THE EXPRESSION AND ROLE OF PROTEIN KINASE C ISOFORMS IN TAMOXIFEN RESISTANT BREAST CANCER

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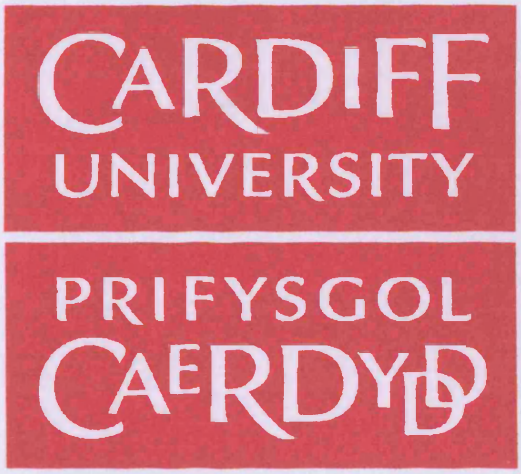

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"Pick yourself up, dust yourself off, start all over again."

-Dorothy Fields (1905-1974)

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ABBREVIATIONS

β-Gal	β-Galactosidase
ADP	Adenosine Diphosphate
AF-1	Activating Function-1
AF-2	Activating Function-2
AKAP79	Protein A-Kinase Anchoring Protein
APES	3-aminopropyltriethoxysilane
aPKC	Atypical Protein Kinase C
APS	Ammonium Persulphate
ARD	Acute Respiratory Disease
ATAC	Anastrozole, Tamoxifen and Combination
ATP	Adenosine Triphosphate
bis	Bisindolylmaleimide IX
bp	Base Pairs
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB Binding Protein
CPE	Cytopathic Effect
cPKC	Conventional Protein Kinase C
csFCS	Charcoal Stripped Foetal Calf Serum
DAB	Di-aminobenzidine Dihydrochloride
DAG	Diacylglycerol
DBD	DNA Binding Domain
DCCM	Dendritic Cell Conditioned Medium
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DN	Dominant Negative
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
E_2	Oestradiol
EDTA	Ethylenediaminetetracetic Acid
EGF	Epithelial Growth Factor
EGF-R	Epithelial Growth Factor Receptor
EGTA	Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic Acid
ER	Oestrogen Receptor
ERE	Oestrogen Response Element
ERK	Extracellular-Signal-Regulated Kinases
FCS	Foetal Calf Serum
FDA	US Food and Drug Administration
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GSK-3	Glycogen Synthase kinase
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylases
HEK	Human Embryonic Kidney
HRG-β	Heregulin Growth Factor-β
HRP	Horse Radish Peroxidase

Hsp	Heat Shock Protein
ICC	Immunocytochemistry
IES	Intergroup Exemestane Study
IGF	Insulin-Like Growth Factor
IGFBP	Insulin-Like Growth Factor Binding Protein
IGF-IR	Insulin-Like Growth Factor I Receptor
IgG	Immunoglobulin G
IP	Immunoprecipitate
IP_3	Inositol 1,4,5-Trisphosphate
IRS	Insulin Receptor Substrates
ITA	Italian Trial
kDa	Kilo Dalton
MAPK	Mitogen-Activated Protein Kinases
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
NCoR	Nuclear Receptor Coregulator
NFκB	Nuclear Factor κB
NID	Nuclear Receptor Interaction Domain
NLS	Nuclear Localisation Sequence
nPKC	Novel Protein Kinase C
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDK-1	Phosphoinositide-Dependent Protein Kinase 1
pfu	Plaque Forming Units
PI3K	Phosphatidyl Inositol 3-Kinase
PIP_2	Phosphatidylinositol Bisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PKI	Protein Kinase Inhibitor
PLC	Phospholipase C
PMSF	Phenylmethylsulphonylfluoride
PS	Phosphatidylserine
PtdIns	Phosphatidyl Inositol
PTEN	Phosphatase and Tensin Homologue Deleted on Chromosome 10
PTGS	Post-transcriptional Gene Silencing
r/t	Room Temperature
RACK	Receptors for Active C-Kinase
REA	Repressor of Oestrogen Activity
RICK	Receptors for Inactive C-Kinase
RISC	RNA-Induced Silencing Complex
RNAi	Ribonucleic Acid Interference
RTK	Receptor Tyrosine Kinase
SD	Standard Deviation
SDS	sodium dodecyl sulphate or lauryl sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel
SEM	Standard Error Mean
Ser	Serine

SERD	Selective Oestrogen Receptor Downregulator
SERM	Selective Oestrogen Receptor Modulator
SH2	Src Homology Domain 2
SHP	Short Heterodimer Partner
siRNA	Small Interfering Ribonucleic Acid
SMRT	Silencing Mediator of Retinoic Acid Receptor
Sos	Son of Sevenless
SRC	Steroid Receptor Co-Activator
STICK	Substrates That Interact with C Kinases
TAM	Tamoxifen
TAM-R	Tamoxifen Resistant
TBP	TATA Binding Protein
TEMED	N,N,N,N tetramethylethylenediamine
TF	Transcription Factor
TFIIB	Transcription Factor IIB
TGF-α	Transforming Growth Factor-α
Thr	Threonine
TPA	12-*O*-tetradecanoylphorbol 13-Acetate
Tyr	Tyrosine
v/v	Volume/ Volume
w/v	Weight/ Volume
WT	Wild Type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-Galactopyranoside

ABSTRACT

The development of resistance to anti-oestrogenic therapies such as tamoxifen is a serious clinical problem in the treatment of breast cancer. A specific model of Tamoxifen resistance has been developed in the Tenovus laboratories by maintaining MCF-7 breast cancer cells in Tamoxifen (10^{-7}M) for 4-6 months. The resistant cells that arise from these cultures are termed TAM-R cells. We wished to utilize these cells to test the hypothesis that resistance to tamoxifen is due to changes in protein kinase C (PKC) isoform expression.

Initially we investigated PKC expression in the TAM-R cells and demonstrated that they express significantly more basal and activated protein kinase C (PKC) -α and δ than wild type MCF-7 cells.

To test the implications of this observation, we wished to specifically and selectively ablate these PKCs in the TAM-R cells and assess the outcomes. The limitations of pharmacological inhibitors such as bisindolylmaleimide IX (Ro31-8220) and Rottlerin were highlighted by our studies which concurs with a general discontent in the current literature over their specificity and efficacy. We therefore utilised RNAi and adenovirus mediated molecular technologies to modulate the PKC-α and PKC-δ isoform expression profile in the MCF-7 and TAM-R cell lines. Using both RNAi and adenoviral infection of dominant negative mutants we demonstrated that down regulation of PKC-α and PKC-δ blocks both growth factor and oestradiol induced growth in MCF-7 and TAM-R cells. Thus PKC-α and δ must play an important role in the mitotic pathways utilised by tamoxifen resistant breast cancer cells.

Moreover overexpressing PKC-α and δ in MCF-7 cells allowed them to acquire resistance to tamoxifen and possibly even led to tamoxifen becoming agonistic for these cells, suggesting a role for these isoforms of PKC in inducing the tamoxifen resistant phenotype.

CHAPTER 1.

GENERAL INTRODUCTION

1.1. History of Breast Cancer

Breast cancer results after normal mammary epithelial cells sustain sufficient genetic damage to acquire the phenotypes characteristic of malignancy. These include unregulated proliferation, protection from cell death, and metastasis (Sachdev and Yee, 2001). It has been described in medical treatises discovered in Egypt that date back as far as 3000BC (Odling-Smee, 2001) although no treatment was advocated at this time other than cautery for the ulcerated tumour (Baum, 1988). The disease was later described by the Greek "Father of Medicine" Hippocrites (c.460-370BC) who coined the word "carcinoma" from the word Karkinos- the Greek for crab (Werner, 1990). Hippocrites advised that surgery would be of greater immediate risk to the patient than no treatment at all. Considering the inherent dangers and suffering of surgery in the days before anaesthesia and antisepsis this was probably sound reasoning (Baum, 1998). Despite this considered advice, the Romans were probably practising mastectomy for breast cancer as early as the first century BC (Odling-Smee, 2001).

The advent of aseptic surgery and anaesthetics made the practice of mastectomy a far more viable and acceptable treatment (Odling-Smee, 2001). In 1895 Dr William Halsted of the Johns Hopkins Hospital in Baltimore introduced the radical mastectomy as the standard operative procedure. The hospital records for mastectomy between the period 1889 to 1931 indicate that this procedure was not terribly successful. From a population of around 900 patients operated on by Halsted or his students 6% died soon after the operation, the local recurrence of disease was seen in 30% of patients and the ten-year survival rate was just 12%. However, between 1930 and 1950 there were increases in survival rates which can be attributed to earlier diagnosis and advances in clinical classification techniques of the disease (Baum, 1998).

1.1.1 Breast Cancer Incidence

Breast Cancer is currently the most common cancer in women worldwide, accounting for 25% of all female malignancies with the highest proportion of those accounted for

by women in the developed western countries. The incidence and mortality varies about five-fold around the world (Key *et al.*, 2001). For example, incidence rates in Japan, India and China are only about a quarter of those in the USA. However, breast cancer is increasing in parts of the world that previously had not been associated with a high risk of incidence. This is starkly highlighted in Korea which has seen the incidence of breast cancer rise by 56% between 1980 and 1998 (Kang *et al.*, 2002).

Studies of migrant populations have suggested that the observed differences between countries and ethnic groups are more attributable to social and environmental factors than inherent genetic traits. Migrants from low risk countries to high-risk countries eventually show rates similar to the rest of the population of their adoptive country. A study of migrants from eastern Asia to the USA showed an increase in breast cancer incidence after ten years residence, with a maximum increase in risk was observed once the migrants had been resident in the country for one or two generations (Key *et al.*, 2001).

In the UK, 38,000 women are diagnosed with breast cancer every year. This accounts for 30% of all new female cancers, more than twice as many as the next most common, which is colorectal cancer. The lifetime risk of contracting breast cancer if you are a woman is now 1 in 9. Most women diagnosed are past the menopause, though around 7000 cases each year are in women under 50 years of age. Additionally, around 200 men are diagnosed with breast cancer every year (http://www.statistics.gov.uk/).

1.2. Oestrogens and Breast Cancer

It is now known that around 70% of breast tumours present the receptor for the steroid oestrogen (Harvey *et al.*, 1999). The possibility that oestrogen could be functioning as a mitogenic factor in breast cancer was first alluded to in 1896 by the British physician George Beatson who published a paper in the Lancet describing the regression of breast cancer in premenopausal women who had undergone complete oopherectomy (Beatson, 1896). Four years later, a study reported that a third of patients could expect regression of their disease following this treatment (MacGregor

and Jordan, 1998). However, it would take another 60 years for the mechanism through which the steroid hormone was acting to begin to be elucidated.

In 1962, Jensen and Jacobson first described the oestrogen receptor (ER) in the uterus of rats. Jensen and colleagues went on to formulate the first ER assay. This assay was used to test which patients' breast tumours would respond to cessation of endocrine stimulation by oopherectomy in premenopausal women or adrenalectomy in postmenopausal women. It was thus established that women with ER rich tumours responded to endocrine ablation, whilst women with ER negative breast tumours had a poor prognosis (MacGregor and Jordan, 1998).

1.2.1. The Oestrogen Signalling Pathway

Oestrogens act in many tissues including the sex accessory tissues, bone, pituitary and cardiovascular cells (White and Parker, 1998). They exert a wide variety of effects on these tissues including stimulation of growth, differentiation, as well as important regulatory functions (Klinge, 2000). Oestrogens are lipophilic steroid hormones that are classically thought to diffuse freely across the plasma membrane into cells, where they bind to oestrogen receptors (ER) in the nuclei of responsive cells causing a change in conformation and concurrent homodimerization (MacGregor and Jordan, 1998). Novel pathways for the action of oestrogen, which have more recently been elucidated, will be discussed in detail later.

The profound and varied effects of estrogens are mediated through two distinct but related oestrogen receptor subtypes that regulate the transcription of target genes, through binding to specific DNA target sequences, termed the oestrogen response elements (EREs). The ERE consists of inverted repeats of the sequence GGTCA separated by three variable nucleotides, e.g. 5'- CAGGTCAnnnTGACCTG-3' where n = any nucleotide. However, most oestrogen-regulated genes contain imperfect, non-palindromic ERE sequences (Klinge, 2000).

1.2.2. Oestrogen Receptor Structure

The ER belongs to the family of nuclear receptors, a large group of ligand inducible transcription factors that total approximately 150 different proteins. This superfamily, which includes receptors for steroid hormones, thyroid hormones, retinoids, fatty acids and prostaglandins and a number of orphan receptors, are grouped due to their highly conserved primary structures and the organisation of their functional domains (White and Parker, 1998).

It was discovered in 1996 that two different forms of ER exist and that they were encoded by separate genes (Hopp and Fuqua, 2001). The conventional ER was designated ERα and the newly discovered ER isoform, ERβ. The human ER α gene resides on chromosome 6q sub band 25.1 and is transcribed as a single mRNA of 6.5kb that encodes a protein of 595 amino acids, with an approximate molecular mass 66kDa. The ERβ gene is located on chromosome 14q22-24 and encodes a protein of 530 amino acids, with a molecular mass of around 60kDa (Hopp and Fuqua, 2001).

Both ERα and ERβ share a high degree of structural homology and display the typical structural characteristics of the steroid receptor family. Both ER subtypes consists of six functional domains which are designated A – F (from N- to C terminus) and encoded by 8-9 exons (Klinge, 2000). The amino terminal A/B domains show the greatest variability between all the steroid receptors (Sommer and Fuqua, 2001) with even ER-α and β sharing poor homology (approximately 17%). The A/B domains mediate transcription in a cell and promoter context specific manner through their ligand independent transcription activating function (AF-1) region (Klinge, 2001; MacGregor and Jordan, 1998).

The middle, C domain is highly conserved between ERα and β (97%), within the nuclear receptor family and between species (i.e. 100% amino acid identity between man and chicken) (Kumar, 1987). This high degree of conservation indicates the region's importance in the functionality of these receptors. The C region contains both the DNA binding domain (DBD) and the dimerisation domain (MacGregor and Jordan, 1998). Dimerisation appears to be vital for receptor functionality as mutations

that prevent or impede dimerisation result in receptors that are insoluble or transcriptionally inactive (Tamrazi *et al.*, 2002). The DBD consists of two zinc motifs through which the receptor interacts directly with the DNA helix (Klinge, 2001). Differences in these zinc fingers between different steroid hormone receptors account for differences in their specificity. When these zinc fingers are not present the ER cannot bind DNA either *in vitro* or *in vivo*. The C region may also have a role in nuclear localisation of the receptor and also may be required for hsp90 binding. The role of hsp90 and other chaperones may be to maintain the receptor in the correct conformation to allow rapid responses to hormone signals (White and Parker, 1998).

The D domain is a 40-50 amino acid sequence that functions as a hinge region between the DBD and the ligand-binding domain (LBD). This region shares moderate homology between ERα and ERβ (around 30%) and is poorly conserved in length and sequence between species and other members of the nuclear receptor family (Kumar, 1987). The hinge region contains sequences for dimerisation and nuclear localisation sequences (NLS). It has also been shown to have important binding sites involved in interactions with receptor co-activator and co-repressor proteins (Klinge, 2001).

The C- terminal E and F domains comprise the LBD, a hsp90 binding region, a ligand dependent NLS, a dimerisation domain, and a ligand dependent trans-activational function (AF-2) (MacGregor and Jordan, 1998). Although region E shows only moderate homology between ERα and ERβ (59% amino acid identity) they both bind oestradiol and other natural and synthetic ligands with the same affinity (Hopp and Fuqua, 2001). The LBD can be thought of as a specific and selective ligand dependent switch. The LBD has a compact three layer structure comprising of 12 α-helices which form a hydrophobic ligand binding pocket (Sommer and Fuqua, 2001) and one β turn arranged as an antiparallel helical 'sandwich' (Moras and Gronemeyer, 1998). Binding of ligand alters the conformation of the LBD with helix 12 forming a lid over the pocket that secures the ligand within the pocket (Klinge, 2001). Hydrophobic residues on the surface of the helix 12 lid form a surface with which receptor co-activating proteins can interact. These conformational changes, induced upon the binding of ligand, affect the recruitment of co-factors leading to a modulation of the receptor's transcriptional activity (Sommer and Fuqua, 2001).

The E region has been shown through deletion studies to be essential for efficient activation of transcription (Kumar, 1987). The AF-2 is localised to the most C terminal end of the E domain and is highly conserved within the nuclear receptor superfamily. It is recognised by a variety of transcriptional co-activators. A third transactivational function, termed AF-2a, has been characterised towards the N-terminal end of the LBD (Klinge, 2001). It has been shown that AF-2a can activate gene transcription in a ligand independent manner even in the absence of AF-1 or AF-2 (Hopp and Fuqua, 2001). The F domain is thought to play a role in distinguishing between oestrogen agonists and antagonists, through interactions with cell specific factors (Klinge, 2001).

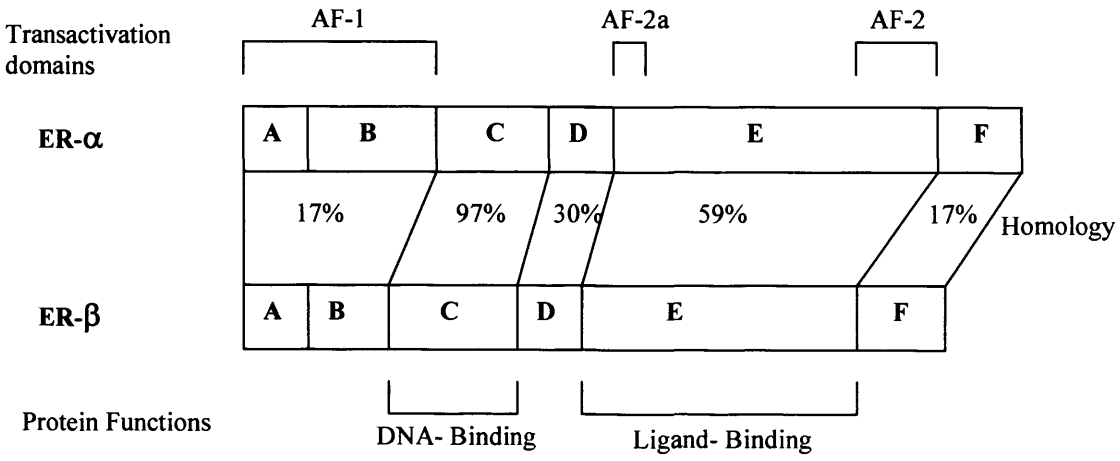


Figure 1.1. Comparison of the ER-α and ER-β functional domains.

The diagram illustrates the relative locations of the transactivation domains AF-1, AF-2, and AF-2a. Also indicated are the functional domains involved in DNA-binding and ligand-binding The degree of homology between the two isoforms is shown as a percentage.

1.2.3. Oestrogen Receptor Co-regulators

The ER is known to interact with many nuclear proteins *in vivo* and *in vitro*. Early experiments showed that the over-expression of one type of nuclear receptor

displayed a dose-dependent inhibitory effect on the transcriptional activation of other nuclear receptors. This observed 'squelching' implicated a finite amount of a factor or group of factors that are necessary for transcription and that the nuclear receptors must compete for binding of these co-activators. There have now been around 20 transcription enhancing co-activators identified that interact with ERα (Klinge, 2000).

1.2.3.1. Co-Activators

Interactions between the ER and co-activators are mediated through the ligand-dependent AF-2 of the LBD and a distinct region on the co-activator called the nuclear receptor interaction domain (NID) or the 'NR box'. The NR box comprises of an α helical LxxLL motif (where L represents leucine and x represents any amino acid) that is present in single or multiple copies (McKenna, 2002). One of the common functional properties of co-activators is histone acetyltransferase (HAT) activity. HATs acetylate lysine residues on the N-terminal tails of histones H3 and H4, resulting in a weaker association of the histones with DNA, providing a more transcriptionally permissive environment (McKenna, 2002). Thus, nucleosomal conformation and stability are altered, enhancing the pre-initiation complex, facilitating transcriptional activation by RNA polymerase II (Klinge, 2000).

