO-treated) group ($P < 0.05$) as determined by the ANOVA balanced model followed by Fisher's least significant difference *post hoc* test.

course. To ensure that even a small amount of cleaved product was detected if present in the HBL100 cell line, a high amount of protein was loaded onto the gel, and a long film exposure time was used. The data were interpreted with respect to cleavage or noncleavage of the protein and not to any change in the relative amount of full-length protein.

Effect of I3C on PKB Levels and Phosphorylation. We next investigated whether the induction of apoptosis by I3C in the MDA MB468 cell line could be due to inhibition of signaling via the prosurvival protein kinase PKB. The tumor cells were found to have approximately 3-fold higher basal levels of phospho-PKB than the HBL100 cell line, although the latter had higher total PKB protein levels (Fig. 4).

Whereas there was no intentional stimulation of PKB phosphorylation, it should be noted that in the MDA MB468 cell line only, the level of phosphorylated PKB (both sites) was increased in the DMSO-treated control compared with untreated cells ($P < 0.05$).

In these and subsequent experiments, we used higher concentrations of I3C than in the longer-term apoptosis and growth studies to elicit a fast response and investigate the early events that precede induction of apoptosis in response

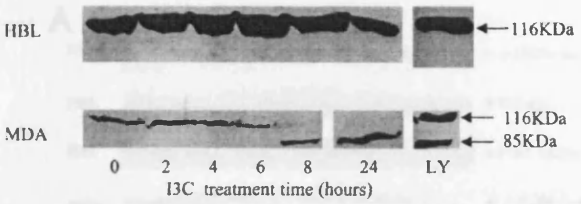

Fig. 3. Cleavage of PARP in HBL100 and MDA MB468 cells in response to I3C (500 μM; 0–24 h) or LY294002 (50 μM; 24 h). The blots shown are representative of two or more independent experiments. In these experiments, to ensure detection of potentially very low levels of cleaved PARP (if present) in the HBL100 cell line, a high amount of protein was loaded onto the gel, and a long film exposure was used.

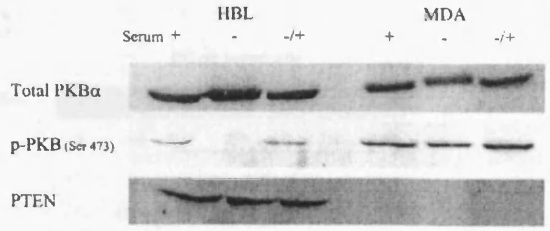

Fig. 4. Representative blots showing comparative basal levels of total PKBα protein, phospho-PKB (Ser 473), and PTEN in the HBL100 and MDA MB468 cell lines. *Lane 1* (+), protein levels from cells cultured under normal growth conditions (10% FCS); *Lane 2* (−), protein levels from cells after 24 h of serum starvation; *Lane 3* (−/+), protein levels 4 h after replenishment of serum (10%) to starved cells.

to the agent. Concentrations of up to 1 mM were used in some instances to confirm any apparent absence of treatment-related effects on components of a pathway. Treatment with I3C for 5 h resulted in a dose-dependent decrease in basal levels of phosphorylated PKB in the MDA MB468 cell line compared with the DMSO control but had no effect in the HBL100 cell line (Fig. 5*A*). This was detected using antibodies specific to either the Ser-473 or Thr-308 phosphorylation sites. In the HBL100 cell line, whereas there was clearly no decrease in phosphorylation at the Ser-473 site, phospho-PKB was not detectable using the antibody to the Thr-308 phosphorylation site. Thus, the Ser 473 antibody was used in subsequent experiments. The decrease in phospho-PKB was also time dependent, and treatment with 500 μM I3C resulted in a gradual decrease in phospho-Akt (Ser 473) levels to approximately 50% after 5 h (data not shown). I3C (0–1000 μM, 5 h) did not decrease PKB protein levels in either cell line, indicating that the decrease in phospho-PKB observed in the MDA MB468 cells under the same conditions was due to a decrease in the phosphorylation status of the protein (Fig. 5, *A* and *B*). Treatment with the phosphatase 2A inhibitor okadaic acid resulted in a dose-dependent increase in phospho-PKB levels in both breast cell lines, but pretreatment with okadaic acid (10, 25, or 50 nM, 2 h before I3C treatment for 5 h) did not protect against the I3C-induced decrease in phosphorylated PKB in the MDA MB468 cell line (data not shown). It is noteworthy that treatment with okadaic acid caused severe toxicity to the MDA MB468 cells at concentrations above 25 nM, whereas the HBL100 cells tolerated concentrations up to 100 nM.

Effect of I3C on PKB Activity. PKB activity, as determined by *in vitro* kinase assay, was also found to be decreased in a dose-dependent manner after I3C treatment (5 h) in the MDA MB468 cell line, but not in the HBL100 line (Fig. 5, *C* and *D*, data shown for MDA MB468 only), corresponding to the effect of I3C on the levels of phosphorylated PKB in each cell line. I3C (up to 750 μM) had no effect on the kinase activity when added directly to the assay, although at the higher concentration (1000 μM) there was slight evidence for direct inhibition of PKB activity (Fig. 5*C*).

Effect of I3C on the PTEN Tumor Suppressor. The tumor suppressor PTEN has been shown to dephosphorylate PI3K-generated phosphatidylinositol 3,4,5-trisphosphate molecules (45), which are required for the activation of PKB (reviewed in Refs. 33 and 44). We therefore examined the levels of PTEN in the cell lines and found that they were inversely correlated with phospho-PKB, with the HBL100 cell line expressing substantial levels, whereas in the MDA MB468 line, PTEN was not generally detected (Fig. 4). I3C (0–1000 μM, 24 h) had no significant effect on PTEN levels in either cell line (data not shown).

Inhibition of PKB and Induction of Apoptosis by LY294002. PKB is activated via a PI3K-generated phospholipid-dependent mechanism, leading to the phosphorylation of PKB by at least one 3-phosphoinositide-dependent kinase (reviewed in Ref. 33). To determine whether inhibition of PKB was sufficient for the induction of apoptosis in the MDA MB468 line, we investigated the effect of the PI3K inhibitor LY294002 on PKB phosphorylation and induction of apoptosis. In contrast to I3C, LY294002 (50 μM) inhibited PKB phosphorylation in both cell lines (Fig. 6). In the MDA MB468 cell line, LY294002 treatment clearly resulted in induction of apoptosis after 24 h, as detected by PARP cleavage, whereas very little evidence of apoptosis was observed in the HBL100 cell line (Fig. 3).

Effect of I3C on the Prostate Cell Lines DU145 and LNCaP. To investigate whether the proapoptotic and PKB-inhibitory effect of I3C was specific to cell lines with undetectable or very low levels of PTEN, we investigated the effect of I3C on LNCaP and DU145 prostate cell lines, which express different levels of phospho-PKB and PTEN (Fig. 7*A*). Densitometric analysis of Western blots showed the DU145 cells to have the highest levels of PTEN (twice that of HBL100 cells and approximately 6-fold higher than LNCaP cells) with barely detectable levels of phospho-PKB, whereas the LNCaP cells, like the MDA MB468 cells, expressed much higher levels of phospho-PKB with low PTEN (Fig. 7*A*). Although less marked than in the breast cells, I3C inhibited growth of the high phospho-PKB-expressing LNCaP cell line to a greater extent than the DU145 cell line, with approximate IC_{50} values of 22 and 48 μM (data not shown).

I3C (5 h of treatment) caused a dose-dependent decrease in phospho-PKB levels in the LNCaP cell line, with no obvious effect in the DU145 line (Fig. 7*B*). LY29004 completely abolished phospho-PKB in both cell lines. In agreement with results obtained with the breast cells, the high phospho-PKB/low PTEN-expressing cell line LNCaP, but not DU145