The first steroid receptor co-activators to be described were the SRC family of related proteins, comprising of SRC-1, SRC-2 and SRC-3. These co-activators are able to promote oestradiol-mediated transcription and promote interaction between AF-1 and AF-2. These co-activators are also believed to form a ternary complex with other proteins to promote hormone responsive gene transcription. These co-integrator proteins, such as CBP and p300, possess intrinsic HAT activity and have been implicated in the co-activation of several transcription factors (TFs) including steroid receptors, NF-κB and p53. The SRC family has also been shown to interact with other TFs such as fos and jun and basal TFs TBP and TFIIB. Additionally, SRC-1 interacts with cyclin D1, a key cell cycle regulator (Hopp and Fuqua, 2001).

1.2.3.2. Co-Repressors

Along with the activation of target genes in response to hormones, an important part of gene regulation involves the silencing of these genes in the absence of stimulation. Basal transcription can be suppressed via the recruitment of nuclear receptor co-repressors such as SMRT (silencing mediator of retinoic acid receptor) and NCoR (nuclear receptor coregulator). Both these are found complexed with the co-repressor mSin3 and histone deacetylases (HDACs). Histone deacetylation may be involved in transcriptional repression by maintaining chromatin in a more condensed state that impairs the ready access of TFs to the DNA. Ligand binding triggers the release of these repressor proteins and stimulates the recruitment of co-activators via a drastic conformational change in the AF-2 domain (Leo and Chen, 2000). There have been several co-repressors of ER reported to date. These include REA (repressor of oestrogen activity) which competes with co-activators for the ligand binding domain, SHP (short heterodimer partner) interferes with DNA binding as well as competing with co-activators and the BRCA-1 (breast cancer susceptibility genes) which can downregulate the p300 co-integrator protein (Dobrzycka *et al.*, 2003).

1.2.4. Phosphorylation of the ER

All steroid receptors, including the ER, exist as phosphoproteins in the absence of ligand binding. Upon the binding of oestrogen, the ER undergoes additional phosphorylation at several residues including serines 104, 106, 118, 154, 167, 294 and tyrosine 537 (Castano *et al.*, 1998). The hyperphosphorylation of steroid receptors is believed to be important in DNA-binding, transcriptional activation and stability (Leclercq, 2002). The ER does not undergo phosphorylation solely through activation by oestrogen. The ER can also be phosphorylated in the absence of ligand binding by a number of growth factors and/or protein kinases. One well-studied example of ligand-independent phosphorylation is a serine residue at position 118, which is required for full activation of the AF-1 region (Kato *et al.*, 1995). Growth factor receptors for epidermal growth factor (EGF) or insulin-like growth factor (IGF) can activate serine 118 through the activation of ERK, a member of the MAPK family. ERK activation has been shown to be a contributing factor in both proliferation and

cell survival of the MCF-7 breast cancer cell line (Levin, 2002). Phosphorylation of ER at Ser118 has also been shown to enhance the interaction of the receptor with the p68 RNA helicase that results in an increase in AF-1 activity (Hopp and Fuqua, 2001). The AF-1 region contains phosphorylation sites for a number of other kinases including cyclin A/cdk2 and PI3/AKT, though less is known about their specific ER function (Hopp and Fuqua, 2001). Another major ligand-independent phosphorylation site within the ERα is a conserved tyrosine 537 residue and the homologous tyrosine 443 residue present in ERβ (Hopp and Fuqua, 2001). Phosphorylation at these sites activates the receptor, possibly through the realignment of helix 12, forming the interacting surface required for the recruitment of co-activators (White and Parker, 1998). It may also be important in the ligand inducible changes in ERα conformation (Hopp and Fuqua, 2001). Growth factors can also activate $pp90^{rsk-1}$ via ERK, resulting in Ser 167 phosphorylation of ER. This provides a further route for ER activation in the absence of oestrogen (Levin, 2002).

1.2.5. Alternative signalling through the ER

Over the past two decades the classical mechanism of oestrogens action has been augmented by an increasing wave of literature that describes alternative pathways through which oestrogen can exert it's influence. The classical model describing steroid hormones as transcriptional activators that act through their nuclear receptors has been central to their perceived mechanism of action. However this classical model can only account for changes in protein expression that occur over a typical time scale of hours whilst many effects of steroid hormones stimulation have been shown to occur within minutes (Valverde and Parker, 2002). The onset of these actions are too expeditious to be attributable to either RNA synthesis or protein translation (Nadal *et al.*, 2001). These rapid responses have been attributed to oestrogen exerting it's effect not only on nuclear receptors but also in cytosolic and plasma membrane locations (Nadal *et al.*, 2001) (Figure 1.2).

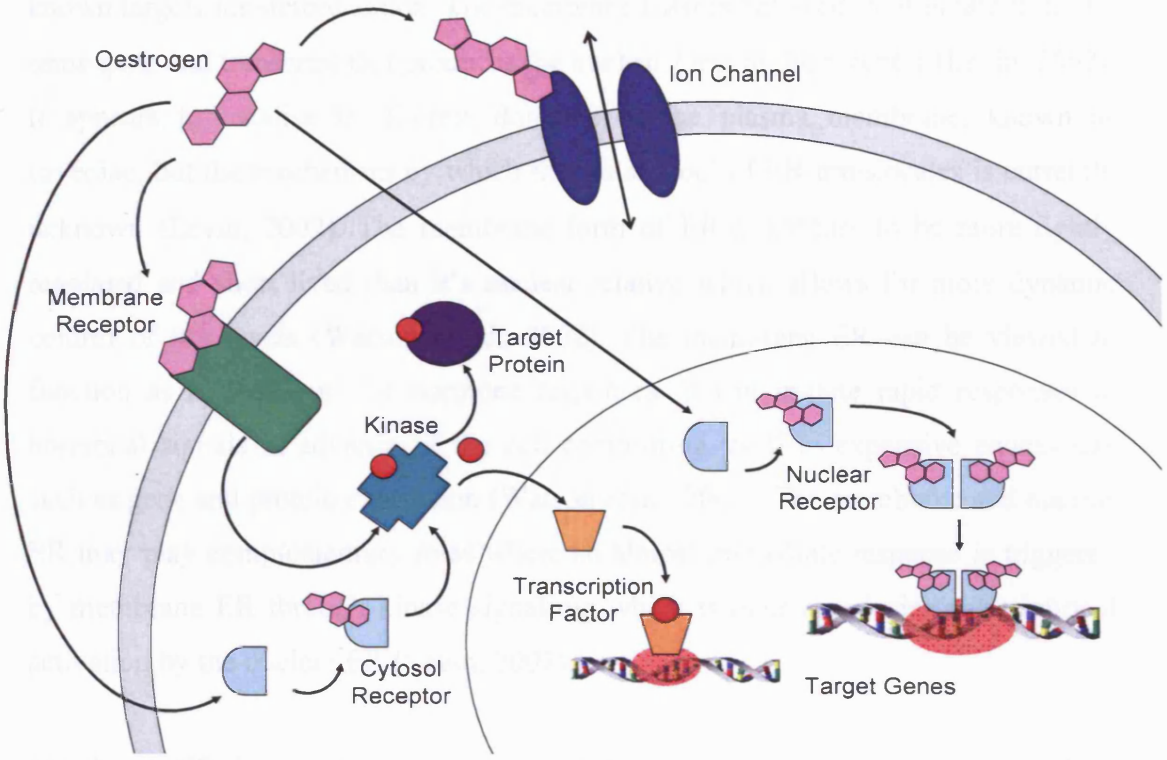


Figure 1.2. Mechanisms of action of steroids at three different cellular levels: membrane, cytosol and nucleus.

Membrane targets include steroid receptors similar to the nuclear receptors, non-classical steroid receptors, and ligand- and voltage-activated ion channels. Cytosolic targets are believed to be the classical `translocating' receptors. Nuclear targets effect the direct modulation of gene expression by the interaction of receptor complexes with the hormone response element in the DNA or with other transcription factors (Adapted from Valverde and Parker, 2002).

Membrane proteins involved in rapid oestrogen actions include specific ER, ion channels, membrane enzymes, and other ligand receptors (Nadal *et al.*, 2001). There is now strong evidence for the presence of a plasma membrane ERα in cells that are known targets for steroid action. The membrane ERα is believed to originate from the same gene and transcript that produces the nuclear form of the receptor (Levin, 2002). It appears to localise in discrete domains of the plasma membrane, known as caveolae, but the mechanism by which this small pool of ER translocates is currently unknown (Levin, 2002). The membrane form of ER-α appears to be more tightly regulated and short lived than it's nuclear relative which allows for more dynamic control of it's levels (Watson *et al.*, 2002). The membrane ER can be viewed to function as a 'look-out' for hormone responses. It can initiate rapid responses to hormonal signals in advance of the cell committing itself to expensive endeavours such as gene and protein expression (Watson *et al.*, 2002). The membrane and nuclear ER may play complementary roles where an almost immediate response is triggered by membrane ER through kinase signalling which is maintained via transcriptional activation by the nuclear ER (Levin, 2002).

Membrane ER functions can be stimulated through the binding of oestrogen which initiates signalling through G-protein activation and calcium flux (Kely and Levin, 2001). They can also be facilitated via cross talk with signalling molecules including the epithelial growth factor receptor (EGFR) (Levin, 2002). This leads to cascades that can activate cAMP, phospholipase C and IP_3 which in turn causes the activation of kinases such as protein kinase A (PKA) and protein kinase C (PKC) (Levin, 2002).

Oestrogen can also exert their influence through binding to cytosolic ERα and ERβ receptors. Upon the binding of oestrogen, the cytosolic ER interact with the Src kinase changing it's conformation to an active state and thereby activating the ERK MAPK signalling cascade (Nadal *et al.*, 2001).

1.3. Anti-Oestrogenic Therapy

Since Beatson's experiments over 100 years ago, it has been known that manipulation of the endogenous levels of oestrogen can be an effective treatment in the

management of hormone responsive breast tumours. Although oopherectomy can still be used as an effective treatment for the cessation of oestrogen stimulated growth, the administration of anti-oestrogenic drugs is now the most widely used treatment. The proposal for using an antagonist to the action of oestrogen as an anti breast cancer agent was suggested by Lacassagne in 1936 (Lacassagne, 1936). Anti-oestrogenic drugs are now used in both pre-menopausal and postmenopausal women and in metastatic, adjuvant and chemopreventive settings. The drugs available are generally well tolerated with a low incidence of dose-limiting toxicities, and responses are seen in approximately 70% of patients selected on the basis of the hormone receptor expression profile (Clarke *et al.*, 2001).

1.3.1. Tamoxifen

The most successful and widely used anti-oestrogenic treatment is the selective oestrogen modulator (SERM) Tamoxifen (TAM) (Figure 1.3). Originally, and unsuccessfully, designed as a contraceptive, TAM was first approved for the treatment of breast cancer in 1973 in the UK and later by the US Food and Drug Administration (FDA) in 1977. Since then the drug has also been approved for use as an adjuvant treatment with chemotherapy (1986) as an adjuvant alone in postmenopausal patients found to have axillary lymph nodes containing evidence of tumour spread (1988) and in pre- and post-menopausal node negative women that are ER positive (1990). Tamoxifen is now also used in women thought to have a high risk of suffering from the disease (1990) (Park and Jordan, 2002). Clinical experience with TAM now exceeds 10 million patient years and is the most widely used anti-cancer drug (Clarke *et al.*, 2001).

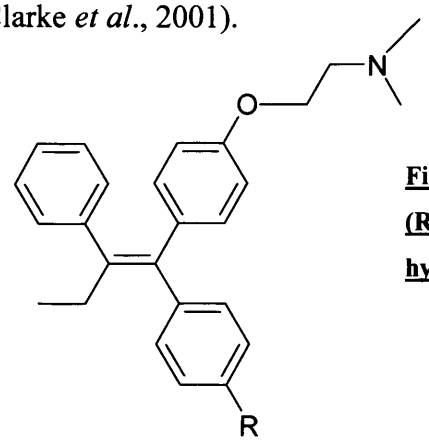


Figure 1.3. Molecular structure of Tamoxifen (R=H) and its active metabolite 4-hydroxyTamoxifen (R= OH).

1.3.1.1. Selective Oestrogen Receptor Modulation by TAM

The original model describing how TAM inhibited the growth stimulatory effects of oestrogen, proposed a mechanism by which TAM acted as an antagonist that competed with oestrogen for binding to the ER. It was therefore thought that TAMs anti-oestrogenic properties would have undesirable effects in other tissues where oestrogen is required for normal growth and regulation. For example, oestrogen is vital for the maintenance of bone density in post-menopausal women so a compound with anti-oestrogenic properties would be thought to compromise skeletal integrity. Paradoxically female patients who were administered TAM actually displayed a beneficial increase in bone mineral density (McDonnel, 1999). It was discovered that far from acting as an antagonist in bone, it actually acted as an agonist. However, this agonist activity is also displayed in the endometrium where it has the undesirable effect of increasing the risk of endometrial cancer. In women who are prescribed TAM this increase in risk is about 2-3 fold (Jordan, 1999). Much has been made of this increased risk but it can be put into some perspective by the fact that TAM has been shown to reduce the risk of breast cancer by around 35-50% compared to 1% potential increase in risk of endometrium cancer (http://www.breastcancer.org/).

1.3.1.2. Mechanism of TAM Action

This discovery that SERMs such as TAM can function as both agonist and antagonists in cell specific contexts has meant that the classical model of ER pharmacology has had to be re-evaluated. The classical models of ER action described the ER as an inactive receptor that was switched to an active conformation by binding an agonist such as oestradiol. This action could be blocked by the introduction of an antagonist that would simply compete for binding with oestrogen at the binding site. However, it has since been shown that antagonists such as TAM do not solely function by blocking activation of the ER but rather by inducing a conformational change in the ER itself that interferes with its ability to bind oestradiol at multiple steps in the signal transduction pathway (McDonnell, 1999).

TAM binds a hydrophobic pocket in the LBD of the ER. *In vitro* assays have demonstrated that TAM binding allows the ER to dimerise and bind to DNA with high affinity but blocks transcriptional activity mediated through the ligand dependent AF-2 (White and Parker, 1998) (Figure 1.4). This inhibition is caused by the bulky alkylaminoethoxyphenyl side chain of TAM interacting with the Asp 351 residue of the LBD. This interaction prevents the re-orientation of helix 12, the most C terminal helix of the LBD. For a functional AF-2 response it is essential that helix 12 seals the ligand into the receptor to form an active AF-2 domain with other parts of the LBD and bind the necessary co-activators to form a viable transcription complex (Levenson and Jordan, 1999). When the receptor has bound TAM, rather than forming a functional AF-2 conformation, helix 12 binds to co-activator recognition sites by mimicking the interaction between the 'NR box' and the LBD (Shiau *et al.*, 1998).

The inhibition of AF-2 activity is contrasted by the ERs AF-1 domain which is active even when Tamoxifen is bound to the receptor (White and Parker, 1998). Therefore it has been proposed that in contexts where AF-2 is required for ER transcriptional activity TAM functions as a pure antagonist. Conversely, in contexts where AF-2 is not required and AF-1 is sufficient for ER transcriptional activity TAM can act as an agonist (McDonnell, 1999).

The identification of steroid receptor co-activators and repressors that interact with the ER has provided further mechanisms by which oestrogen and SERMs exert their influence in a cell and context specific manner. It has been shown that the ability of co-activators and repressors to bind the ER is influenced by the structure of the receptor-ligand complex. Because SERMs induce different conformational changes within the ER, it is likely that the resulting receptor-ligand structure would not interact with all the co-activator and co-repressor proteins in an equivalent manner. Therefore the nature of the SERM activity could be dependent on the interactions of these proteins with the ER-SERM complex (McDonnell, 1999).

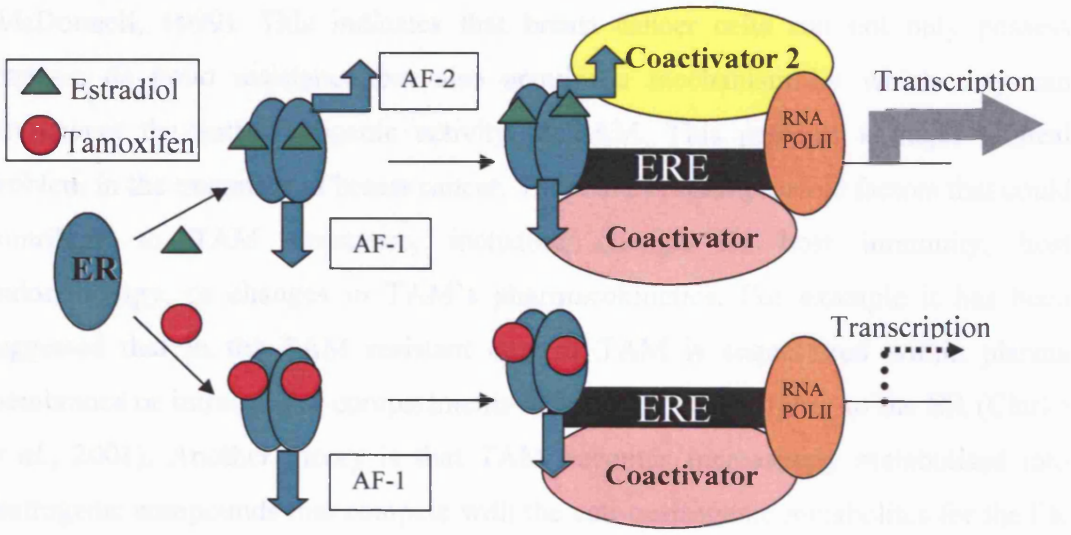


Figure 1.4 Oestradiol induced activation and tamoxifen (TAM) mediated attenuation of the oestrogen response element

Oestradiol binds the oestrogen receptor (ER) causing receptor homodimerisation. The homodimer localises to the cell nucleus where it binds the oestrogen response element (ERE). Transcription is initiated through the two activation functions, AF1 and AF2, interacting with transcriptional co-factors to stimulate the activity of RNA polymerase II (RNA POLII). Upon binding TAM the ER still dimerises but the AF2 function is blocked. This allows some partial agonist activity through AF1 but attenuates overall transcription.

1.3.1.3 Tamoxifen Resistance

The absence of oestrogen receptor is the greatest cause of TAM treatment failure in breast tumours. In more than 90% of these cases anti-oestrogenic therapies have no effect (Clarke *et al.*, 2001). However, even patients with oestrogen receptor positive breast tumours that are initially responsive to the anti-proliferative effects of TAM will experience a recurrence of their disease within 2-5 years of the treatment (McDonnell, 1999). This indicates that breast cancer cells can not only possess intrinsic de novo resistance but also acquire a mechanism by which they can circumvent the anti-oestrogenic activity of TAM. This presents a major clinical problem in the treatment of breast cancer. There are several possible factors that could contribute to TAM resistance, including changes in host immunity, host endocrinology, or changes in TAM's pharmacokinetics. For example it has been suggested that in the TAM resistant context TAM is sequestered within plasma membranes or intracellular compartments reducing it's availability to the ER (Clarke *et al.*, 2001). Another theory is that TAM becomes increasingly metabolised into oestrogenic compounds that compete with the anti-oestrogenic metabolites for the ER (Lonning *et al.*, 1992). It was originally thought that the acquisition of antioestrogen resistance was caused by a loss or mutation of the ER. However, it has since been shown that breast cancer cell lines that have lost anti-oestrogen sensitivity retain an ER positive phenotype with normal ER functionality (Brunner *et al.*, 1993). Indeed Tamoxifen resistant breast tumours are still sensitive to other hormonal therapies that target the ER such as the pure anti-oestrogen ICI 182,780 (Fulvestrant) (Robertson, 2001).