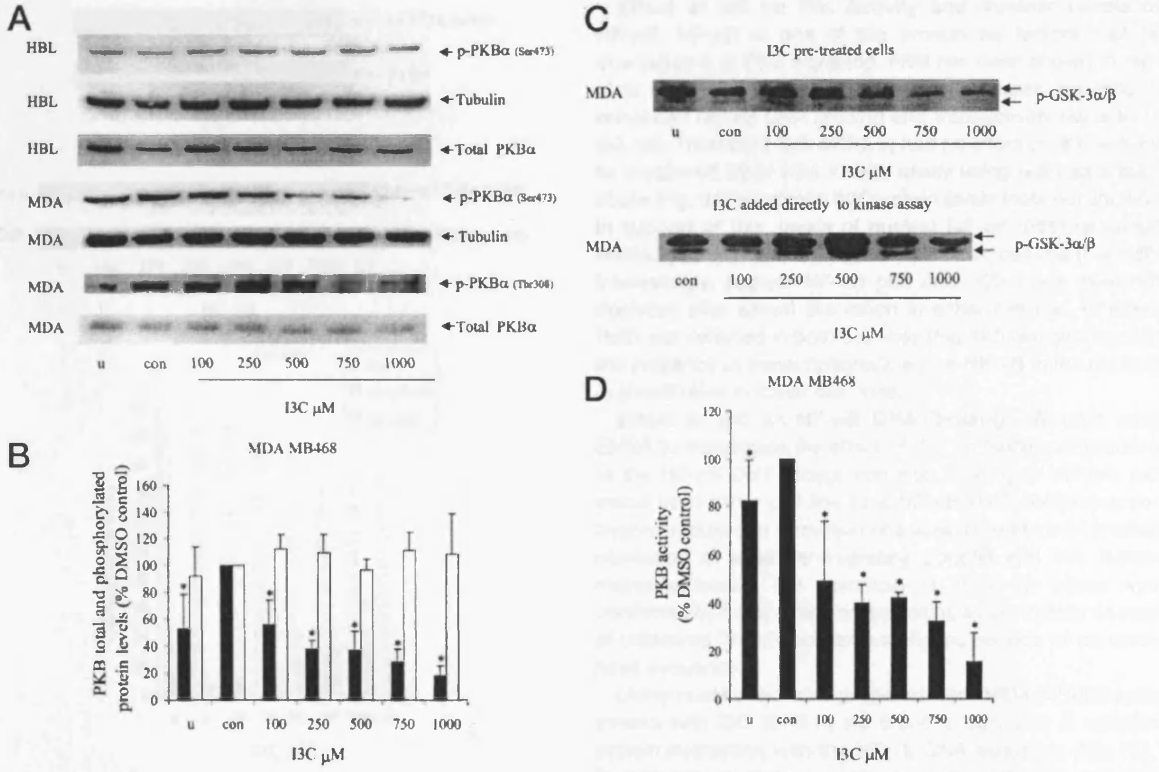

Fig. 5. Effect of I3C on PKB levels (*A* and *B*) and activity (*C* and *D*). *A*, representative Western blots for HBL100 and MDA MB468 showing the effect of increasing concentrations of I3C (5-h treatment) on levels of phospho-PKB (Ser 473 and Thr 308) and total PKB [blot shown is with an antibody to total (non-phospho-specific) PKBα, which gave results in agreement with an antibody that detects all three isoforms (data shown in *B*)]. Tubulin loading controls are shown for HBL100 and MDA MB468 phospho-PKB (Ser 473) blots. *B*, effect of I3C on total (□) and phospho-PKB levels (■) in the MDA MB468 cell line. Data shown are mean ± SE ($n = 3$) of values obtained by densitometric analysis of Western blots and are expressed as a percentage of the DMSO control. *C*, representative Western blots showing PKB activity determined by phosphorylation of a glutathione *S*-transferase-GSK-3α/β substrate in an *in vitro* kinase assay. PKB activity was assayed in extracts from MDA MB468 cells treated with I3C as described above (*top panel*) and in extracts from untreated MDA MB468 cells when I3C was added directly to the kinase assay (*bottom panel*). *D*, effect of I3C on PKB activity. Data shown are mean ± SE ($n = 3$) of values obtained by densitometry as described in *B*. The blots shown in *A* and *C* are representative of three independent experiments. It should be noted that the exposure times of the blots shown in *A* were not identical. *Bars* in *B* and *D* marked * are significantly different ($P < 0.05$) from the control levels, as determined by the ANOVA balanced model followed by Fisher's least significant difference *post hoc* test. *Lanes* marked *u* and *con* indicate no treatment and the DMSO control, respectively.

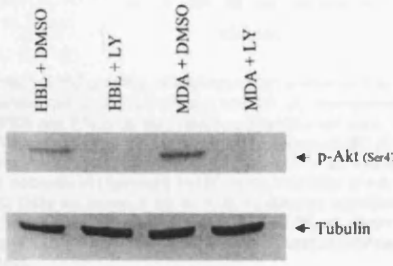

Fig. 6. Effect of LY294002 (5 h, 50 μM) on levels of phospho-PKB (Ser 473) in HBL100 and MDA MB468 cell lines. The blot shown is representative of results obtained on more than three occasions. The *bottom panel* shows a tubulin loading control for the phospho-PKB blot.

cells, underwent significant apoptosis after treatment with LY294002 for 24 h (Fig. 7*C*). Furthermore, the LNCaP cell line was also more sensitive to induction of apoptosis by I3C (24 h), showing a consistently greater fold increase in the proportion of apoptotic cells relative to control with each treatment (Fig. 7*C*). The LNCaP cells undergo a higher basal rate of apoptosis (approximately 9% in control cells compared with 6% in control DU145 cells), suggesting that they may be predisposed to cell death via this mechanism. The increase in necrosis that occurred alongside the increase in apoptosis in the LNCaP cells after I3C treatment may represent secondary necrosis of previously apoptotic cells.

Effect of I3C on Bcl-2, Bcl-xL, and Bax. In a recent study, Rahman *et al.* (23) showed an increase in the ratio of Bax to Bcl-2 in T47D cells in response to I3C, thus favoring apoptosis. PKB has been implicated in the transcriptional regulation of Bcl-2 (41), and therefore a decrease in Bcl-2 seemed a likely candidate for mediating the apoptotic effect of PKB inhibition by I3C. We found no evidence of a decrease in Bcl-2 or an increase in Bax after 5 or 24 h of treatment with I3C (Fig. 8*A*, 5 h data). However, at 48 and 72 h, levels of Bcl-2 were decreased in both cell lines, although the data did not show a clear dose response (Fig. 8*B*, 48 h data). Levels

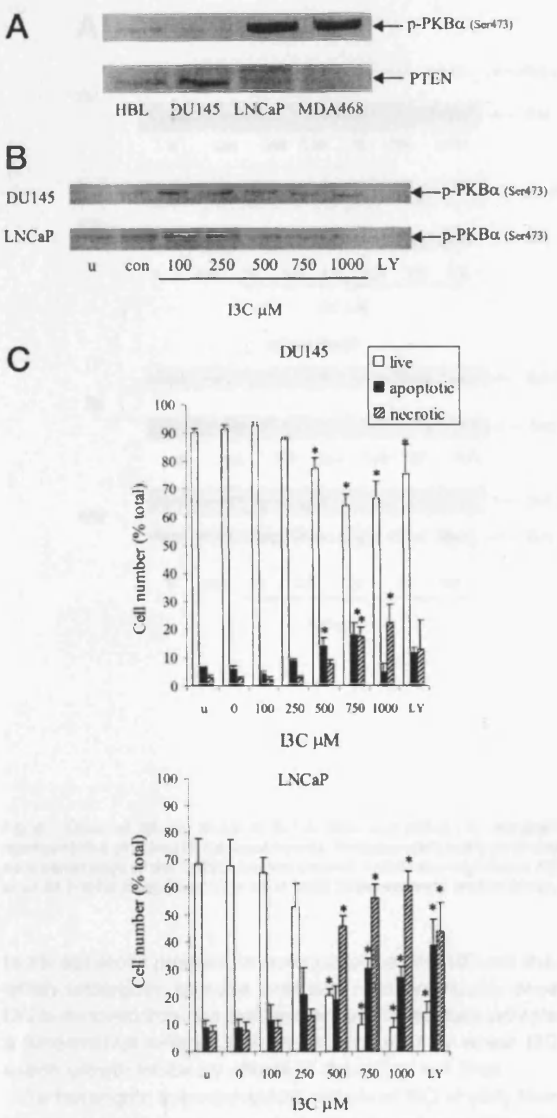

Fig. 7. Effect of I3C on PKB phosphorylation and induction of apoptosis in the prostate cell lines DU145 and LNCaP. *A*, representative blots of phospho-PKB and PTEN levels in the two prostate cell lines, together with the MDA MB468 and HBL100 cell lines. The effect of I3C (5-h treatment) on phospho-PKB in the prostate cells is shown in *B*. Induction of apoptosis and necrosis in response to I3C and LY294002 (24-h treatment) is shown in *C*. Data are mean ± SE ($n = 3$); * indicates significant difference from the control (DMSO-treated) group ($P < 0.05$) as determined by the ANOVA balanced model followed by Fisher's least significant difference *post hoc* test.