1.3.1.4 Growth Factor Receptors and Tamoxifen Resistance

There is a growing wealth of literature that has established the importance of growth factor signalling and its cross talk with the oestrogen receptor as a vital mechanism by which breast cancer cells are able to evade the effects of extended treatment with Tam. This paradigm has been established due, in part, to the observation that several growth factor stimulated cell signalling pathways are elevated in the resistant disease state.

Growth factor mediated responses are only possible in cells that possess highly sensitive and selective receptors. There are several different types of transmembrane receptor including G-protein coupled receptors, ion channel linked receptors and the receptor tyrosine kinase family (RTK). The family of RTK receptors are transmembrane receptors with an intrinsic intracellular kinase activity. Upon binding ligand these receptors undergo dimerisation to produce either a homodimer with another same receptor or a heterodimer with a closely related receptor of the same family (Heldin, 2003). Dimerisation allows the receptor to undergo autophosphorylation on intracellular tyrosine residues. These phosphorylated tyrosines are recognised by proteins containing Src homology 2 (SH2) domains. There are two groups of SH2 containing protein. There are those that contain intrinsic enzymatic ability such as the tyrosine kinase Src and phospholipase C γ and there are proteins without intrinsic activity that serve as adaptors that mediate interactions between other molecules. One such adaptor protein is Grb2 which associates with guanosine exchange factor Son of Sevenless (Sos) which activates the small G protein Ras. This subsequently activates Raf-1, an important component in the MAPK pathway which has been shown to play direct roles in many cellular processes including proliferation and survival (Santen, 2002) (Figure 1.5).

As well as having direct effects on proliferation, MAPK can also phosphorylate key sites in the AF-1 domain of the ER such as on the serine 118 and 167 residues leading to ligand independent activation of the receptor (Kato *et al.*, 1995). It has been shown in our models of Tamoxifen resistance that basal phosphorylation of serine 118 is elevated and can be further enhanced by the activation of tyrosine kinase growth factor receptors (Britton *et al.*, 2002).

Another pathway downstream of RTKs that is now achieving greater scrutiny is the phosphotidylinositol 3-kinase (PI3K)–AKT pathway. PI3Ks are a family of heterodimeric lipid kinases activated by RTKs. They can be grouped into three classes, referred to as I, II and III. PI3Ks phosphorylate the 3'-hydroxyl of phosphotidylinositol (PtdIns) to produce four species of phosphorylated inositides: PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3 (Neri *et al.*,2002). These

lipid products of PI3K function as second messengers by recruiting cytoplasmic proteins with phosphoinositide-binding domains to specific sites on the cellular membrane (Fruman, 2003). One such protein recruited to the plasma membrane is AKT where it becomes fully activated by phosphorylation at threonine 308 and serine 473. Activation of AKT by PI3K is known to trigger mechanisms of tumour progression such as growth, proliferation, survival and motility (Vivanco and Sawyers, 2002). Termination of PI3K downstream signalling occurs by dephosphorylation. This is carried out by a phosphatase encoded by the tumour suppressor gene PTEN (phosphatase and tensin homologue deleted on chromosome 10). In a significant number of tumours PTEN is mutated or inactive and the PI3K pathway is constitutively activated (Neri *et al.*, 2002). Whilst the specific role of PI3K in TAM resistance has not been established, it has been shown that there is a strong association between the downregulation of PTEN expression in ERα positive tumours and failure to TAM treatment (Shoman et al., 2005).

1.4. ErbB Receptors

The first tyrosine kinase receptor to be characterised was the epidermal growth factor receptor (EGFR). The EGFR was subsequently found to be one of a member of related transmembrane tyrosine kinase receptors called the ErbB receptor family (Marmor and Yarden, 2003). There are four members of this family: the EGF receptor itself (also termed ErbB1), ErbB2, ErbB3 and ErbB4. The ErbB receptors are single chain membrane spanning proteins with significant sequence homology. The importance of the tyrosine kinase domains in their function is highlighted by ErbB1, ErbB2 and ErbB4 sharing 80% sequence homology in this region. The ErbB3 receptor is distinct in that it lacks this tyrosine kinase functionality due to substitutions at four important amino acid residues (Vereb *et al.*, 2002). The ErbB2 receptor is also distinct from the other ErbB receptors as it is the only family member not to bind a known physiological ligand. As ErbB2 is an orphan receptor and ErbB3 lacks tyrosine kinase functionality, and therefore represent the deaf and the dumb of the ErbB receptors, they can have no downstream effects as homodimers. Interestingly however, the heterodimer they form is the most potent signal transducer of all the ErbB combinations (Yarden and Sliwkowski, 2001).

Overexpression of the EGFR and other ErbB family members is frequently seen in human breast cancers and is an indicator of poor prognostic outcome (Verbeek, 1998) and elevated levels of proliferation (Nicholson *et al.*, 1999). This is especially evident

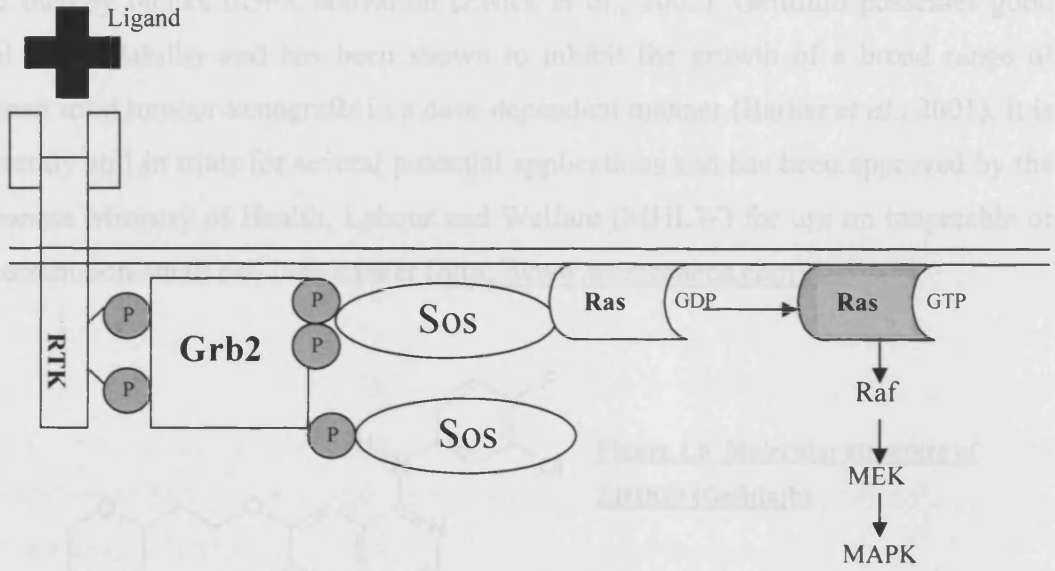


Figure 1.5. Diagrammatic representation of growth factor signalling resulting in the activation of MAPK.

Growth factor ligand binds to its transmembrane receptor and activates tyrosine kinases inherent in the receptor molecule. A series of protein-protein complexes form involving Grb-2, and Sos that lead to GDP-Ras being converted to GTP-Ras which activates Raf, MEK and then MAPK (Adapted from Santen *et al.*, 2002).

in the failure of 95% of ER negative / EGFR positive breast tumours to show endocrine responsiveness and the poor survival characteristics of these patients (Nicholson and Gee, 1996). EGFR has also been shown to be over-expressed in both ER negative and ER expressing tumours that have developed anti-oestrogen resistance (Chrysogelos, 1994). This could be due to the inverse relationship that exists between the levels of ER and EGFR in breast cancer cells or could be the result of a direct role of the EGFR. For example, in vitro transfection of EGFR or ErbB2 into hormone dependent breast cancer cells results in hormone independent proliferation and Tamoxifen resistance respectively (Nicholson *et al.*, 1999).

The significance of EGFR signalling in TAM resistant breast cancer cells is highlighted by an increased sensitivity to the highly specific EGF-R tyrosine kinase

inhibitor Gefitinib (ZD1839) (Figure 1.6). This compares with a minimal growth inhibitory effect observed following Gefitinib treatment of oestrogen sensitive cells (Gee *et al.*, 2003). Gefitinib is an ATP analogue of the quinazoline family that competes with ATP for the ATP binding site at the receptor tyrosine kinase domain and thereby blocks EGFR activation (Zwick *et al.*, 2002). Gefitinib possesses good oral bioavailability and has been shown to inhibit the growth of a broad range of human solid tumour xenografts in a dose-dependent manner (Barker *et al.*, 2001). It is currently still in trials for several potential applications and has been approved by the Japanese Ministry of Health, Labour and Welfare (MHLW) for use on inoperable or recurrent non-small cell lung cancer (http://www.astrazeneca.com)/.

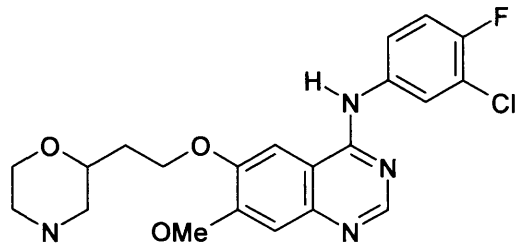


Figure 1.6 Molecular structure of ZD1839 (Gefitinib)

Whilst the *in vitro* data with Gefitinib demonstrates that EGF-R signalling is important in Tamoxifen resistance it has also become clear that it's favoured heterodimerisation partner ErbB2 also plays a role. As with EGF-R activation, breast cancer patients with ErbB2 receptor gene amplification and overexpression display reduced survival and reduced time to relapse highlighting ErbB2 as a potential drug target. In 1998 a humanised monoclonal anti-ErbB2 antibody called Trastuzumab was approved by the FDA. Trastuzamab binds to ErbB2 causing downregulation by causing receptor internalisation, inhibition of cell cycle progression and induction of immune response (Fischer *et al.*, 2003). Two simultaneous clinical trials combining Trastuzumab and chemotherapy in high-risk ErbB2 positive breast cancer were reported early due to a 52% decreased risk of recurrence in patients treated with Trastuzumab compared to control groups (Moyer, 2005). To evaluate the importance of ErbB2 in cells that have developed TAM resistance, TAM sensitive and TAM resistant cell models were treated with Trastuzumab. It was shown that Trastuzumab was effective in reducing the growth of the TAM resistant cell line whilst having little

significant effect on the TAM sensitive cells indicating a possible role for ErbB2 in acquired TAM resistance (Nicholson *et al.*, 2004).

1.5. IGF Signalling

Another widely studied RTK is the insulin-like growth factor I receptor (IGF-IR). The IGF-IR carries out multiple functions in many normal human tissues, including the mammary gland. There is now growing evidence that IGFs can affect breast cancer growth through mitogenic and anti-apoptotic signals and act in synergism with oestrogen to increase proliferation (Sachdev and Yee, 2001). IGFs are generally more potent mitogens in oestrogen responsive cells than either TGFα or EGF (Nicholson and Gee, 1996) and have been shown to have effects *in vitro* on several breast cancer cell lines at picomolar to nanomolar concentrations (Lee and Yee, 1995). Also stimulation of the IGF-IR can enhance the phosphorylation of serine residues 118 and 167 in the ER of our Tamoxifen resistant model (Nicholson *et al.*, 2002).

The IGF system utilises two ligands that possess a high degree of structural homology with insulin, IGF-I and IGF-II. Both IGF-I and IGF-II perform endocrine, paracrine and autocrine roles. There are two high affinity receptors for IGF-I and II. The IGF-I receptor (IGF-IR) is a transmembrane tyrosine kinase receptor whilst IGF-IIR is not and the mechanisms it employs are unclear, however it is thought to be important in the interaction between IGF-II and IGF-IR (Sachdev and Yee, 2001). The IGFs and IGF-IR have been shown to stimulate transformation, proliferation and inhibit apoptosis, thereby promoting survival, in breast cancer cells. Their proliferative effects are thought to be heavily dependent on interactions with a family of six specific binding proteins (IGFBPs) that are known to sequester IGFs. IGFs are usually complexed to circulating IGFBPs which serve as a reservoir for IGFs, extending their half-lives and regulating their contact with their receptors. IGFBPs can have stimulatory or inhibitory effects depending on their concentration, phosphorylation status and proteolytic fragmentation (Lee and Yee, 1995). Tamoxifen has been shown to influence the delivery of IGF-I to the breast cancer cell *in vivo* by decreasing plasma levels of IGF-I, possibly through the inhibition of gene expression, and elevating plasma IGFBP-I (Lonning *et al.*, 1992).

Unlike other growth factor RTKs, IGF-IR employs a family of soluble adapter proteins, called insulin receptor substrates (IRS), to initiate its downstream signalling cascades (Whitehead *et al.*, 2000). As with signalling through the EGFR, IGFR via IRS acts as an activator of downstream Ras-MAPK signalling cascades (Sachdev and Yee, 2001) and PI3K (Whitehead *et al.*, 2000). The major IRS adapter protein used by IGF-I in ER expressing cells to transduce signals is IRS-1. It has been reported that IRS-1 is over-expressed in some primary breast tumours (Sachdev and Yee, 2001) and that its expression can be induced by oestradiol leading to enhanced IGF-IR signalling (Dupont and Le Roith, 2001).

1.6. Protein phosphorylation

Activation of receptor tyrosine kinases, such as the EGF-R or IGF-R, by their respective ligands can lead to activation of their intrinsic enzymatic activity and the modulation of downstream effector proteins. There are several types of modulation that can occur including isoprenylation, which is often important in protein localisation, and glycosylation which can alter the activity of a protein (Schenk and Snaar-Jagalska, 1999). Probably the most important and widely-studied of these covalent modifications is protein phosphorylation mediated by protein kinases and protein dephosphorylation mediated by protein phosphatases. (Manning *et al.*, 2002). Phosphorylation of a protein can either activate it or inhibit it. For example the AP-1 transcription factor c-Jun possesses a site at its N-terminus that is phosphorylated by MAPK family members. Phosphorylation at this site is involved in c-Jun activation. Conversely, c-Jun also contains a site near its DNA binding domain that when phosphorylated by glycogen synthase kinase-3 (GSK-3) causes silencing of c-Jun activity (Schenk and Snaar-Jagalska, 1999).

Studies of the human genome have identified 518 putative protein kinases genes which constitute about 1.7% of all human genes (Manning *et al.*, 2002). The importance of phosphorylation is highlighted by the discovery that even the genome of the budding yeast *saccharomyces pombe* contains around 120 different protein kinases (Gomperts *et al.*, 2002). Kinases function by adding the γ-phosphate of ATP

to the hydroxyl group of specific serine, threonine or tyrosine residues. The residues phosphorylated in any given protein are dependent on the specificity of the kinase for that protein and the accessibility of the residues to the kinase, though generally the residues phosphorylated are exposed on the surface of a protein and often located in the interface between the subunits of regulatory proteins (Gomperts *et al.*, 2002).

The amino acid that undergoes phosphorylation defines the two major classes of protein kinases: Serine/Threonine (Ser/Thr) kinases and tyrosine (Tyr) kinases. The Ser/Thr kinases are present in all eukaryotes whilst the Tyr Kinases evolved later and are mostly associated with multicellular organisms (Young and Kuriyan, 2003). The activity of protein kinases are themselves regulated by multiple signalling molecules that can modulate the kinase by several different mechanisms. These include changes in the conformational shape of its catalytic core by phosphorylation or dephosphorylation, ligand coupled allosteric activation or inhibition, or the by changes in the localisation of the kinase (Young and Kuriyan, 2003).

When active, the multitude of different protein kinases retain significant structural similarity in their catalytic domains. In the inactive state however the different classes of kinase have developed different structural mechanisms to prevent the unregulated induction of a catalytically competent conformation (Huse and Kuriyan, 2003). The three dimensional structure of an eukaryotic protein kinase domain was first elucidated when the molecule cyclic AMP-dependent protein kinase, also known as protein kinase A (PKA), was visualised by X-ray crystallography. The catalytic domain was visualised in a complex with magnesium adenosine triphosphate (Mg-ATP) and a naturally occurring heat stable protein kinase inhibitor (PKI) to capture the PKA in a conformation that was primed and ready to carry out phosphorylation but without an available acceptor for the γ-phosphate (Knighton *et al.*, 1991). This structure has subsequently been used as the model of the catalytic domains of active protein kinases.

The catalytic domain of a protein kinase comprises of two lobes of around 275 residues. The N-terminal lobe which contains an anti-parallel β-sheet and an α-helix and the larger C-terminal lobe which is primarily α-helical in composition. The

manner in which the kinase binds the two substrates of the phosphorylation reaction, ATP and the polypeptide phosphate acceptor, is believed to be highly conserved between all the protein kinases. Both substrates bind in the cleft between the N-terminal and C-terminal lobes. There is not thought to be a set order for substrate binding, but due to it's high concentration within the cell ATP is thought to bind first (Taylor *et al.*, 2004). The ATP is bound deep within the cleft between the two lobes in a highly conserved region called the phosphate binding loop. This phosphate binding loop contains a conserved glycine rich sequence motif (GXGXnG) where n is usually a tyrosine or phenylalanine. The glycine residues allow the loop to get into very close proximity and interact with the ATP. In the absence of ATP the glycine residues render the phosphate binding loop very flexible. This allows the binding of small molecule inhibitors that can bring about large structural distortions to the catalytic domain (Huse and Kuriyan, 2002). The peptide substrate molecule binds to the front end of the binding pocket close to the γ-phosphate of the ATP molecule on a centrally located "activation loop" that provides a platform for the peptide substrate. When the kinase is active the loop is phosphorylated stabilising it in an open and extended conformation that aids peptide binding (Huse and Kuriyan, 2002). Catalysis of the phosphorylation reaction occurs in two stages. Firstly a bond is formed between the phosphate group of the ATP and the amino acid to be phosphorylated. Then the phosphodiester bond between the β and γ phosphates is broken, resulting in a phosphorylated peptide and ADP which are then released from the active site (Lodish *et al.*, 2001).

Protein kinases play an integral role in nearly every aspect of regulation in the mammalian cell including transcription, cell cycle progression, apoptosis and differentiation. Their importance in these cellular processes and ubiquity in the cell implicates protein kinases as targets for investigation in oncogenic as well as normal cellular processes. In recent years, the deregulation of certain protein kinases have become synonymous with certain types of cancers This has lead to a great deal of financial and intellectual investment in the development of protein kinase inhibitors as possible treatments. However, common catalytic mechanisms, structural similarity and the importance of protein kinases to multiple physiological processes represents a

challenge in identifying selective inhibitors as potential treatments or modes of study in disease states (Dancey and Sausville, 2003).

1.7 Protein Kinase C

One of the most prominent protein kinases is the serine/threonine kinase protein kinase C (PKC). PKC is almost ubiquitously activated in signal transduction mechanisms involving polypeptide hormones, cytokines and growth factors (Martinez-Lacaci and Dickson, 1996). PKC has been shown to function in amazingly diverse cellular processes ranging from growth, proliferation and apoptosis to learning and memory (Mellor and Parker, 1998). There is now a growing body of evidence that PKC has a role in the growth and differentiation of breast cancer cells. PKC has been shown to be present at elevated levels in human breast tumour biopsies compared with relative normal tissue (O'Brian *et al.*, 1989) and *in vitro*, a positive correlation has been found between elevated levels of PKC and a more aggressive phenotype (Lee *et al.*, 1992). Additionally, it has been shown that inhibitors of PKC can inhibit the growth of MCF-7 breast cancer cells (Seynaeve *et al.*, 1993).