of Bcl-xL were decreased in the MDA MB468 cells at ≥375 μM and in the HBL100 line at 500 μM after only 24 h (Fig. 8*C*). Because the effects on these apoptotic regulatory proteins were not observed until the 24 or 48 h time points, occurred in both cell lines, and were not always clear cut, it is unlikely that they constitute a major mechanism by which I3C initiates apoptosis in the MDA MB468 cell line, but they could well contribute to its progression.

Effect of I3C on IKK Activity and Nuclear Levels of NF-κB. NF-κB is one of the prosurvival factors that lie downstream of PKB signaling. PKB has been shown to regulate NF-κB via activation of the IKK complex, resulting in enhanced NF-κB DNA binding and transactivational activity (53, 54). Treatment with I3C (5 h) had no effect on IKK activity as measured by *in vitro* kinase assay using IκBα as a substrate (Fig. 9*A*) or on total IKK protein levels (data not shown). In support of this, levels of nuclear NF-κB (p65) remained unchanged after a 5-h treatment of either cell line (Fig. 9*B*). Interestingly, nuclear NF-κB p65 and p50 levels were not depleted after serum starvation in either cell line, whereas RelB was reduced in both cell lines (Fig. 9*C*), suggesting that the presence of transcriptionally active NF-κB in the nucleus is constitutive in these cell lines.

Effect of I3C on NF-κB DNA Binding. We next used EMSA to investigate the effect of I3C on binding of proteins to the NF-κB DNA recognition site. Binding of nuclear extracts from either cell line to a NF-κB DNA consensus sequence resulted in formation of a specific pattern of binding consisting of a slower-migrating doublet and two faster-migrating bands. The specificity of all of the bands was confirmed by competition assays using a 200× molar excess of unlabeled NF-κB consensus oligonucleotide or an unrelated sequence.

Using nuclear extracts prepared from MDA MB468 cells treated with I3C for 5 h, we found a decrease in specific protein interaction with the NF-κB DNA sequence (Fig. 9*D*). In contrast, nuclear extracts from the HBL100 cell line showed an apparent increase in protein binding (Fig. 9*D*). In both cases, this suggests that I3C can cause alteration in DNA binding of NF-κB protein family members independently of changes in nuclear NF-κB levels and IKK activity. In separate supershift experiments, we confirmed the presence of both p65 and p50 in the DNA-binding protein complex (Fig. 9*D*).

Discussion

Results presented in this study show clearly that the antiproliferative effect of the chemopreventive agent I3C is cell type specific even between cells from one tissue but that modulation of cell signaling pathways and induction of apoptosis can contribute to its mechanism of action in breast and prostate tumor cells. We showed that the MDA MB468 breast tumor cell line underwent apoptosis after treatment with I3C in a dose- and time-dependent manner, as assessed by measurement of phosphatidylserine externalization and confirmed by PARP cleavage. Apoptosis was detected by 72 h in MDA MB468 cells treated with 10 μM I3C and as early as 6 h in MDA MB468 cells treated with 500 μM I3C. In contrast, the nontumorigenic HBL100 cell line was resistant to induction of apoptosis by concentrations of I3C up to 1 mM but did show an increase in the percentage of necrotic cells after 96 h of treatment with 200 μM I3C and above. It is likely that this differential induction of apoptosis accounts for the approximate 4-fold difference in sensitivity in terms of growth inhibition between the two cell lines. This would also explain the inability of the MDA MB468 cells to recover from treatment once they have become committed

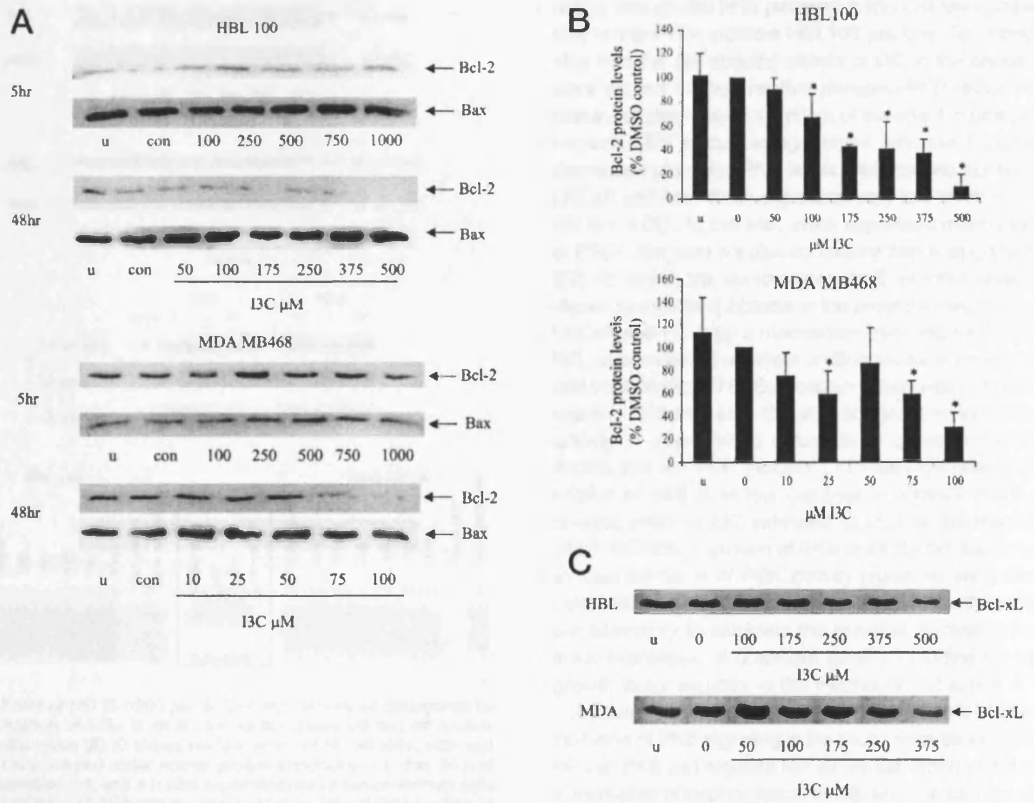

Fig. 8. Effect of I3C on levels of Bcl-2, Bax, and Bcl-xL. *A*, representative blots of Bcl-2 and Bax after 5 or 48 h of treatment with I3C. Blots are representative of at least three experiments. Densitometric analysis of data from the 48 h time point is shown in *B*. Data shown are the mean ± SD expressed as a percentage of the DMSO-treated control. * indicates significant difference from the DMSO-treated control. *C*, effect of I3C on Bcl-xL protein levels after 24 h (blot is representative of at least three separate experiments).

to the apoptotic process, in contrast to the HBL100 cell line, which undergoes necrosis only and recovers readily once I3C is removed from the culture medium. These data indicate a fundamental difference in the mechanism by which I3C exerts growth-inhibitory effects in these two cell lines.

To investigate the mechanistic effects of I3C at early time points and also, in some cases, to rule out any potential mechanistic role of a pathway, particularly in the more resistant HBL100 cell line, we used concentrations of the agent above 500 μM. It should be noted that whereas these experiments provided useful mechanistic data, concentrations of I3C above 500 μM are unlikely to have any relevance to a physiological situation.

To investigate the differential response of the two cell lines, components of the PKB pathway were examined. Despite the higher levels of total PKB protein found in the HBL100 cell line, the MDA MB468 cell line had higher levels of the phosphorylated form. Whereas PTEN, which blocks activation of PKB by dephosphorylation of PI3K signaling intermediates, was expressed in the HBL100 cell line (lower phospho-PKB expression), we did not detect it in the MDA MB468 cell line (high phospho-PKB). These data are in agreement with a report by Lu *et al.* (55), in which it was shown that loss of function of PTEN in breast cancer cell lines, including MDA MB468, resulted in increased basal phosphorylation of multiple components of the PI3K signaling pathway. We conclude therefore that the presence of PTEN in the HBL100 cell line accounts for its lower level of phospho-PKB in comparison with the MDA MB468 cell line. Together, these observations would seem to signify a heightened importance of signaling through the PKB pathway in the MDA MB468 cell line relative to the HBL100 cell line.