The protein kinase C (PKC) family at present incorporates 12 distinct members (Gomperts *et al.*, 2002). These isoforms can be grouped together on the basis of their structure and co-factor regulation. The first group to be discovered were the conventional PKCs (cPKCs) comprising of isoforms α, βI and βII (two alternatively spliced variants), and γ. The cPKCs are activated by the phospholipid phosphatidylserine (PS) in a Ca^{2+} dependent manner. The cPKCs also bind the second messenger diacylglcerol (DAG) which increases specificity of the enzyme for PS and shifts the affinity for it into the physiological range (Mellor and Parker, 1998). DAG is produced through the cleavage of phosphotidylinositol bisphosphate (PIP_2) by phospholipase C (PLC) in response to extracellular stimuli such as growth factor or hormone signalling. The cleavage of PIP_2 also creates the second messenger inositol 1,4,5-trisphosphate (IP_3) which initiates a rise in intracellular calcium levels increasing opportunity for PKC activation (Blobe *et al.*, 1996). PKC is also activated by other products of the PI cycle including PIP_2 itself. PIP_2 is also the primary *in vivo*

substrate of PI3K which converts it to phosphotidylinositol trisphosphate (PIP_3) which can also activate some isoforms of PKC (Vivanco and Sawyers, 2002).

cPKC and nPKC enzymes are targets for the tumour-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) which has been a commonly used tool in PKC research. Phorbol esters such as TPA appear to bind the same sites as DAG though with a lower requirement for elevated Ca^{2+} concentrations. However phorbol esters also appear to have greater metabolic stability than DAG which leads to a more prolonged activation (Blobe *et al.*, 1996). Although phorbol esters initially induce activation of PKC they then cause depletion following prolonged treatment. Therefore the tumour promoting effect could be a consequence of PKC activation or its subsequent deletion (Lu *et al.*, 1997).

The next family of PKCs to be discovered were termed the novel PKCs (nPKC) comprising δ, ε, η, θ, and μ. As with the cPKCs, the nPKCs are activated by DAG and phorbol esters such as TPA in the presence of PS but differ in that they are insensitive to Ca^{2+} concentration (Mellor and Parker, 1998). The least understood PKC isoforms are the atypical PKCs (aPKCs) ζ, ι, and λ (which appears to be the mouse homologue of ι) (Liu and Heckman, 1998). The aPKCs are unresponsive to Ca^{2+}, DAG, and phorbol esters but rather appear to be primarily regulated through lipid co-factors such as PIP_3 (Moscat *et al.*, 2001). aPKCs have been implicated in important downstream steps of the PI3K pathway and interestingly their overexpression has resulted in the enhancement of activator protein-1 (AP-1) promoter activity leading to increased transcriptional activity (Moscat *et al.*, 2001).

1.7.1. PKC Structure

All PKC isoforms consist of a regulatory and a catalytic domain with four conserved (C1-C4) and 5 variable (V1-V5) regions. C1 and C2 regions are situated in the N-terminal regulatory domain with regions C3 and C4 found within the C-terminal catalytic domain. The C1 domain contains one or two cysteine-rich domains which act as the DAG or phorbol ester binding site. The C2 region serves as the Ca^{2+} binding domain in the cPKCs but is absent in the Ca^{2+} independent isoforms (Schenk

and Snaar-Jagalska, 1999). This regulatory domain also contains a pseudosubstrate site that inhibits the kinase activity of the catalytic domain in the absence of activating co-factors. Its inhibitory action is exerted through a sequence motif that resembles a consensus phosphorylation site in PKC substrates but presents an alanine residue rather than the phosphorylatable serine/threonine residue of the substrate. Therefore, interaction of the pseudosubstrate site with the substrate binding pocket in the C4 region of the catalytic domain results in an inactive conformation and suppression of kinase activity (Dekker and Parker, 1994).

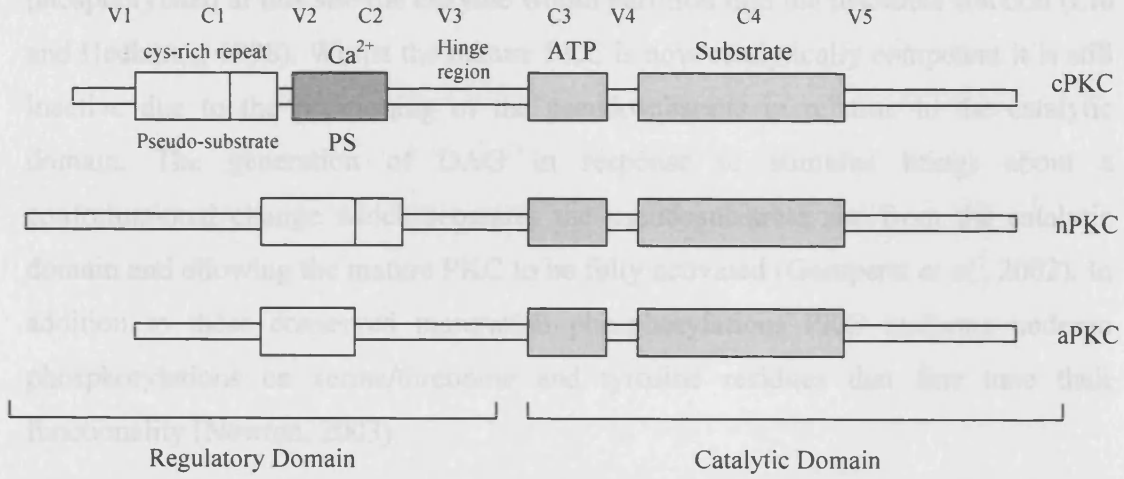


Figure: 1.7 Diagram illustrating the structures of the different sub-families of PKC .

The isoforms of PKC can be grouped into sub-families by their structure and co-factor regulation. The diagram illustrates of the structural differences between the conventional (cPKC); novel (nPKC); and atypical (aPKC) PKC isoforms.

1.7.2 Phosphorylation of PKC

PKC is synthesised within the cell as an inactive precursor which can be located in the detergent insoluble fraction. Whilst the catalytic site is accessible the enzyme is catalytically incompetent (Gomberts *et al.*, 2002). For the classical and novel PKC isoforms to become catalytically viable they undergo the sequential phosphorylation of three specific residues. The first phosphorylation is at a threonine residue on the activation loop of the kinase. This transphorylation is carried out through phosphoinositide-dependent protein kinase 1 (PDK1) which is also known to phosphorylate the activation loop of the PKB family of kinases (le Good *et al.*, 1998)

as well as p70 s6 kinase (Belham *et al.*, 1999). The naming of PDK-1 was somewhat unfortunate since it appears to be constitutively active and therefore can act on substrates, such as the conventional PKCs, independently of phosphoinositide (Newton, 2004). The phosphorylation of the activation loop by PDK-1 leads to two further rapid autophosphorylations near the carboxyl terminus in a 65-70 amino acid segment of the V5 domain that immediately follows the catalytic domain (Belham *et al.*, 1999). The first of these are thought to stabilise the enzyme as it replaces the requirement for a negative charge at the activation loop (Newton, 1995). The final phosphorylation allows the enzyme to be released into the cytosol. Unless the PKC is phosphorylated at this site the enzyme would partition into the insoluble fraction (Liu and Heckman, 1998). Whilst the mature PKC is now catalytically competent it is still inactive due to the positioning of the pseudosubstrate in relation to the catalytic domain. The generation of DAG in response to stimulus brings about a conformational change which separates the pseudosubstrate site from the catalytic domain and allowing the mature PKC to be fully activated (Gomperts *et al.*, 2002). In addition to these conserved maturation phosphorylations PKC isoforms undergo phosphorylations on serine/threonine and tyrosine residues that fine tune their functionality (Newton, 2003).

1.7.3 PKC Distribution

There has been many studies into the tissue-specific variations in PKC isoform content and distribution. This has not been easy though as many tissues are heterogenous and comprise many different cell types. Also whilst the variations in PKC between different cell types has been widely described as a consequence of gene expression, there has been little evidence in most cases to show that the differences are caused by variation at the transcriptional level (Liu and Heckman, 1998). Despite these limitations certain isoforms have been found to be more plentiful in certain cell types. Whilst PKC α, βI/ βII, δ, ε and ζ appear to be ubiquitously expressed, PKC θ is predominantly present in skeletal muscle, PKC μ is strongly expressed in the thymus and lung and PKC γ expression is almost solely the preserve of central nervous system and brain (Liu and Heckman, 1998). The variation in isoform distribution between different cell types is further complicated by variations in the distribution of

these isoforms within the cell itself. These differences in subcellular localisation may confer isoform specific functions within specific cell types following activation and translocation to specific anchoring proteins (Way *et al.*, 2000).

In the unstimulated resting state the conventional PKCs are present in the cytosol. Whilst they do transiently interact with the plasma membrane this is only due to diffusion within the cytosol and the inherent affinity the conventional PKC isoforms have for the membrane is very low. When the C2 region of conventional PKC binds calcium following elevation of levels within the cell, it causes PKC's affinity for the plasma membrane to be greatly enhanced. This causes the PKC to translocate and associate strongly with the plasma membrane via interactions that orientate the binding face of the conventional PKC toward negatively charged phospholipids in the plasma membrane (Schaefer *et al.*, 2001). This robust direct association with the two-dimensional plane of the plasma membrane greatly increases the chance of PKC colliding with and binding the much less abundant second messenger DAG than would occur due to the transient encounters they enjoy whilst PKC traverses the three-dimensional region of the cytosol. The binding of DAG by PKC allows the release of the pseudosubstrate sequence from the catalytic domain and therefore allows the activation of the PKC (Newton, 2003). This association of PKC with the plasma membrane can be maintained for a time after calcium levels have subsided. As the novel PKCs lack the calcium binding C2 domain they cannot benefit from calcium induced translocation to the plasma membrane and therefore translocate 5 to 10 fold slower than conventional PKCs (Schaefer *et al.*, 2001). As previously noted, the atypical PKC isoforms are insensitive to both calcium and DAG. However they have been shown to be activated downstream of the PI3-K pathway and form complexes with specific binding partners that target the isoforms to the correct location to elicit their responses (Ohno, 2001).

1.7.4 PKC Binding Proteins

The ubiquity, broad specificity and promiscuity of the PKC isoforms has lead to the opinion that the association with specific binding proteins and subsequent localisation must play a role in PKC facilitating the correct specific response to a stimulus or

convergence of separate stimuli. Genetic, biochemical and cell based methods have elucidated a large number of heterogeneous PKC binding proteins each with their own specific mechanisms of binding and isoform specificity. Some of these binding proteins bind multiple isoforms whilst others are responsible for the distribution of a single specific isoform (Jaken and Parker, 2000). These binding proteins can also display specificity for isoforms in different phosphorylation states and can facilitate a specific isoforms to display activity or inhibitory functions in response to different stimuli (Newton, 2003).

Whilst these specific binding proteins are heterogenous in nature and display a broad range of functions they share common functions within the cell. Many function as targeting proteins that position specific PKCs to appropriate locations in response to a stimulus whilst equally importantly preventing inappropriate events occurring. This change in localisation could function to bring the PKC closer to a substrate, a regulator of activity such as a kinase or phosphatase or into a different intracellular compartment (Newton, 2003). The modulation of localisation and the over-lapping specificities of some of the binding proteins may also provide a mechanism through which multiple signals integrate at PKCs, altering the type and fidelity of response elicited (Jaken and Parker, 2003). For example it has been shown that the scaffolding protein A-kinase anchoring protein (AKAP79) can co-localise PKC with PKA and the calmodulin stimulated protein phosphatase calcineurin (Faux and Scott, 1997).

One class of binding protein that can associate with and direct PKCs are the STICKs (substrates that interact with C kinase). STICKs are all phospholipid binding proteins that generally bind PKCs in an isoform non-specific manner. Whilst they bind to the regulatory domain of PKC with a very high affinity, this interaction appears to be transient and/or dependent on specific conditions as the endogenous PKC and STICKs are difficult to co-immunoprecipitate *in vivo* (Jaken and Parker, 2000). The STICKs include the PKC substrate STICK72 which can be found in membrane protrusions and ruffles and is associated with a role in cell motility. Another is γ-adducin which dissociates from its binding to the cytoskeleton upon phosphorylation by PKCs (Dong *et al.*,1995). Since the interactions between STICKs and PKCs are generally not isoform specific it can be postulated that additional mechanisms must be

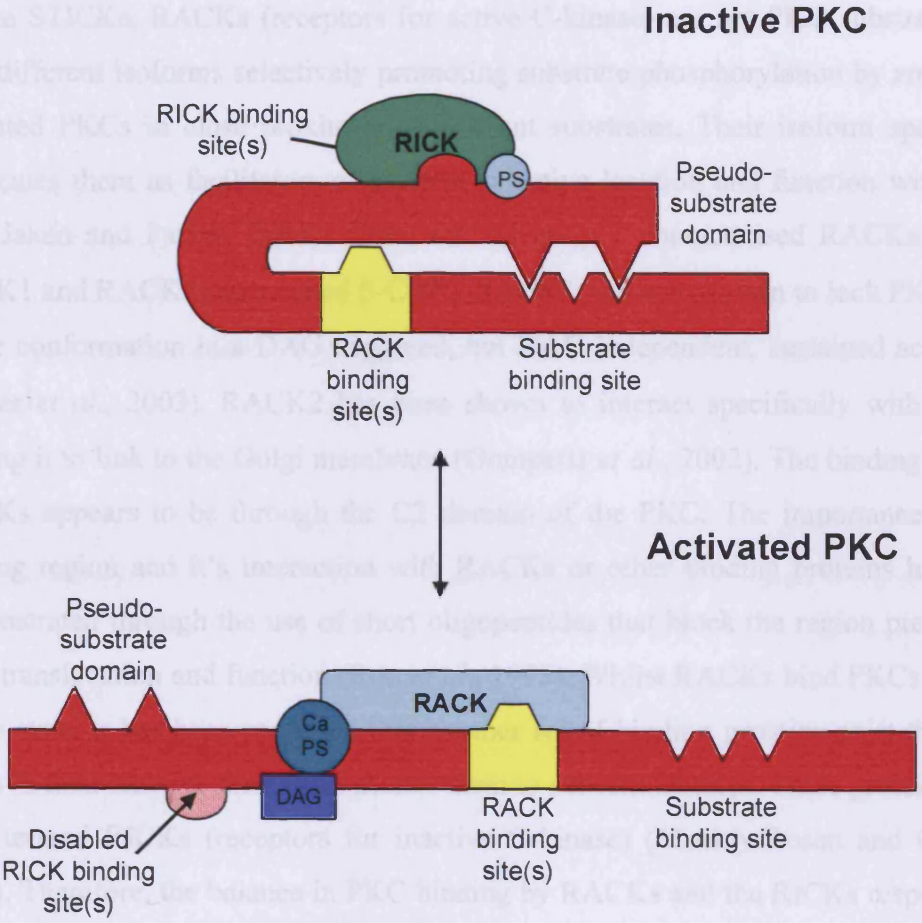


Figure 1.8 A model of the interaction between the RICK anchoring proteins with inactive PKC and the RACK anchoring proteins with activated PKC.

In the inactive form, the PKC is folded with the amino terminus pseudo-substrate site associated with the substrate binding site in the catalytic domain and an exposed binding site for RICKs. On activation the PKC unfolds exposing the RACK binding site and the substrate binding site whilst leaving the RICK binding site unavailable (adapted from Mochly-Rosen and Gordon, 1998)

employed to assure correct associations occur and potentially damaging erroneous targeting is avoided.

Unlike STICKs, RACKs (receptors for active C-kinase) are not PKC substrates and bind different isoforms selectively promoting substrate phosphorylation by anchoring activated PKCs in close proximity to relevant substrates. Their isoform specificity implicates them as facilitators of isoform selective location and function within the cell (Jaken and Parker, 2003). There are currently 2 characterised RACKs termed RACK1 and RACK2 (also called β-COP). RACK1 has been shown to lock PKC in an active conformation in a DAG triggered, but DAG independent, sustained activation (Parker *et al.*, 2003). RACK2 has been shown to interact specifically with PKC-ε causing it to link to the Golgi membrane (Gomperts *et al.*, 2002). The binding site for RACKs appears to be through the C2 domain of the PKC. The importance of this binding region and it's interaction with RACKs or other binding proteins has been demonstrated through the use of short oligopeptides that block the region preventing PKC translocation and function (Ron *et al.*, 1995). Whilst RACKs bind PKCs in their active state, it has been proposed that another set of binding proteins exist that bind PKCs in their inactive forms and anchor them to subcellular sites. These proteins have been termed RICKs (receptors for inactive C-kinase) (Mochly-Rosen and Gordon, 1998). Therefore, the balance in PKC binding by RACKs and the RICKs respectively determines which isoforms are placed in close proximity with a particular group of substrates and which are kept away.

1.7.5. PKC in Breast Cancer

The study of PKC involvement in cancer is now focused on the interplay between specific PKC isoforms in specific tumours rather than overall PKC levels. For example, studies have shown that there is a correlation between isoform expression and the ER status of breast cancer cells. It has been shown that abundant levels of PKC δ are present in ER positive breast cancer cells with little detectable PKC-α. Conversely, ER negative breast cancer cells expressed abundant levels of PKC-α with little or no PKC-δ (Assender *et al.*, 2005). Since there exists an inverse relationship between ER status and PKC expression, it is possible that PKC could play a role in

the shift from a hormone-dependent to hormone-independent phenotype in breast cancer cells. Many other studies now support this hypothesis, for example it has been shown that stable transfection of PKC-α into hormone-dependent breast cancer cells causes the down-regulation of the ER while elevating the basal activity of the AP-1 complex (Tonetti *et al.*, 2000). Elevated AP-1 signal transduction has been implicated in both hormone-independence and Tamoxifen resistance (Smith *et al.*, 1999). Interestingly, elevated PKC-α and AP-1 levels have also been described in endometrial carcinoma tumour models that display growth stimulation by Tamoxifen (Fournier *et al.*, 2001). Furthermore, it has been demonstrated that in the ER positive MCF-7 breast cancer cell line, the predominant isoform is PKC-δ (Shanmugam *et al.*, 1999). PKC-δ participates in signalling pathways that lead to ER phosphorylation in a cell type and promoter specific manner (Lahooti *et al.*, 1998). PKC-δ down-regulation has been implicated as the mechanism by which phorbol esters such as TPA exert their tumour promotion. This has been consolidated by the observation that PKC-δ down-regulation by either a specific inhibitor or the use of a dominant negative PKC-δ mutant stimulated anchorage independent growth (Lu *et al.*, 1997). Interestingly it has been proposed that PKC-δ could be part of a priming mechanism for apoptotic signalling. It has been shown that cells over-expressing EGF-R, which is common in human tumours, can be transformed by the down-regulation of PKC-δ (Zhong *et al.*, 2002). However, it has also been shown that PKC-δ is a substrate of IGF-IR and is required for IGF-IR mediated cell transformation (Li *et al.*, 1998). The importance of PKC-δ in IGF-I signalling in the breast cancer cell is highlighted by performing studies that show an increase in PKC-δ activity on stimulation with IGF-I and that down-regulation of PKC-δ inhibits IGF-I induced growth (Assender *et al.*, 2000). As IGF-I is such a potent mitogen, therapies directed towards the association between IGF-IR and PKC-δ could prove useful in the treatment of endocrine responsive breast cancers.

1.8 Alternative Antioestrogenic Therapies-Tamoxifen Derivatives

The relative success of tamoxifen as an anti-oestrogenic therapy has validated the modulation or ablation of the oestrogen receptor as a viable and efficacious strategy in

the therapy of breast cancer. However, as with any therapy, tamoxifen is not without its drawbacks, not least of which is the acquisition of resistance. However the discovery of any pharmacologically effective candidate is not a research *cul de sac* but rather a starting point from which a myriad of successive therapies can emanate.