PKB has been well documented as a prosurvival factor (26, 32, 33, 37, 39, 41–43) and has also been implicated in oncogenesis (39, 44, 56, 57). I3C (5-h treatment) decreased basal levels of phosphorylated PKB and PKB activity in the MDA MB468 cell line, but not in the HBL100 cell line. I3C did not inhibit PKB activity when added directly to an *in vitro* kinase assay at concentrations up to 750 μM, indicating that it was not acting as a direct enzyme inhibitor. Over an 8-h treatment period, no decrease in total PKB protein level was observed, strongly suggesting that I3C acts on signaling pathways upstream of PKB. Because the effect was not prevented by pretreatment with the phosphatase 2A inhibitor okadaic acid, it seems most likely that I3C acts by preventing phosphorylation of the protein rather than by promoting its dephosphorylation. Recent results reported by Meng *et al.* (58) showed induction of PTEN by I3C in the T47D breast cell

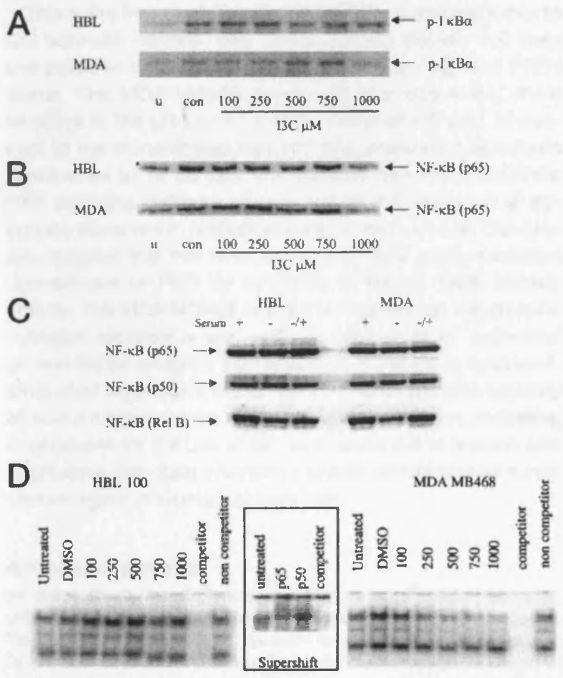

Fig. 9. Effect of I3C (0–1000 μM; 5 h) on IKK activity as determined by phosphorylation of IκBα in an *in vitro* kinase assay (*A*) and on nuclear NF-κB (p65) levels (*B*). *C* shows nuclear levels of NF-κB (p65, p50, and RelB) in cells cultured under normal growth conditions (+), after 24 h of serum starvation (−), and 4 h after replenishment of serum-starved cells with 10% FCS (−/+). *D* shows the effect of I3C on NF-κB DNA binding as measured by EMSA in HBL100 and MDA MB468 cell lines. The blots shown are representative of three independent experiments. Supershift of p65 and p50 in control HBL100 cells is shown in the *boxed area* of *D*.

line. However, in our study, I3C did not appear to stimulate PTEN levels in either MDA MB468 or HBL100 cell lines.

Inhibition of PKB phosphorylation in both breast cell lines by the specific PI3K inhibitor LY294002 still only induced apoptosis significantly in the MDA MB468 cell line. This observation led us to two conclusions: (*a*) first, because inhibition of PKB by LY294002 induced apoptosis in the MDA MB468 cell line, it is likely that such inhibition contributes to the I3C-induced apoptosis observed in this cell line; and (*b*) second, the HBL100 cell line was resistant to induction of apoptosis by LY294002, despite a decrease in phospho-PKB levels, indicating that other survival signaling pathways predominate in this cell line. We also have preliminary data (not shown here) for two additional human breast tumor-derived cell lines, T47D and MCF7, both of which are positive for PTEN. Levels of PTEN were similar in MCF7 and HBL100 cell lines and higher in the T47D line. Levels of phospho-Akt in the T47D cell line were similar to those in the HBL100 cell line, and lower than those in the MCF7 cell line. I3C inhibited the growth of these cell lines with an approximate IC_{50} of 60 μM and, as in the HBL100 cell line, did not show inhibition of phospho-PKB levels after a 5-h treatment.