The derivation of tamoxifen has been carried out for over a decade in the hope that an agent could be found that improves on the positive outcomes the drug can yield whilst reducing the risks associated with its undesirable side effects. This evolution of the tamoxifen structure has lead to the investigation of several compounds with either modified side chains in the case of the first generation SERMs or altered triphenylethylene ring structures producing the second generation 'fixed ring' SERMs. Toremifene is a first generation SERM that only differs from tamoxifen by the addition of a single clorine atom at position 4 (Howell *et al.*, 2004). It was developed in the hope of reducing uterotrophic effects whilst maintaining the efficacy of tamoxifen. However whilst Toremifene displayed similar beneficial effects in postmenopausal women as tamoxifen it was also comparable in the incidence of subsequent cancers, including endometrial. Also, patients displaying tamoxifen-refractory cancer subsequently treated with Toremifene displayed very little incidence of benefit indicating that the two SERMs display cross resistance. Other first generation SERMs include Droloxifene and Idoxifene. Both these drugs showed reduced uterogenic effects compared to tamoxifen with higher binding efficiencies with regard the ER in preclinical studies. However at phase III trials both treatments were inferior to Tamoxifen and clinical development was halted (Robertson, 2004).

The second generation 'fixed ring' SERMs initially were the cause of much optimism as they displayed great affinity for the ER with potent anti-oestrogenic effects and agonist action on bone mineral density whilst being devoid of the uterotrophic effects displayed by tamoxifen. One such second generation SERM is Raloxifene. Whilst there is limited data on Raloxifene's effectiveness in advanced breast cancer it has been shown to be extremely effective in increasing bone mineral density whilst displaying a less oestrogenic effect on endometrial cells. This agonistic effect on bone and improved safety profile over tamoxifen has lead to Raloxifene being developed as a treatment for osteoporosis. Raloxifene has also shown some promise as a

chemopreventive agent and is currently the focus of the STAR (Study of tamoxifen and Raloxifene) chemoprevention trial (Howell *et al.*, 2004). Currently a second generation SERM designated ERA-923 is being developed as a possible second line therapy for Tamoxifen-refractory metastatic breast cancer. A further study is planned to assess its use in ER/PgR-positive metastatic disease (Robertson, 2004).

1.8.1 Selective Oestrogen Receptor Downregulators (SERDs)

Whilst the new generation of SERMs have currently failed to supersede tamoxifen a new class of anti oestrogen has emerged that target the ER but are distinct in terms of their pharmocology and molecular mechanism of action. These compounds are call selective oestrogen receptor downregulators (SERDs). The most promising SERD currently being studied is Fulvestrant which is currently in phase III trials. SERDs are steroidal anti-oestrogens that compete with oestrogen for binding to the ER. Unlike SERMs however, SERDs are 'pure anti-oestrogens' in that they possess no oestrogenic properties. This is due to the fact that even though the SERDs bind the ER they possess long bulky side chains that sterically prevent dimerisation. This causes an increase in ER turnover and a reduction in detectable levels of ER both *in vivo* and *in vitro*. Consequently SERD treatment inactivates both AF-1 and AF-2 signalling pathways of the ER, disrupts nuclear localisation and prevents the expression of oestrogen regulated genes (Howell *et al.*, 2004). Since SERDs display no oestrogenic effects it removes the risk of partial agonist activity of associated with SERMs in tissues such as the endometrium. It also negates a possible mechanism of drug resistance utilised by the breast cancer cell since the partial agonist activity of Tamoxifen has been implicated in the development of resistance. In fact cells that have acquired resistance to tamoxifen still display sensitivity to SERDs (Robertson, 2001). However the lack of oestrogenic properties in all cell contexts and reduction of ER activity may have implications on patients bone density though pre-clinical animal data has been conflicting and inconclusive (Howell *et al.*, 2004)

1.8.2 Aromatase Inhibitors

Whilst the ovaries are the principal source of oestrogen in the premenopausal woman there exists additional sites of oestrogen biosynthesis. These include mesenchymal cells in adipose tissue and skin, osteoblasts and perhaps chondrocytes in the bone, vascular endothelial and aortic smooth muscle cells and several sites in the brain such as the hypothalamus (Simpson, 2000). These extragonadal sites of oestrogen production gain greater significance in the post-menopausal woman as they become the main source of oestrogen, though not as a major systemic hormone but rather at a local level in the sites where it is produced. Interestingly postmenopausal women retain a level of oestradiol within breast tissue that is 10 to 20 times higher than the circulatory oestradiol levels, a level comparable with premenopausal women despite a far lower level of systemic hormone (Lonning, 2004). It has also been shown that there are significantly higher levels of oestradiol in malignant tissue compared to surrounding non-malignant tissues and that this difference is independent of oestrogen receptor content (van Landeghem *et al.*, 1985) suggesting potential local regulation of oestradiol biosynthesis by hormones, growth factors and interleukins acting upstream of tissue specific promoters (Lonning, 2004).

Oestrogen biosynthesis is catalysed by the enzyme complex aromatase (aromatase cytochrome P450). Aromatase binds a C_{19} steroid substrate and catalyses a series of reactions that leads to the formation of a phenolic A ring that is characteristic of oestrogens (Simpson *et al.*, 1994). In postmenopausal women the principal source of the C19 precursors is the adrenal cortex which produces androstenedione, dehydropiandrosterone (DHEA) and DHEA sulphate which is converted to androstenedione for aromatisation (Simpson, 2000). The primary substrate, androstenedione, is aromatised into oestrone which is the major unconjugated circulating oestrogen in postmenopausal women. Probably half of the circulating oestradiol in the postmenopausal woman is converted from circulating oestrone. The other half is produced by the direct aromatisation of testosterone, although aromatase has a lower affinity for testosterone and the level of testosterone is roughly a quarter of that of androstenedione (Lonning, 2004). Studies on the regional variations in aromatase expression in the breast have identified that the highest expression occurs

in adipose tissue proximal to a tumour. This correlates with the increased local concentration of oestradiol in breast tumours and suggests that cross talk exists between the breast tumour and the surrounding adipose tissue (Simpson, 2000).

Whilst the treatment of breast cancers with SERMs and SERDs provide a way of subverting or degrading the oestrogen receptor, the discovery of aromatase provides a potential therapeutic target to reduce the concentration of oestrogen at local sites such as the breast as well as the serum levels in post menopausal women suffering from breast cancer. The first generation aromatase inhibitor aminoglutethimide was compared with Tamoxifen showed no difference in rate of response or duration of response. The second generation of inhibitors formestane and fadrozole were able to inhibit aromatase activity by around 90% at clinical doses and showed promise that they may be superior to Tamoxifen as a first line therapy. However the results of clinical trials showed no statistically significant benefit over Tamoxifen. A third generation of more potent aromatase inhibitors have now been developed that inhibit aromatase activity by 99%. These include the steroidal aromatase inhibitor Exemestane which binds to the p450 site of the aromatase complex. There are also the non-steroidal aromatase inhibitors Anastrazole and Letrozole that bind to the enzymes substrate binding pocket (Lonning, 2004). These inhibitors are now showing real promise as a possible second-line therapy following tamoxifen treatment or even in usurping tamoxifen's status as the first-line therapy of choice for postmenopausal women with oestrogen receptor positive breast cancer. This assessment can be made based on patient data obtained from four randomised phase III trials. The ATAC (anastrazole, tamoxifen and combination) trial indicated that initial treatment with anastrazole lead to a reduction in events such as recurrence, contralateral breast cancer, or death compared to tamoxifen alone or when both were combined. The Italian trial (ITA) and the Intergroup Exemestane Study (IES) randomised women treated with tamoxifen for 2-3 years and either continued treatment with tamoxifen for a full 5 years or switched them to Exemestane for the IES study or anastrazole for the ITA study. Both trials indicate an increase in the incidence of disease free survival in the groups switched to the aromatase inhibitor (Howell and Buzdar, 2005). The use of an aromatase inhibitor as a second-line therapy was also tested in the MA-17 trial which randomised patients treated with tamoxifen for 5 years to either letrozole or

placebo. The trial was halted after 2 years as the group treated with letrozole displayed a significant reduction in events (Winer *et al.*, 2005).

Whilst apparent reduction in events and increase in disease free survival suggest that there are advantages in the use of aromatase inhibitors in the treatment of post menopausal women with oestrogen receptor positive breast cancer, it is still not clear whether they would be most effectively applied as a first-line therapy or following a period of treatment with tamoxifen. There are also questions over the duration of treatment possible with the aromatase inhibitors and their long term risks and toxicities. As data emerges from the studies mentioned above and others these questions should have more definitive answers and whether the disease free advantage observed with aromatase inhibitors can be translated into an overall increase in survival.

1.9 Aims

Previous observations on PKC isoform expression in breast cancer cells have demonstrated that PKC-δ expression is indicative of an ER positive phenotype (Shanmugam *et al.*, 2001) and is therefore associated with a good prognostic outcome to Tamoxifen treatment (Assender *et al.*, 2005). Conversely, it has been shown that expression of PKC-α is associated with a loss of oestrogen responsiveness, lack of Tamoxifen efficacy and a more aggressive and invasive neoplastic phenotype (Ways *et al.*,1995; Morse-Gaudio *et al.*, 1998; Tonetti *et al.*, 2000). This project aims to expand on these observations by determining:

- the PKC-α and PKC-δ expression profile of tamoxifen resistant cells
- whether changes in the expression / activation of the PKC-α and PKC-δ isoforms are a consequence of anti-oestrogen resistance or under-lie its acquisition.

The Tenovus Centre for Cancer Research within Cardiff University has developed a robust and clinically relevant tamoxifen resistant cell model (TAM-R) through the long term tamoxifen treatment of the tamoxifen sensitive MCF-7 human breast cancer

cells (Nicholson and Gee, 2000). To determine the PKC isoform profile of these cells, protein will be extracted and assayed by Western analysis using isoform and phosphorylation state specific antibodies. We can then compare the isoform profile to the MCF-7 cells from which they are derived and elucidate the differences in PKC activation and expression brought about by the acquisition of the TAM-R phenotype. We will also use this technique to ascertain the importance of the PKC-α and PKC-δ isoforms on growth factor signalling in the tamoxifen sensitive and resistant cell lines and what roles these isoforms play in ER signalling. This will be carried out through the use of Western analysis and also through growth studies utilising the technique of Coulter counting which provides an accurate and reproducible way of counting cell numbers.

One of best ways to analyse the function of a molecule is to ablate its expression or inhibit its function. To this end, we will attempt a variety of strategies to selectively and efficaciously inhibit the PKC-α and PKC-δ isoforms and try to un-pick their function from the other isoforms of the PKC family and the multitude of other protein kinases which function within the cells. The most commonly used strategy in this endeavour is the use of small molecule chemical inhibitors, and there are several that have been widely reported to possess selectivity for PKCs and even for individual isoforms. These inhibitors will be tested for their efficacy and selectivity in our model cell lines. Alternatively a variety of molecular protocols have been developed to selectively and specifically inhibit and target enzyme targets. We will investigate the techniques of RNAi through the use of siRNAs, to disrupt mRNA expression of PKC-α and PKC-δ, and the use of adenovirally delivered PKC-α and PKC-δ dominant negative expressing plasmids to disrupt the isoforms function. The effect of PKC-α and PKC-δ inhibition will be tested on basal growth of the cell lines but also their effect on cell growth when treated with a range of growth factors and oestradiol.

We will also utilise an adenoviral system to introduce wild type overexpressing PKC-α and PKC-δ into the cell lines. We can therefore overexpress these isoforms in the MCF-7 cell line to determine if this can confer resistance to tamoxifen or increase sensitivity to the growth factor pathways implicated in tamoxifen resistant breast cancer.

CHAPTER 2.

MATERIALS AND METHODS

2.1. General Materials and Equipment

2.1.1. Materials

The chemicals, reagents and disposables required for general use in this study are listed alongside their suppliers in Table 2.1.

Table 2.1. Materials/chemical reagents and their suppliers

Materials	Supplier
GAPDH siRNA (with scrambled control)	Ambion, Huntington,UK
Anti-Rabbit Horseradish Peroxidase (HRP) linked IgG (from donkey), Anti-Mouse HRP linked IgG (from sheep), Rainbow Marker (10-250 Kda), dNTPs and random hexamers, PD-10 desalting column (containing G-25 Medium)	Amersham, Little Chalfont, England
Virkon	Antec International Ltd, Suffolk, UK
Fulvestrant	AstraZeneca Pharmaceuticals, Cheshire, UK
Bromophenol Blue, Glass cover slips (thickness number 2, 22 mm^2)	BDH Chemicals Ltd, Poole, UK
Isoton® II azide-free balanced electrolyte solution (sodium chloride at 7.9$g.l^{-1}$, disodium hydrogen orthophosphate at 1.9$g.l^{-1}$, EDTA disodium salt at 0.4$g.l^{-1}$, sodium dihydrogen orthophosphate at 0.2$g.l^{-1}$ and sodium fluoride at 0.3$g.l^{-1}$)	Beckman Coulter Ltd, High Wycombe, UK
Sterile Syringe Needles (BD microbalance™ 3 characteristics: 25 $G^{5/8}$, 0.5 X 16)	Becton Dickinson (BD) Biosciences Ltd, Oxford, UK

Bijou tubes (5ml)	Bibby Sterilin Ltd., Stone, UK
Supersensitive Concentrated Detection Kit (consisting of Biotinylated anti-mouse immunoglobulin and Streptavidin peroxidase), StrAviGen Multilink Kit (consisting of multilink concentrated Biotinylated anti-mouse, rabbit, rat and guinea pig immunoglobulin and Streptavidin peroxidase)	Biogenex, San Ramon, USA
Arklone P	The Basic Chemical Co Ltd, High Wycombe, UK
The liquid DAB^+ substrate chromogen system (K3468), Mouse/Rabbit Envision system HRP DAB^+ kits, Goat serum	DAKO, Cambridgeshire, UK
Eppendorf tubes	Elkay Laboratory Products Ltd, Basingstoke, UK
Methanol, acetone, ethanol, formaldehyde, hydrochloric acid (HCl), sucrose, dipotassium hydrogen orthophosphate anhydrous (HK_2O_4P), potassium dihydrogen orthophosphate (H_2KO_4P), glycerol, chloroform, isopropanol	Fisher Scientific, Loughborough, UK
0.2μm Supor membrane VacuCap® 60 filter unit	Gellman Laboratory Pall, Ann Arbour, USA
MXB Autoradiography Film (Blue Sensitive; 18 X 24 cm)	Genetic Research Instrumentation (GRI), Rayne, UK
Pipette Tips	Greiner Bio-One Ltd, Gloucestershire, UK

RNase Erase	ICN Biomedicals Inc., Ohio, USA
RPMI 1640 phenol red-free, DCCM, Phenol Red RPMI, Opti-MEM reduced serum media, Dulbecco's modified Eagle medium (DMEM), phosphate buffered saline (PBS), streptomycin/penicillin, fungizone, foetal calf serum and L-glutamine, MMLV reverse transcriptase, Lipofectamine 2000 transfection reagent, oligofectamine transfection reagent, Lipofectin transfection reagent	Invitrogen, Paisley, UK
Bovine Trypsin	Lorne Laboratories Ltd, Reading, UK
RiboJuice Transfection Reagent, GeneJuiceTransfection Reagent	Novagen, Nottingham, UK
Tissue culture plasticware (6, 12 and 14 well plates, flasks, 35mm, 60mm and 100mm dishes)	Nunc Int., Roskilde, Denmark
Perbio Chemiluminescent Supersignal® West Pico, Dura and Femto	Pierce and Warriner Ltd, Cheshire, UK
RNasin Ribonuclease inhibitor	Promega, Southampton, UK
PKC δ specific siRNA, PKC-α specific siRNA	Qiagen, Crawly, UK
Xylene Soluble Mountant	Raymond A Lamb Ltd, Eastbourne, UK
Western Blocking Reagent	Roche Diagnostics, Mannheim, Germany

Sterile disposable pipettes (5ml, 10ml and 25ml), Falcon tubes (50ml), Coulter Counter lids and cups	Sarstedt AG and Co., Nümbrecht, Germany
Nitrocellulose membrane BA 85 (0.45μM)	Schleicher and Schuell, Dassell, Germany
Syringes (5ml and 10ml)	Sherwood Medical Davis and Geck, Gosport, UK
4-Hydroxytamoxifen (TAM), Insulin-like growth factor I (IGF I) and II (IGF II), Epithelial growth factor (EGF), aprotinin, Heregulin growth factor β (HRG β), Oestradiol (E2), Bisindolylmaleimide IX (bis), Dimethyl sulfoxide (DMSO), Rottlerin, Pipette tips (polypropylene micro-capillary round), bacitracin, Acrylamide/bisacrylamide 30% v/v solution, ammonium persulphate (APS), bovine serum albumin (BSA), glycine, dithiothreitol (DTT), pepstatin A,gelatine, glycerol, ethylenediaminetetraacetic acid (EDTA), HEPES, leupeptin, N,N,N,N tetramethylethylenediamine-(TEMED), ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), phenylmethylsulphonylfluoride (PMSF), , phenylarsine oxide, potassium chloride, sodium chloride (NaCl), sodium dodecyl sulphate or lauryl sulphate (SDS), sodium fluoride (NaF), sodium molybdate (Na_2MoO_4), sodium orthovanadate (Na_3VO_4), Triton X-100, Trizma base, Trizma-HCl, Tween 20, Ezview™ Red Protein G affinity gel, glutaraldehyde, 3-aminopropyltriethoxysilane (APES), TRI reagent, ethidium bromide, potassium chloride (KCl), magnesium chloride ($MgCL_2$) anhydrous, Tris-base, mineral oil, acetic acid, methyl green, Potassium ferricyanide, Potassium ferrocyanide, 5-bromo-4-chloro-3-indolyl-β-D-Galactopyranoside (X-Gal)	Sigma Chemical Co Ltd., Dorset, UK

Filter Paper (No. 4), Filter Paper (grade 3; 460 X 370mm)	Whatman, Maidstone, UK
Fixer (X-0-fix), Developer (X-0-dev)	X-0-graph Imaging System, Tetbury, UK

2.1.2. Growth Factors and Inibitors

Stocks of TAM were prepared by dilution to a concentration of 10^{-3} M in ethanol and added at 1/10,000 (v/v) to obtain the 10^{-7}M final concentration in the media. Insulin-like growth factor I (IGF I) and II (IGF II), epithelial growth factor (EGF) and heregulin growth factor β (HRG β) were all stored at -20°C as stocks of 100ng/μl. The final concentration used in experimental media was 10ng/ml. Stock solution of 10^{-5}M Oestradiol (E2) diluted with ethanol, was stored at -20°C and used in the experimental media at a final concentration of 10^{-9}M. Bisindolylmaleimide IX (bis) was stored as a stock solution of 10^{-3}M dissolved in Dimethyl sulfoxide (DMSO) and used in the experimental media at a final volume of 500nM. Rottlerin was diluted in DMSO and stored at -20°C at a stock concentration of 5mM. Fulvestrant was a kind gift from AstraZenenca Pharmaceuticals (Cheshire, UK). It was stored at -20°C as a 10^{-3}M stock solution with ethanol as the diluent and utilised in experiments at a final concentration of 10^{-7}M.

2.1.3. Antibodies

All antibodies used in this study are listed alongside their supplier in Table 2.2.

Table 2.2. Antibodies and their suppliers

Antibody	Supplier
GAPDH	Abcam Ltd, Cambridge, UK
Total PKC-α Total PKC-δ	Becton Dickinson (BD) Biosciences Ltd, Oxford, UK
Phosphorylated PKC-δ (Thr505) Phosphorylated PKC δ (Ser643) Phosphorylated ERK 42/44	Cell Signalling Technology, New England Biolabs Ltd, Hertfordshire, UK
β-Actin antibody	Sigma Chemical Co Ltd, Dorset, UK
Phosphorylated Ser657 PKC-α	Upstate Ltd, Milton Keynes, UK.

2.1.4. Equipment

All tissue culture work was carried out under sterile conditions, in a vertical circulating air, class II biological safety cabinet (MDH Intermed Airflow from Bioquell, Andover, UK). All disposable materials coming into contact with the cells, that didn't come sterile packed for single use, were sterilised at 119°C prior to use using a Denly BA852 autoclave from Thermoquest Ltd, Basingstoke, UK. Cells were grown in a humidified atmosphere at 5% CO_2 and 37 °C in a BB16 Function Line incubator from Haraeus Instruments (Hanau, Germany) and visualised prior to commencement of tissue culture work using a phase contrast microscope (Nikon Eclipse TE200; Kingston-upon Thames, UK). Cells were counted using the Beckman Coulter ® Counter Multisizer II (High Wycombe, UK). Finn Pipiettes (1-10µl, 5-50µl, 20-200µl, 100-1000µl and 500µl-5ml) were supplied by Thermo Labsystems (Helsinki, Finland). Cell lysates were centrifuged using an IEC Micromax RF micro-centrifuge (Thermo Electron Corporation, Hampshire, UK). Protein sample concentrations were analysed at 750nm on a CECIL CE 2041 spectrophotometer (Cambridge, UK). Gel electrophoresis was performed on a Mini-Protean ® 3 electrophoresis apparatus from BioRad Laboratories Ltd (Hertfordshire, UK). Electric current was applied to the apparatus by the powerpack 300 also from BioRad. Blots were incubated on a platform rocker STR6 from Stuart Scientific, Bibby Sterilin Ltd. (Stone, UK).

2.2. Tissue Culture

2.2.1. Routine Cell Seeding from Stock

The human mammary-carcinoma MCF-7 wild type cell line was kindly given to our laboratory by AstraZeneca Pharmaceuticals (Cheshire, UK). MCF-7 cells were removed from liquid nitrogen storage and routinely grown in phenol red containing RPMI medium supplemented with 5% foetal calf serum (FCS), antibiotics [streptomycin (100mg/ml) and fungizone (5µg/ml)].

2.2.2. Cell passage

All cells were routinely cultured in 75cm^2 sterile flasks and were passaged approximately once a week upon reaching confluency. First the cell culture medium

was removed with an aspirating pump and replaced by trypsin solution (10ml). The trypsin solution used to passage the cell consisted of $0.2g.l^{-1}$ EDTA and $0.5g.l^{-1}$ Bovine Trypsin in PBS. The flask was placed in an incubator at 37°C for 5 minutes until the cells become detached. The trypsinised cells were then mixed with an equal volume of medium (10mls) and centrifuged at 1350g for 5 minutes at room temperature (r/t). The supernatant was then removed and discarded and the cells resuspended in 10ml of culture medium through repeat pipetting through a 10ml pipette until no clumping of cells were visible. Taking 1ml of the cell suspension (1/10), cells were then seeded in 15ml of medium in a $75cm^2$ flask and put in an incubator (5% CO_2 at 37°C) until required or until confluency, when they were again passaged.

2.2.3. Experimental medium

To avoid the unwanted oestrogenic properties of the phenol red in standard RPMI medium, prior to each experiment, cells were set up in a phenol red free equivalent medium called white RPMI 1640. It was also necessary to carry out the experiments in the absence of steroids, so steroid-depleted charcoal stripped foetal calf serum (csFCS) was used. The csFCS was prepared by firstly aliquoting the foetal calf serum (FCS) (100ml) and adjusting the pH to 4.2 using 5M HCL. This was then allowed to equilibrate for 30 minutes at 4°C. A charcoal/dextran solution was prepared using distilled water with Norit A (charcoal, 11.1%) and Dextran C (0.06%). This mixture was then stirred vigorously for 1 hour. 5ml of charcoal solution (5% v/v) was added to each 100ml aliquot of FCS and incubated with gentle agitation for 16 hours at 4°C. The charcoal was then removed by centrifugation (12,000g for 40 minutes) and the solution filtered with Whatman N° 4 paper to remove any traces of charcoal. The pH of the solution was then readjusted to pH7.2, sterilised and then filtered again with 0.2μM membrane filter Supor Vacucap® 60 to remove fine impurities and contaminating micro-organisms.

The experimental media utilised for the MCF-7 cells comprised the following: phenol red free RPMI containing 5% (v/v) csFCS, antibiotics [streptomycin (100mg/ml), penicillin (100IU/mL) and fungizone (2.5μg/ml)] and glutamine (4mM). This media will now be referred to as white and 5% (W +5%).

In experiments where serum free media is required the experimental media comprised the following: DCCM-1, streptomycin (100mg/ml), penicillin (100IU/mL), fungizone (2.5μg/ml) and glutamine (4mM).

2.2.4. Plating of Cells for Tissue Culture Based Experiments

To ensure that equivalent cell numbers were utilised for each experiment the cells were counted prior to plating. Firstly the cells were detached from the flask by trypsinisation as outlined previously (2.2.2). Once the pellet was obtained by centrifugation the supernatant was removed using an aspirator and the cells resuspended in 10ml of the experimental media. The cells were resuspended using a syringe with a 25 $G^{5/8}$ 0.5 X 16 needle. The cells were pushed through the needle to separate the cells and provide a single cells suspension. 100μl of this solution was then added to 10ml of Isoton in a counting cup and then counted using the Beckman Coulter counter Multisizer II. A volume of experimental medium was then mixed with a known number of cells to seed each experiment appropriately.

2.2.5. Establishment of the Tamoxifen Resistant Cell Line (TAM-R)

The Tamoxifen resistant cell derivative of the MCF-7 cell line was established through routine maintenance of the MCF-7 cell line in experimental medium (as outlined in 2.2.3) containing 4-OH Tamoxifen (10^{-7}M) (TAM). Cells were continuously cultured in this TAM-containing medium for a period of 6 months, during which time the cells were routinely passaged when necessary. The first two months of this treatment regime caused a signifigant growth inhibition of the cells. After this time, the cell growth began to gradually increase until it reached growth rates comparable to the untreated wild type MCF-7 cell line. This indicated that the cells were able to overcome the growth inhibitory effects of TAM and had developed a Tamoxifen resistant (TAM-R) phenotype. These TAM-R cells were then routinely cultured for a further four months and characterised before being utilised for experimental work.

2.2.6. Cell counting for growth experiments

Cells were grown in 24 well plates with each condition being assessed set up in triplicate wells. After removal of the medium with an aspirator, 1ml of trypsin was

added to each of the wells to be counted and the plate returned to the incubator until the cells were visibly detached (usually 3-5 minutes). Using a 5ml syringe with a 25 $G^{5/8}$ 0.5 X 16 needle, the detached cells were drawn up and down twice to separate the cells and provide a single cells suspension which was drawn into the syringe. Then 1 ml of isoton was added to the well and drawn into the syringe and repeated twice more to give a total volume of 4ml. This cell suspension was then added to a Coulter counting pot containing 6ml of isoton to give a final volume in the pot of 10ml. The number of cells in each pot was then counted twice using the Coulter counter with gentle agitation between each count to to resuspend the cells. The average of the two counts were then multiplied by the dilution factor of 20 to give the number of cells per well.

2.2.7. Statistical analysis of cell counts

In all growth experiments each condition was carried out in triplicate and counted in duplicate (i.e. 3 pairs of counts for each condition). To analyse the counts, the mean of each pair was taken and the mean of those three values expressed ± SEM. In cases where the experiment was further repeated, the average of all the paired means were used. Results were subjected to paired t-test analysis using the SPSS (Statistical Package for the Social Sciences) version 12.0.2 software to determine significance.

2.3. SDS-PAGE and Western Blotting

Cell lines, cultured as a monolayer in a flask as previously outlined (2.2.4), were resuspended by trypsinisation, re-seeded at a density appropriate for the time scale of the experiment into 60mm or 100mm dishes and allowed time to attach and commence growth. Once the cells commenced log phase growth the media was aspirated and replaced with the experimental media containing the required treatments at the stated doses and time periods. If the experiment required the cells to be serum starved prior to treatment the cells were grown to around 70% confluency, then the medium was aspirated and replaced with DCCM for 24 hours prior to treatment. The medium was then aspirated and replaced by DCCM containing the experimental treatments at the required doses and time periods. All experiments contained a control arm in which the media was replaced with the corresponding medium minus any

treatments. All studies involving TAM-R cells were performed with 4-OH Tamoxifen (10^{-7}M) present in the experimental media.

2.3.1. Cell Lysis

After the stated treatments, cells were washed three times with PBS (37°C) and excess PBS removed by aspiration. 150µl ice cold lysis buffer (50mM Tris-HCL, 5mM EDTA, 150mM NaCl, 1% Triton X-100 (v/v) in distilled water, pH7.5) supplemented with protease and phosphatase inhibitors (2mM Na_3VO_4, 20mM NaF, 1mM PMSF, 10 µg/ml leupeptin, 20µM phenylarsine oxide, 10µg/ml aprotinin and 10mM sodium molybdate) was then added to the 60mm dishes. The dishes were scraped using a cell scraper to fascilitate the removal of as many cells as possible. Lysate was incubated on ice for 10 minutes then the cellular contents transferred to 1.5ml Eppendorf tubes and centrifuged at 15,800g for 15 minutes at 4°C. Supernatants were aliquotted and stored at -20°C until required.

2.3.2. Sample Preparation

The total protein concentration of the samples was determined using the BioRad assay using known concentrations of bovine serum albumin (BSA) to construct a standard curve for spectrophotometric quantification. BSA was diluted in sufficient lysis buffer (without the protease inhibitors) to obtain 50µl of solution at protein concentrations of 0, 0.25, 0.5, 0.75, 1 and 1.45mg.ml^{-1}. The curve was then constructed after reading their absorbances at 750nm. The BSA samples at the above concentrations and protein samples (12.5µl of sample and 37.5µl of lysis buffer to give a final volume of 50µl) were prepared in spectrophotomic cuvettes. To each sample (protein and BSA) 250µl of reagent A (from BioRad kit) was added, supplemented with substrate S (20µl in 1ml of reagent A) (from BioRad kit). Finally 2ml of reagent B (from BioRad kit) was added to each cuvette and the colour was allowed to develop for a minimum of 15 minutes. The absorbance at 750nm for each BSA sample of known concentration was then read and a calibration curve plotted of absorbance versus concentration. Then each protein sample was processed, spectrophotometrically assayed at 750nm and its concentration determined from the standard curve. 50µg of protein from each sample under investigation, were then mixed with 10µl of loading

buffer (4% (w/v) SDS, 20% (v/v) glycerol, 120mM upper buffer (pH6.8), 0.1% (W/V) bromophenol blue, plus 100mM DTT).

2.3.3. SDS-Page

SDS-PAGE was performed under reducing conditions following the method of Laemmli (1970). Resolving gel and stacking gels, containing 7.5% (w/v) and 5% (w/v) acrylamide respectively, were routinely used. The gels were prepared as follows: resolving gel comprised 7.5% acrylamide/bisacrylamide, 375mM lower buffer (pH8.8), 0.1% (w/v) SDS, 0.1% (w/v) APS and 70μM of TEMED. The upper stacking gel comprised 5% acrylamide/bisacrylamide, 125mM upper buffer (pH6.8), 0.1% (w/v) SDS, 0.05% (w/v) APS and 116μM TEMED. These gels were routinely prepared as outlined in table 2.3.

Table 2.3. Composition of resolving and stacking gels used for SDS-PAGE

GEL COMPONENT	7.5% (W/V) RESOLVING GEL	5% (W/V) STACKING GEL
ddH_2O	4.8ml	6.1ml
0.5M Upper Buffer	-	2.5ml
1.5M Lower Buffer	2.5ml	-
Acrylamide/Bisacrylamide (30%)	2.5ml	1.25ml
10% (w/v) APS	100μl	100μl
10% (w/v) SDS	100μl	50μl
TEMED	6μl	10μl

Electrophoresis was carried out using the Mini-Protean ® 3 electrophoresis apparatus from BioRad Laboratories Ltd with the gel apparatus assembled as outlined by the manufacturer. The resolving gel was pipetted to within 1.5cm of the upper edge of the inner glass plate and water gently layered on top of the solution to ensure the formation of a level gel front and the exclusion of any air bubbles. The gel was allowed to polymerise for 30-40 minutes. Once the gel was set the water was removed using a strip of Whatman grade 3 filter paper and the stacking gel overlayed with the well-forming comb inserted. After the stacking gel had polymerised, the comb was gently removed and the upper and lower chamber of the tank filled with running buffer (pH8.8) (250mM Trizma base, 2M Glycine, 40mM SDS). The protein samples combined with loading buffer were heated to 100°C for 10 minutes and allowed to

cool before loading onto the gel. The rainbow protein size markers (10-250 kDa) were loaded into the first lane of each gel. Electrophoresis was carried out at a constant voltage between 150-200 volts until the dye front had reached the base of the gel.

2.3.4. Western Blotting

Seperated proteins from the gel were transferred onto nitrocellulose membrane for 1 hour at 100V in transfer buffer (0.2M of glycine, 25mM of Trizma base, 20 %(v/v) of methanol in distilled water, pH 8.3) using the BioRad transfer aparatus. Afterwards, the blots were blocked in a solution of 5% skimmed milk (w/v) and TBS-Tween (10mM Tris, 0.1M NaCl, 0.05% (v/v) Tween 20, pH 7.5) for at least 1 hr to prevent non specific binding of antisera. Blots were then incubated in the appropriate primary antibodies.

After exposure to antibodies the membranes were washed three times during a 15 minutes interval in TBS-Tween and then incubated for 1 hour with the required secondary antibody labelled with horseradish peroxidase (donkey anti rabbit or sheep anti mouse) diluted 1/10000 in BM Chemiluminescence Blotting Substrate, made up in TBS-Tween (1:20mls). Membranes were then washed three times in TBS-Tween for a total of 30 minutes. Detection was performed by applying a thin film of Supersignal® WEST DURA or PICO chemiluminescent substrate to the membrane for 5 minutes. Hyperfilm ECL film was then exposed to the membrane until a satisfactory level of exposure could be obtained. The film was then processed using a x-ray developing machine. Results were scanned using a BioRad model GS-700 densitometer.

2.3.5. Immunoprecipitation

Cells, cultured as a monolayer in a flask, were removed by trypsination and re-plated at 1.5 x 10^6 cells per 100mm dish in W+5% medium and grown to 70%-80% confluency (usually after four days). The medium was then aspirated and the cells lysed as previously described in section 2.3.1, except that 1ml of lysis buffer was used per 100mm dish. Total protein concentrations of samples were again determined using the BioRad assay and CECIL spectrophotometer. Cell lysates were adjusted to contain 1mg protein and immunoprecipitated using either 1µl or 2µl of a specific antibody and

incubated for 1hr whilst gently rotating in a cold room (4°C). 40µl of Ezview™ Red Protein G (Sigma) was added to the mixture and rotated gently in a cold room over night. The immune complex was centrifuged at 3500g at 4°C for 5 minutes, the supernatant removed and the complex washed with PBS (r/t). This procedure was repeated three more times and the resulting pellet re-suspended in 50µl of loading buffer. Samples were heated to 100°C for 10 minutes to release and denature the bound proteins before gel loading. Proteins were run on a SDS-PAGE gel and Western blotted according to the standard protocol (2.3.4).

2.3.6. Statistical Analysis

Where the data allowed, the densitometry numbers obtained by scanning the bands obtained by Western blot were analysed by paired t-test using the SPSS software to determine significance.

2.4. Immunocytochemistry (ICC)

Slides were analysed under a phase contrast microscope (Olympus BH-2) and photographed on a digital camera (DP-12, Olympus) from Olympus (Oxfordshire, UK). All other equipment used in this technique have been listed in previous sections.

2.4.1. Experimental tissue culture

Each cell line, cultured as a monolayer in a flask, were removed by trypsination and re-suspended in W+5% and seeded onto 22-mm^2 3- aminopropyltriethoxysilane (APES)-coated glass coverslips contained in 35mm culture dishes at a density of 1 x 10^5 cells/dish. Once the cells have reached a confluency of around 70-80% the media was removed and the cells were fixed by the method appropriate to the immunocytochemical assay to be performed (see below).

2.4.2. ER-ICA Fixation

Coverslips are immersed in 4% (in PBS) Formaldehyde solution at room temperature for 15 minutes then immersed in PBS at room temperature for at least 5 minutes. Next the coverslips were immersed in Methanol (-10°C to -30°C) for 5 minutes, then Acetone (-10°C to -30 °C) for 3 minutes. The coverslips were then washed in PBS at

room temperature for at least 5 minutes and stored at -20°C in sucrose storage medium.

2.4.3. Acetone: Methanol Fixation

Coverslips were immersed in a 1:1 (v/v) mixture of acetone and methanol at a temperature of between -10°C and -30°C for 10 minutes. The coverslips were then removed and allowed to air dry for 30 minutes. Once dry, the coverslips were stored at-80°C.

2.4.4. PKC δ Assay

The sucrose storage medium was removed from the coverslips, which had been fixed by the ER-ICA method, and then washed twice for 5 minutes in PBS. The coverslips were blocked with 10% normal goat serum for 10 min to prevent non-specific antibody binding then excess serum removed. PKC-δ mouse monoclonal IgG2b antibody (1:100 dilution in PBS) was added to the coverslips and left overnight at room temperature. The following day the coverslips were washed in PBS (3 mins) and then in detergent buffer wash (DPC) (2x 5 mins). The coverslips were then incubated for 45 mins in peroxide conjugated goat anti-mouse secondary antibody at a dilution of 1:25 in PBS. Coverslips were then washed as before and a mouse peroxidase-anti-peroxidase (PAP) conjugated antibody diluted 1:250 in PBS, was added for 45 mins at r/t. Staining was visualised by applying DAB to the coverslips for 6 mins and counterstaining with methyl green (0.5%) for 30 seconds.

2.4.5. PKC α Assay

The coverslips, fixed using the acetone : methanol method, were rehydrated for 5 minutes in PBS. The coverslips were then blocked with 10% normal goat serum for 10 min to prevent non-specific antibody binding. Excess serum was then removed and PKC-α mouse monoclonal IgG2b antibody (1:100 dilution in PBS) added to the coverslips and left overnight at room temperature. The following day the coverslips were washed in PBS (3 mins) and then in detergent buffer wash (DPC) (2x 5 mins). The coverslips were then incubated for 30 mins in peroxide conjugated goat anti-mouse secondary antibody at a dilution of 1:25 in PBS. Coverslips were then washed as before and a mouse peroxidase-anti-peroxidase (PAP) conjugated antibody diluted

1:250 in PBS, was added for 30 mins at r/t. Staining was visualised by applying DAB to the coverslips for 10 mins and counterstaining with methyl green (0.5%) for 30 seconds.

2.5. Reverse transcription-Polymerase Chain Reaction (RT-PCR)

All bench work was carried out in the Labconco purifier PCR enclosure, supplied by GRI, Essex, UK. All reverse transcription and PCR procedures were carried out in the BioRad iCycler machine. Agarose was melted in a 950 Watt Microwave purchased from Curries (UK). Electrophoresis apparatus (Sub-Cell® Agarose Electrophoresis systems, Biorad, UK) were run on a BioRad 1000 Powerpac. The trans-illuminator and Polaroid Camera used to visualise results were supplied by GRI. Unmentioned equipment used in this technique are listed in previous sections.

2.5.1. Cell lysis and RNA extraction

To ensure that RNA/DNA remain stable throughout the entire reverse transcriptase experiment all solutions purchased were graded RNA/DNase free, all disposables were autoclaved and gloves worn at all times. Cells were lysed by adding 1ml of TRI lysis reagent for 5 minutes at room temperature. To ensure all the cells had detached the dish was scraped using a cell scraper. The lysed solution was then transferred to a sterile eppendorf to which 200µl chloroform was added. The tube was gently but thoroughly mixed for 15 seconds by shaking and left to stand for up to 10 minutes at room temperature before centrifugation at 15,800g for 30 minutes at 4°C. 400µl of the top aqueous phase (containing RNA) was carefully removed to a fresh sterile tube to which an equal volume of isopropanol was added. The tube was then mixed gently again, left to stand for 10 minutes at room temperature and centrifuged at 15,800g for 10 minutes at 4°C. The precipitated RNA (white pellet) was washed with 75% ethanol, gently vortexed, re-centrifuged (10 minutes), pellet dried (but not totally as this decreases solubility) and re-suspended in sterile water (30µl).