We propose that the sensitivity of the MDA MB468 cell line to I3C stems at least in part from the relative importance of signaling through the PKB pathway in this cell line compared with that in the PTEN-positive HBL100 cell line. To strengthen the idea that the cell-specific effects of I3C in the breast cell lines were related to their relative phospho-PKB levels and PTEN status, we determined the effect of the agent in prostate cells of known PTEN status. In agreement with our hypothesis, I3C decreased phospho-PKB levels and induced apoptosis in the LNCaP cell line, which expresses very low levels of PTEN, but not in the DU145 cell line, which expresses much higher levels of PTEN. Our data are also consistent with a report by Hsu *et al.* (59), in which the cyclooxygenase-2 inhibitor celecoxib was shown to induce apoptosis in the prostate carcinoma cell lines LNCaP and PC-3 by a mechanism involving PKB (59). As with I3C, celecoxib had no effect on Bcl2 levels at short time points, and the inhibition of PKB phosphorylation was not prevented by okadaic acid. Hsu *et al.* (59) also observed no inhibition of PI3K activity in response to celecoxib in prostate cell lines. Our finding that the PI3K inhibitor LY294002 decreased phosphorylation of PKB in all four cell lines in contrast to the cell line-specific effect of I3C indicated to us that the mechanism by which I3C acts upstream of PKB does not consist of a straightforward inhibition of PI3K activity (again showing similarity to celecoxib). Extensive investigations are currently under way in our laboratory to elucidate the possible involvement of differential expression of upstream targets including the epidermal growth factor receptor in the mechanism of action of I3C.

A possible candidate for mediating the effects of I3C-induced inhibition of PKB signaling is the prosurvival transcription factor NF-κB. PKB can regulate NF-κB via activation of IKK, resulting in increased phosphorylation of IκB and consequent release of NF-κB from the inhibitory complex (53, 54). Despite inhibition of PKB, we found no decrease in IKK activity in either cell line in response to I3C treatment. In support of this, nuclear levels of NF-κB (p65) also remained unchanged. However using EMSA, we found that binding to an NF-κB consensus oligonucleotide was decreased using nuclear protein extracts from I3C-treated MDA MB468 cells, but not in those from the HBL100 cell line. From these results, we concluded that I3C alters DNA binding of NF-κB protein family members, including p65 and p50, by a mechanism that does not involve inhibition of IKK activity. The constitutive expression of NF-κB in the nucleus of HBL100 and MDA MB468 cells points to the possibility that in these cell lines translocation of the protein to the nucleus may not be the major limiting step in NF-κB-induced transactivation. The cell line specificity of the inhibition of DNA binding by I3C corresponded with that observed for inhibition of PKB, suggesting that the decrease in NF-κB DNA binding may occur as a consequence of inhibition of PKB activity or of a common upstream regulatory step. Recently, Heiss *et al.* (60) showed that sulforaphane, an aliphatic isothiocyanate also found in cruciferous vegetables, inhibited NF-κB DNA binding in macrophages. The mechanism by which sulforaphane exerted this effect was independent of IκB degradation or translocation of NF-κB to the nucleus, and a redox-dependent mechanism was proposed (60). There are other examples in the literature in which modulation of nuclear NF-κB DNA binding has been found to occur independently of effects on IKK or nuclear translocation (61–64). The mechanism by which I3C affects NF-κB DNA binding in the MDA MB468 cell line remains to be confirmed.

This is the first study to show selectivity in the response to I3C between normal- and tumor-derived human cell lines and between cell lines of differing phospho-PKB and PTEN status. The MDA MB468 tumor cell line was 4-fold more sensitive to the growth-inhibitory effects of I3C and, in contrast to the immortalized HBL100 line, underwent apoptosis in response to 10 μM I3C. We propose that inhibition of the PKB signaling pathway contributed to the induction of apoptosis observed in response to I3C in this cell line. Our data also suggest that this effect may be at least partly mediated downstream of PKB by inhibition of NF-κB DNA binding activity. The MDA MB468 cell line is regarded as negative for estrogen receptor-α and with its high level of epidermal growth factor receptor expression, it is therefore representative of an aggressive breast tumor phenotype that typically affects younger women. This finding therefore has promising implications for the use of I3C as a treatment in women with aggressive, estrogen-insensitive breast cancer and as a preventive agent in women at high risk.

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