Concentration of the RNA was measured using a spectrophotometer at 260 and 280nm wavelength, using 1:500 dilution of RNA in water. A preparation was considered satisfactory if the ratio of the absorbance values obtained at these

wavelengths was 1:2 and not lower than 1:1.6. The RNA integrity and concentration was checked by running RNA through a 2% agarose gel.

2.5.2. Agarose gel electrophoresis

2% agarose in Tris EDTA acetate (TEA) buffer was prepared by heating in a microwave at full power for 1 minute with periodic mixing and left to cool to approximately 40°C. Following addition of 1µl Ethidium bromide, the gel was poured into a gel tray and well comb added. When the gel set, a solution of RNA (equivalent to 1µg) was made up to 6µl in loading buffer and loaded into the lanes of the gel and run for 30 minutes at 70 volts. The gels were then visualised under UV in a dark room and photographed using a polaroid camera.

2.5.3. Reverse transcription (RT)

Total RNA (1µg) was diluted to a final volume of 7.5µl using DEPC treated Sterile Water. This RNA solution was then added to 11µl of RT master mix comprising 5µl dNTP (2.5mM), 2µl 10X PCR buffer (containing $MgCl_2$ (25mM)), 2µl DTT (0.1M) and 2µl random hexamers (RH) (100µM). The tube was placed in a thermal cycler (PTC-200, M J Research) and denatured at 95°C for 5 minutes, removed and cooled on ice for 5 min. MMLV (reverse transcriptase enzyme, 1µl) was then added, followed by RNase inhibitor (0.5µl) to give a final volume of 20µl. The mixture was centrifuged briefly to collect volume and then placed in the thermal cycler and reverse transcribed using the following cycle program:

Step 1	22°C	10 min	(annealing time)
Step 2	42°C	42 min	(RT extension time)
Step 3	95°C	5 min	(denaturing time)

The resulting cDNA was stored at -20°C until required.

2.5.4. Polymerase Chain Reaction

To amplify the cDNA produced in the RT step exponentially, 1µl of cDNA from each sample (equivalent to 50ng mRNA starting material assuming 100% efficiency of RT reaction) was added to a PCR master-mix solution (37.25µl of sterile distilled water,

5µl of PCR buffer (10X), 4µl of dNTPs (2.5mM), 1.25µl forward primer (20µM), 1.25µl of reverse primer (20µM) and 0.2µl of the Taq polymerase) to give a final volume of 50µl per sample.

The reaction mix was placed in a heated lid BioRad iCycler PCR machine and amplified using the parameters outlined in Figure 2.1. Upon completion of the cycles, 5µl of sample was added to 6µl of loading buffer, loaded onto a 2% agarose gel and the bands separated by electrophoresis as outlined in section 2.5.2.

2.5.5. Oligonucleotide-primer design

In order to analyse PKC-δ expression at the mRNA level, oligonucleotide primers were designed (Assender *et al*, 1997) and tested using the Oligo™ Primer Design software package (Medprobe AS, Oslo, Norway) to minimise primer-primer annealing. PKC-δ primer sequence shown in Table 2.3. These primers produced a band at 351bp molecular weight and having a 55°C optimal annealing temperature. To determine the optimum cycle number for these primers they were run with MCF-7 cDNA at cycle numbers of 21, 26, 28, 30, 32 and 34. The intensity of the bands visualised on agarose gel (as described below in section 2.5.2) increased with cycle number (figure 2.2). It can be seen that a cycle number of between 30 and 32 cycles is sufficient to visualise a product whilst not passed the saturation point where subtle differences in expression could be lost.

To analyse PKC-α expression at the mRNA level, oligonucleotide primers were designed using the Primer3 software package (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Possible sequences were checked using the Blast computer program (http://www.ncbi.nlm.nih.gov/BLAST/). Two sets of sequences were selected as possible suitable primers using cDNA obtained from MCF-7 cells. The two sets of primers were run for a period of 21, 24, 28, 30, 32 or 34 cycles with a negative control containing no cDNA. One of the sets of primers produced no bands on an agarose gel whilst the other produced the desired bands at 494bp of increasing intensity as cycle number increased (Figure 2.3). It can be seen that a cycle number of

between 30 and 32 cycles is sufficient for subsequent experiments. This efficacious set of primers are detailed in Table 2.4.

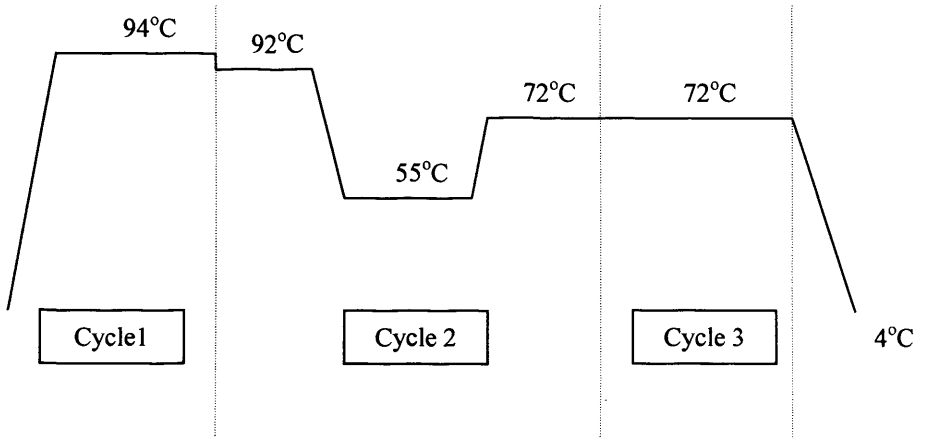
Primers for β-Actin were used as a control, to confirm levels of mRNA expression were uniform in a given experiment (Table 2.3). The primers produced a band at 204 bp after 24 cycles, annealing at 55°C for 1 minute.

2.5.6. *Statistical Analysis*

After photographing the bands produced by PCR, the bands were scanned by densitometry and the raw number used for paired t-test using the SPSS software to determine signifigance.

Table 2.4. Oligonucleotide primer sequences for the RT-PCR of PKC-α, PKC-δ and β-Actin.

PKC-α	5'-AGTGCACCATGGTAGAAAAGC-3'	3'-TAGCTCGTCTTCATCTTCACC-5'
PKC-δ	5'-CACCATCTTCCAGAAAGAACG-3'	5'-CTTGCCATAGGTCCCGTTGTTG-3'
β-Actin	5'-GGA GCA ATG ATC TTG ATC TT-3'	5'-CCT TCC TGG GCA TGG AGT CCT 3'



Cycle 1	(1x)	94°C	5 min
Cycle 2	(30-32 cycles)	92°C 55°C 72°C	1 min 2 min 2 min
Cycle 3	(1x)	72°C	5 min
Cycle 4	End	4°C	-

Figure 2.1 PCR Cycle Profile

Illustration of the PCR cycle sequence utilised when using the PKC-α and δ specific primers.

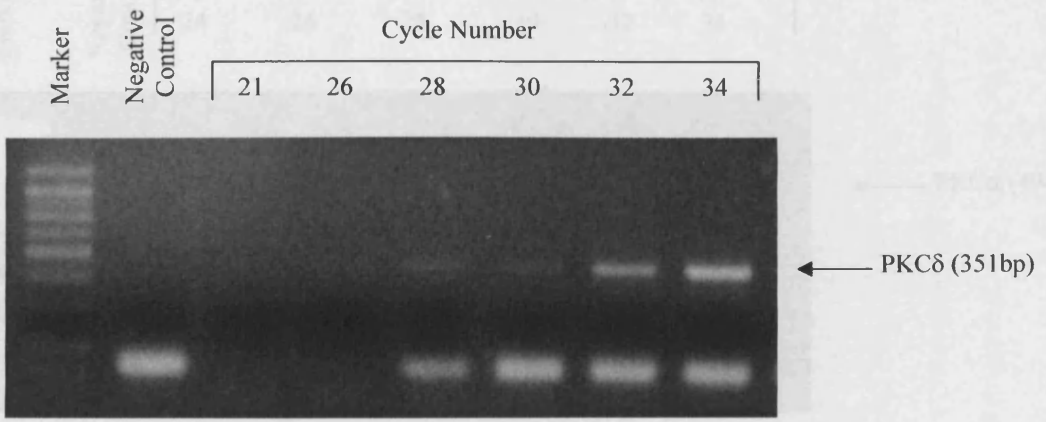


Figure 2.2. PCR optimisation of PKCδ primers

The level of PKCδ expression in cDNA from the WT-MCF-7 cell line was measured using PKCδ specific PCR primers. The number of cycles was varied to ascertain which cycle would be most suitable in future experiments to observe differential expression of the isoform. No cDNA was added to one of the reaction mixes as a negative control.

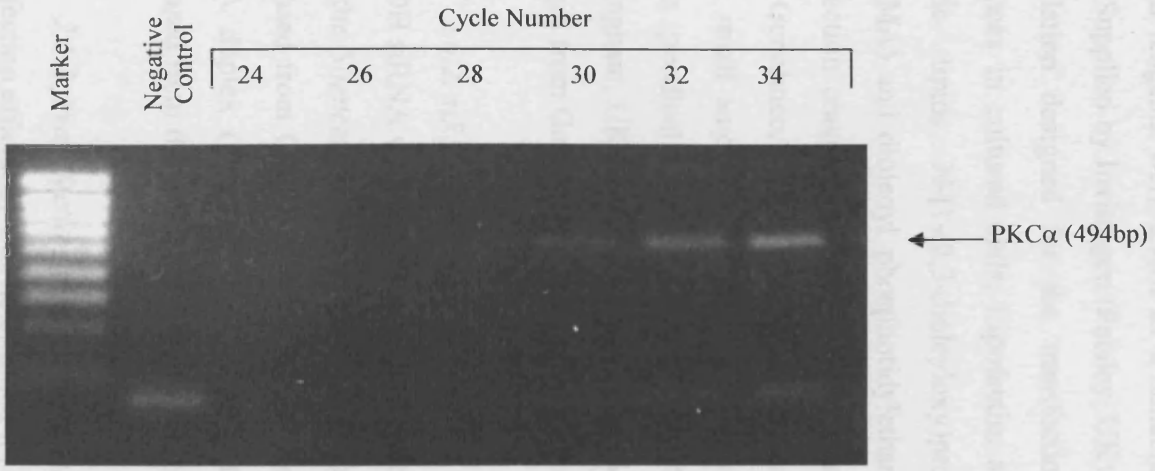


Figure 2.3. PCR optimisation of PKC-α primers

The level of PKCα expression in cDNA from the WT-MCF-7 cell line was measured using PKCα specific PCR primers. The number of cycles was varied to ascertain which cycle would be most suitable in future experiments to observe differential expression of the isoform. No cDNA was added to one of the reaction mixes as a negative control.

2.6. RNA Interference (RNAi)

2.6.1. Transfection reagents

Several reagents were tested as a suitable transfection reagent for the studied cell lines. Supplied by Invitrogen (Paisley, UK) were Lipofectamine 2000, a cationic lipid formulation designed for the transfection and subsequent expression of DNA sequences in cultured cells, Lipofectin, a 1:1 (w/w) liposome formulation of the cationic lipids N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphotidylethanolamine (DOPE); and Oligofectamine, a transfection reagent designed for the transfection of oligonucleotides into cultured cells. GeneJuice, a transfection reagent formulated from a non-toxic cellular protein and a small amount of a novel polyamine, and RiboJuice, an analagous delivery system specifically designed for siRNA delivery, were both purchased from Novagen (Nottingham, UK). β-Galactosidase plasmid used for transfection optimisation was a kind gift from Gavin Wilkinson at University of Wales College of Medicine.

2.6.2. siRNA Duplexes

GAPDH siRNA with scrambled control and siRNA Cy3 Labelling kit were purchased from the *Silencer* range by Ambion (Huntington,UK). PKC δ specific siRNA was purchased from Qiagen (Crawly, UK). The sequence was obtained from a published siRNA duplex (Yoshida *et al.*, 2003). The PKC α specific siRNA utilsed was purchased from the validated HiPerformance range of siRNAs from Qiagen.

2.6.3. Transfection efficiency assessment using β-galactosidase (β-gal)

Transfection efficiency was determined for each cell line by transfecting a set of wells with a β-gal expression vector (β-gal). The β-gal was transfected into the cells as outlined below. The transfected cells were stained for β-gal as follows: Cells were washed with r/t PBS and fixed with 0.5% (v/v) glutaraldehyde in PBS (2mls per well) for 15 minutes at room temperature. After that time, cells were again washed with room temperature PBS. An X-gal staining solution containing potassium ferricyanide and potassium ferrocyanide prepared as below:-

Stocks of 300mM potassium ferricyanide/130mM $MgCl_2$ in PBS, 300mM potassium ferrocyanide/130mM $MgCl_2$ in PBS and 40 mg/ml X-gal (dissolved in di-methyl

formamide) were initially prepared. The staining solution was then made by diluting 1/100 (v/v) each of the two potassium cyanide solutions in PBS (final concentrations of 3mM potassium ferricyanide and 3mM potassium ferrocyanide) with 1/320 (v/v) of stock solution of the X-Gal (final concentration 0.125 mg/ml).
Cells are left in this staining solution (2ml per well) overnight at 37°C. A blue colour stain could be seen the next day in transfected cells only and the percentage transfection could therefore be estimated by counting the relative proportion of stained to non-stained cells.

2.6.4. β-Galactosidase Plasmid Transfection

Initial transfection optimisation was carried out using a β-galactosidase (β-Gal) plasmid to visualise cellular uptake. Cells were seeded out in 12 well plates (with a surface area $4cm^2$) at a seeding density of 3×10^6 cells per plate and grown prior to transfection for 24 hours in W +5% media as described in section 2.2.4. 24 hours prior to transfection the media was replaced with W +5% minus antibiotics and fungizone.

2.6.4.1. Lipofectamine2000

Lipofectamine 2000 (3 µl) was added to 50µl of DCCM culture media and incubated at room temperature for 45 minutes. Separately, 1µg of the β-gal plasmid was added to 50µl of DCCM. The two components were then combined and allowed to incubate for a further 15 minutes at room temperature. Once the complex had been allowed to form, 0.4ml of DCCM containing 1% (w/v) DMSO was added to the transfection mixture. The medium was then aspirated from each of the cell culture wells and overlayed with 0.5ml of the transfection mixture. Cells were then incubated for 6hours at 37°C, after which time the transfection reagent was removed and replaced with ample growth media. The cells were subsequently incubated for a further 24 hours prior to staining.

2.6.4.2. GeneJuice

GeneJuice (1.5µl) was diluted in 50µl of DCCM, vortexed briefly, and incubated for 5 minutes at room temperature. To this mixture, 0.5µg of β-gal plasmid was added and

incubated at room temperature for 10 minutes. The gene juice/plasmid complex was then applied to the cells in one of two ways to test whether the presence of serum affected the transfection efficiency. The 50µl of transfection mixture was either applied to the cells directly without removing their complete growth media or the media was removed and replaced with the transfection mixture made up to a volume of 0.5ml in DCCM containing no antibiotics or fungizone.

2.6.4.3. Lipofectin

β-gal plasmid (400ng) was added along with 600ng of PCR-Script to 60µl of DCCM. Lipofectin (3 µl) was added to 60µl of DCCM and left to incubate at room temperature for 45 minutes after which time the two mixtures were combined and allowed to incubate for a further 15 minutes. Once the complex had been allowed to form, 0.4ml of DCCM containing 1% (w/v) DMSO was added. The medium was then aspirated from the wells and overlayed with 0.5ml of the transfection mixture.

2.6.5. siRNA Transfections

Prior to transfection, the media was changed for 0.5ml of white RPMI/5% csFCS containg no antibiotics.

2.6.5.1. Lipofectamine2000

Lipofectamine 2000 (3.6 µl) was added to 60µl of DCCM and incubated at room temperature for 45 minutes. Separately, 0.71µg of the specific siRNA duplex was added to 60µl of DCCM. The two components were then combined and allowed to incubate for a further 15 minutes at room temperature. Once the complex had been allowed to form, 0.4ml of DCCM containing 1% (w/v) DMSO was added to the transfection mixture. The medium was then aspirated from each of the wells and overlayed with 0.5ml of the transfection mixture. Cells were then incubated for 6hours at 37°C, after which time the transfection reagent was removed and replaced with ample growth media. The cells were subsequently incubated for a further 48 hours prior to assessment/ harvesting.

2.6.5.2. GeneJuice

GeneJuice (3µl) was diluted in 100µl of DCCM, vortexed briefly, and incubated for 5 minutes at room temperature. To this mixture, 0.71µg of siRNA duplex was added and incubated at room temperature for 10 minutes. The gene juice/plasmid complex was then applied to the experimental medium present in the wells.

2.6.5.3. Oligofectamine

Oligofectamine (6 µl) was added to 24µl of Opti-MEM and left to incubate at room temperature for 5 minutes. Separately, the specific siRNA duplex (0.71µg) was added to 100µl of Opti-MEM. After the incubation period the two mixtures were combined and allowed to incubate for 20 minutes. 126µl of the transfection mixture was evenly distributed over the complete growth media in the wells in a drop-wise manner and then rocked gently to ensure even distribution.

2.6.5.4. RiboJuice

RiboJuice (6µl) was diluted in 94µl of Opti-MEM, mixed thoroughly by brief vortexing and allowed to incubate at room temperature for 5 minutes. The specific siRNA duplex (1.3µl) was added to this mixture, mixed gently and incubated at room temperature for 5-15 minutes. A volume of 100µl of the transfection mixture was then evenly distributed in drops over the medium of each well. The plates were then gently rocked to allow even distribution.

2.7. Adenovirus

2.7.1. Passaging HEK-293 cells

Human embryo 293 kidney cells (HEK-293) were kindly provided by Professor David Murphy at Bristol University Research Centre for Neuroendocrinology (URCN) and Dr. Gavin Wilkinson at the University of Wales College of Medicine (UWCN). HEK-293 cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplimented with 10% (v/v) FCS and 5mM l-glutamine in 175cm^2 flasks at 37°C and 5%CO_2. Upon reaching confluency the media was aspirated and 4ml Trypsin/EDTA was added to each flask and allowed to incubate at 37°C for

around 5 minutes until the cells had visibly detached from the flask. The flask may be gently tapped to encourage cells to detach. 10ml of culture media was added to the flask. The cell suspension was then transferred to 50ml Vulcan tubes and centrifuged at 1500g for 5 minutes until a stable pellet was formed. The supernatant was then carefully removed and the pellet was resuspended in 10ml of media by repeated pipetting with a 10ml pipette. A volume of 2ml of each cell suspension was then added to 5 @ 175cm^2 flasks and 30ml of media added to each. The cells were then replaced in the incubator.

2.7.2. Bulking up of adenovirus

The recombinant adenoviruses used in all experiments outlined are incapable of replication in target cells lacking a complementing E1 helper function. Therefore the preparation of viral stocks requires infection and subsequent scaling up of the HEK-293 cells which constituently express the E1 proteins. The adenovirus was then purified from the cells in two different ways outlined below.

2.7.2.1. Arklone P Method

HEK-293 cells were grown in a 175cm^2 flasks till they reached 70% confluency. The media was then aspirated from the flasks and replaced with 25ml of media containing a multiplicity of infection (MOI) of 0.1. The cells were allowed to incubate for 90 minutes after which time the media was removed and replaced with fresh media. After 3 days a clear cytopathic effect (CPE) was visible across the monolayer. The cells could now be readily detached from the flask with the assistance of gentle tapping. The cells were then collected and centrifuged at 2700g for 5 minutes and the supernatant discarded. In case of leakage or tube failure the tubes were removed from detachable centrifuge buckets inside the class II safety cabinet. The cells were resuspended in 10ml PBS and an equal volume of Arklone P by vigorous mixing. The suspension was then centrifuged at 1000g for 5 minutes to separate the mixture into two layers. The top aqueous phase contained the extracted adenovirus. To maximise yield the interface was resuspended with PBS, centrifuged and the top layer extracted as before. The adenovirus containing supernatant was then aliquoted and stored at −80°C.

2.7.2.2. Freeze Thaw Method

HEK-293 cells were grown in 175cm^2 flasks till they reached 70% confluency. The flasks are then inoculated with 20µl of pure virus with a concentration of around 2 X 10^{-10}pfu (plaque forming units). The media containing the inoculum was removed after 16 hours and fresh media added. The cells were then grown till a clear cytopathic effect (CPE) was visible. Once the cells exhibit a gross CPE effect the cells were harvested from the flask rapidly freeze thawed and centrifuged at 1500g for 5 minutes. To ten 175cm^2 flasks of HEK-293 cells, 1ml of the crude viral supernatant suspension was added and incubated at 37°C, 5% CO_2 for around 3 days until a clear cytopathic effect (CPE) was visible across the monolayer. The cells could now be readily detached from the flask with the assistance of gentle tapping. The cells were then collected and centrifuged at 2700g for 5 minutes. The supernatant which contained low levels of the adenovirus was then stored at -80°C for future use. The cell pellet containing the majority of the adenovirus was then resuspended and pooled in a total volume of 2.5ml 0.1M HCl, pH8.0 and freeze thawed to break open the cells. The resultant viral suspension was then sonicated for 4 minutes in an ice bath. Cell debris was then removed by centrifugation at 2700g for 5 minutes. The extracted virus was then ready for aliquoting and storage at -80°C or futher purification by Caesium Chloride (CsCl) gradient centrifugation.

2.7.2.3. CsCl Purification of Adenovirus

Adenovirus was extracted as outlined above from a minimum of 10 175cm^2 flasks to ensure that enough material was available to generate a visible band on the CsCl column following centrifugation. CsCl solution (1.6ml of a 1.45g/ml solution in 5mM Tris HCl, pH7.8) was pipetted into 14 x 89mm Ultra-Clear Beckman centrifuge tubes. Then a less dense CsCl solution (3ml of a 1.33g/ml solution in 1mM EDTA, 5mM Tris HCl, pH7.8) was gently layered on top. The tubes were then filled to within 2.5mm of the top with the adenovirus extract obtained in section 2.7.2.2. The tubes were then spun in an ultracentrifuge at 100,000g overnight at 20°C. The visible viral bands were then collected by piercing the tube with a syringe and passed through a PD-10 column pre-equilabrated with 10mM Tris pH7.5, 1mM $MgCl_2$. The adenovirus was eluated from the columns with 10mM Tris, pH 7.5, 1mM $MgCl_2$. The adenovirus

containing eluate was then filter sterilised and seperated into 20µl aliquots and stored at -80°C.

2.7.3. Titration of Adenovirus by end point dilution

Low passage HEK-293 cells were seeded into 80 wells of a 96 well plate and grown for 24 hours till they reached 50-60% confluence. Serial dilutions of adenovirus were prepared in complete media and 100µl of each dilution added to the appropriate wells and incubated overnight at 37°C, 5% CO_2. Following this incubation period the media containing the adenovirus was replaced with 200µl of fresh media and replaced in the incubator. The media was then changed every 2-3 days as necessary. Once the cytopathic effect was visible in a well it was marked and it's media no longer replaced. After 8 days of incubation the number of wells containing plaques were counted. The titre of the adenoviral stocks were then calculated as plaque forming units (pfu) per ml (pfu/ml) using the Reed & Meünch endpoint calculation formulae (Reed and Meünch, 1938):

Proportional Distance=

$$\frac{\text{\% of wells infected at dilution above 50\% - 50\%}}{\text{\% of wells infected at dilution above 50\% - \% of wells infected at dilution below 50\%}}$$

ID_{50} (Infectivity Dose, 50%)=

log dilution above 50% + (proportional distance x dilution factor)

$TCID_{50}$ / ml (Tissue Culture Infectivity Dose)= $\frac{1}{ID_{50}} \times 10$

$TCID_{50}$/ ml $\simeq$ 0.7 pfu/ml

Final Titre (pfu/ml) = $TCID_{50}$ x 0.7

Using the endpoint dilution method described above, the viral titers for each of the adenoviral vectors was calculated. These titers are listed below in table 2.5.

Table 2.5. Viral titers of adenoviral vectors calculated using the endpoint dilution assay.

ADENOVIRAL VECTOR	VIRAL TITRE (pfu/ml)
Empty Vector	5×10^9
β-gal plasmid	3.6×10^9
PKC δ Dominant Negative	4.8×10^9
PKC δ Wild Type	2.0×10^9
PKC α Dominant Negative	3.2×10^9
PKC α Wild Type	4.8×10^9

To ensure that the relative number of viral particles per cell to be infected is kept constant, the cells are counted and infected at a constant multiplicity of infection (MOI). The volume of viral suspension required to infect with the assigned MOI is calculated using the equation below:

Volume of viral suspension (μl) =

$$\frac{\text{MOI x Number of cells to be infected}}{\text{pfu/ml}} \times 1000$$

2.7.4. Adenoviral infection of the MCF-7 and TAM-R cell lines

The MCF-7 and TAM-R cells were set up in 24 well plates and allowed to grow for 48 hours prior to infection in media containing 5% csFCS. The cells were then infected by addition to the well of the volume of adenoviral suspension calculated to provide the desired MOI (section 2.7.3). The media was then changed 16 hours after infection and the cells allowed to grow to the desired time point with further media changes carried out every three days where necessary.

2.7.5. Establishment of suitable MOI for the PKC-δ WT and DN adenoviruses

MCF-7 cells were set up and infected as outlined in section 2.7.5 with either PKC-δ DN or WT expressing adenovirus at MOI of 20, 50, 100, 200 and 500 in triplicate. Additionally, in each case a triplicate of wells were left uninfected as controls. The media was then changed 16 hours post infection and the cells allowed to grow for a

further 24 hours, after which time the total RNA was extracted and reverse transcribed as described in sections 2.5.1 and 2.5.3 respectively. The cDNA generated from each of the samples was probed for PKC-δ using PKC-δ specific primers (section 2.5.5). The amplification products were size fractionated on a 2% agarose gel containing ethidium bromide and visualised under UV light.

2.7.6. Infection efficiency assessment using a β-gal expressing adenovirus

The MCF-7 and TAM-R cell lines were seeded in 12 well plates and allowed to grow in their home medium containing 5% csFCS for 48 hours prior to infection. The number of cells in each of three wells was then Coulter counted in duplicate (as described in section 2.2.6) and the mean cell number used to calculate the volume of adenoviral suspension required to provide the correct multiplicity of infection (MOI) in each well. The cells were infected with a β-galactosidase expressing adenovirus at MOI of 20, 50, 100 and 200 in triplicate wells with three wells left uninfected as a control. The media was changed 16 hours post infection and the cells allowed to grow for a further 24 hours. The cells were then stained for the presence of β-galactosidase using the chromogenic substrate X-Gal and fixed as outlined in section 2.6.3. The infection efficiency was assessed by visually counting the percentage of cells with the charateristic blue staining relative to the overall cell population.

2.7.7. In Vitro Kinase Assay of Adenovirally Infected Cells

Cells were grown in 24 well plates for 48 hours prior to infection of triplicate wells with adenovirus. The media was then changed 16 hours post infection and the cells allowed to grow for a further 24 hours, after which time the cells were lysed on ice using 200μl of cold lysis buffer containing 20mM Tris pH 7.6, 0.25M NaCl, 3mM EDTA, 3mM EGTA, 10μg.ml^{-1} leupeptin, 2mM sodium vanadate ($NaVO_3$), 25mM sodium fluoride (NaF), 1mM DTT and 0.5% Nonidet (N)P-40 per well. The contents of the triplicate wells were pooled together and cell debris removed by centrifugation at 13,000g for 5 minutes at 4°C. The supernatant was then transferred to a sterile eppendorf and a further 600μl of lysis buffer minus DTT and NP-40 was added. An appropriate volume of anti-PKC isoform specific antibody (α or δ) was added to the samples and gently rotated in a cold room (4°C) for 1 hour. The total volume of

Ezview™ Red Protein G for all the samples was added to a fresh eppendorf and pulse centrifuged for 10 seconds to precipitate the beads and remove the preservative supernatant. The beads were then resuspended to their previous volume in lysis buffer minus DTT and NP-40, 20µl added to each of the samples and the samples rotated gently in a cold room over night. The samples were then centrifuged at 13,000g for 1 minute to pellet the beads, the supernatant discarded and the pellet resuspended with 200µl cold lysis buffer minus DTT and NP-40. The resuspended pellet was then centrifuged at 13,000g for 1 minute and the supernatant discarded. The pellet was then washed in ice cold kinase buffer (137mM NaCl, 5.4mM pottasium chloride(KCl), 0.3mM di-sodium hydrogen phosphate (Na_2HPO_4) 0.4mM pottasium dihydrogen phosphate (KH_2PO_4), 1mg/ml L-glucose, 25mM β-glycerophosphate, 25mM magnesium chloride ($MgCl_2$) and 5mM EGTA. ATP was added to kinase buffer at 100µM and radiolabelled to approximately 500,000 counts per minute (cpm) per 40µl with γ–P^{32} ATP. Samples were centrifuged for 1 minute and the resultant supernatant discarded. Non specific binding control samples for PKC-α and PKC-δ were resuspended in 40µl of labelled kinase buffer minus the substrate peptide and incubated at 37°C for 10 minutes. The reaction was terminated with 5µl of 45% Sodium trichloroacetate (TCA) at 4°C and the tubes placed on ice. The selective PKC substrate peptide MBP (4-14) (Calbiochem) was then added to the remaining labelled kinase buffer to a concentration of 50µM. The kinase reaction was initiated by adding 40µl of the radiolabelled kinase buffer containing the substrate peptide to each tube and the samples mixed by drawing up and down with the pipette. The samples were then incubated at 37°C for 10 minutes after which time the reaction was halted by the addition of 5µl of 45% sodium trichloroacetate (TCA) at 4°C and the tubes placed on ice for 10 minutes. The samples were then centrifuged at 13,000g for 3 minutes and the supernatant spotted onto 2cm x 2cm squares of Whatmann P-81 paper and allowed to dry. Unincorporated P^{32} was washed away with 2 x 1 minute washes with 75mM phosphoric acid (H_3PO_4), 1 x 10 minute wash with 75mM H_3PO_4, and 2 x 5 minute and 1 x 1 minute washes with 75mM phosphate buffer pH 7.4. The paper squares were then placed in scintillation tubes containing 10ml H_2O and P^{32} incorporation measured by scintillation counting using the Cerenkov method. The pmol P^{32} incorporated per sample was defined using the calculation below:-

pmol P^{32} incorporated per sample =

$$\frac{\text{Specific Counts x 5000}}{\text{Total Counts}}$$

where Specific Counts =

$$\text{radiolabel incorporation in presence of substrate} - \text{radiolabel incorporation in absence of substrate}$$

CHAPTER 3.

STUDY INTO THE PKC-α AND PKC-δ PROFILE OF THE MCF-7 CELL LINE AND ITS TAMOXIFEN RESISTANT (TAM-R) DERIVATIVE

3.1. INTRODUCTION

Protein kinases are a major intermediary and facilitator in a multitude of biological functions. Therefore it is not surprising that the deregulation of their expression or activity is implicated in many disease states. Aberrant PKC signalling alone has been implicated in diseases ranging from the most prevalent diseases in western society such as cancer, heart disease and diabetes to a major killer of children in developing countries, enteropathogenic *Escherichia coli* induced diarrhoea (Crane and Vezina, 2005).

For many years since its discovery PKC was studied and described as a single entity. However PKC is actually a blanket term for a series of closely related signalling molecules that can act in a synergistic or antagonistic manner depending on their intracellular location and activation status in a cell specific context. This complexity requires a viable and reproducible assay system to disentangle the various isoforms and accurately assess their function. The advent of PKC isoform and phosphorylation state specific antibodies has greatly eased this endeavour by allowing the total levels of intracellular PKC expression and activation to be visualised and measured by robust techniques such as Western analysis and immunocytochemistry without the need for more involved procedures such as radioactive *in-vitro* kinase assays (Figure 3.1).

Whereas previously it has been reported that overall levels of PKC were elevated in breast cancer compared to the surrounding normal tissue (O'Brian *et al.*, 1989) and in ER- compared to ER+ cell lines (Fabbro *et al.*, 1998); we now know that there is a more complex interplay regarding the individual isoforms. For example elevated levels of PKC-δ and relatively low levels of PKC-α are found in oestrogen receptor positive cell lines and conversely, relatively low expression of PKC-δ and elevated levels of PKC-α are associated with oestrogen receptor negative cell lines (Assender *et al.*, 1997; Morse-Gaudio *et al.*, 1998; Shanmugan *et al.*, 2001). Other studies have also shown that overexpression of PKC-α decreases ER expression in T47D cells but not MCF-7 cells (Tonetti *et al.*, 2000). How closely these cell line models mimic the clinical scenario however still remains to be elucidated, as studies to date have been

conflicting, i.e. Tonetti *et al.*, (2003) showed that PKC-α expression is raised in advanced disease, whilst Kerfoot *et al.* (2004) showed that it is decreased. Although the cell models have tried to address PKC isoforms expression patterns in cases of *de novo* resistance to tamoxifen there has been relatively little work to determine how the expression and activation of these isoforms relate to the acquired resistance phenotype.

To examine the effect of the acquisition of Tamoxifen resistance on PKC expression and utilisation, the MCF-7 cell line and a Tamoxifen resistant derivative (TAM-R) were studied. The MCF-7 cell line was initially derived from a female breast cancer patient with metastatic disease (Brooks *et al.*, 1973). This oestrogen receptor positive breast cancer cell line, one of the most commonly used cancer models in the world, is initially responsive to the growth inhibitory effects of Tamoxifen treatment. However routine maintenance of the MCF-7 cell line in the presence of Tamoxifen produces a cell line which can circumvent the growth inhibitory effects of Tamoxifen and develop a TAM-R phenotype (section 2.2.5) whilst retaining oestrogen receptor functionality, as illustrated by their enduring sensitivity to the pure antioestrogen fulvestrant (Nicholson *et al.*, 2004). Therefore this cell line serves as a useful and relevant model as it mirrors the acquisition of Tamoxifen resistance that eventuates in the clinical setting.

The identification of the physiological substrates and cellular functions of protein kinases within model cell lines has benefited from the development of many low molecular weight, cell permeable chemical inhibitors. With over 500 distinct protein kinases sharing conserved catalytic mechanisms and structural similarities, the greatest challenge in the design of these protein kinase inhibitors is that of selective inhibition (Dancey and Sausville, 2003). Several inhibitors have been reported to have such selective inhibition for the PKC family of protein kinases. These have now become widely used tools in the elucidation of PKC function in the signal transduction pathways involved in normal and neoplastic cells.

A whole class of PKC inhibitors has been derived from the indolocarbozole, staurosporine (Figure 3.2a), a compound discovered during the course of screening

extracts from the bacterium *Streptomyces sp.* for constituent alkaloids with PKC inhibiting properties (Omura *et al.*, 1977). Staurosporine itself is a powerful inhibitor of PKC (IC_{50} 10nM) (Davis *et al.*, 1989) due to its ability to compete with its ATP binding site, however it displays poor selectivity across the various isoforms (Way *et al.*, 2000). This selectivity has been increased through the chemical modification of the basic staurosporine structure to produce functional derivatives such as the 2,3-bisindolylmaleimides, including bisindoloylmaleimide IX (Ro31-8220) (bis) (Figure 3.2b) (Gescher, 1998). For example, these derivatives have much better specificity for PKC over other intracellular kinases such as PKA, and are reported to possess slight selectivity for the PKC-α isoform over the other conventional PKCs (Wilkinson *et al.*, 1993). Since its derivation, Bis has been used in over 600 published studies to demonstrate physiological roles for PKC in many cell systems and species (McGovern and Shoichet, 2003).

The lack of true isoform specificity with Bis causes complications when attempting to elucidate the mechanisms of specific PKC isoforms as different isoforms can have contradictory effects across multiple signalling pathways. Rottlerin (mallotoxin) (Figure 3.2c), a compound derived from the tree *Mallotus philippensis*, has been shown to inhibit protein kinases with some specificity for PKC. More importantly it was originally observed to have the ability to differentiate between the PKC isozymes, and was the first inhibitor to show a greater specificity for PKCδ (IC_{50} values 3-6μM) than for PKCs α, β ,γ (30-42μM) or PKC ε,η and ζ (80-100μM) (Gschwendt *et al.*, 1994). Rottlerin has been used in, at least, hundreds of studies to elucidate the role of PKCδ in a wide spectrum of cellular events including apoptosis, cell differentiation and MAPK activation (Soltoff, 2001).

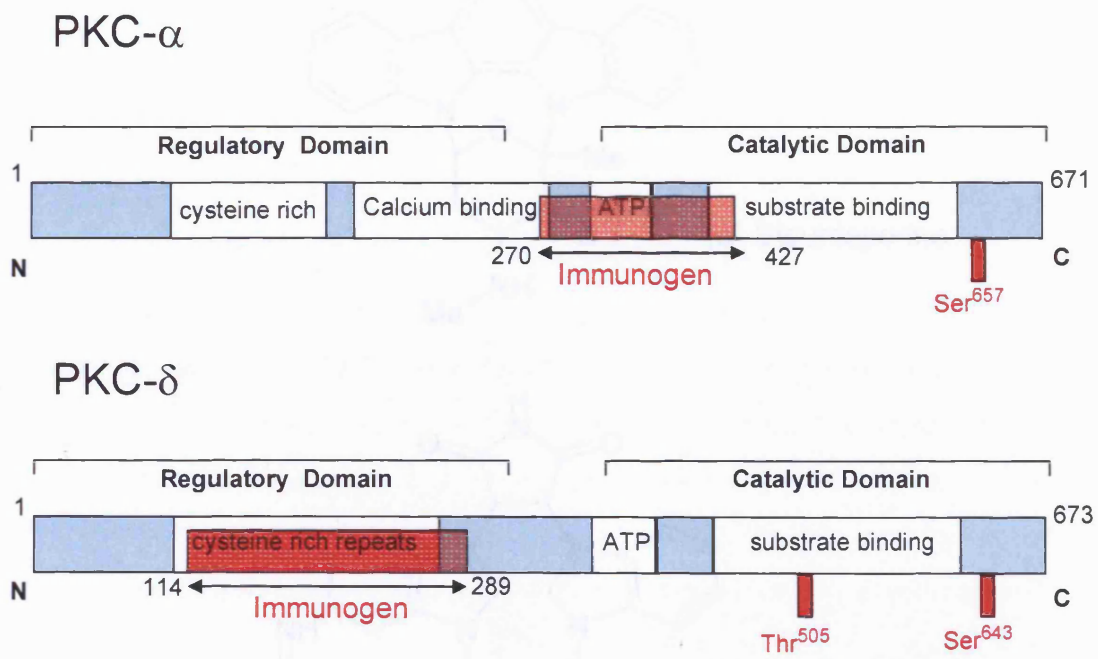


Figure 3.1 Protein fragments of PKC used as immunogen in the production of PKC-α and δ antibodies.

Monoclonal antibodies for PKC-α and PKC-δ were generated using fragments of the human PKC isoforms as immunogens. The PKC-α specific antibody was generated using a protein fragment of human PKC-α comprising amino acids 270 and 427 and the PKC-δ immunogen comprised a fragment of human PKC-δ between amino acids 114 to 289. Both antibodies were supplied by BD Transduction Laboratories (Oxford, UK). Also illusrated are the phosphorylated amino acid residues targeted by phosphorylation specific antibodies for PKC-α and δ. Antibodies directed at phosphorylated PKC-δ residues threonine 505 and serine 643 were supplied by Cell Signalling Technology (Herts, UK). Antibody specific for PKC-α phosphorylated at serine 657 was supplied by Upstate (Milton Keynes, UK).

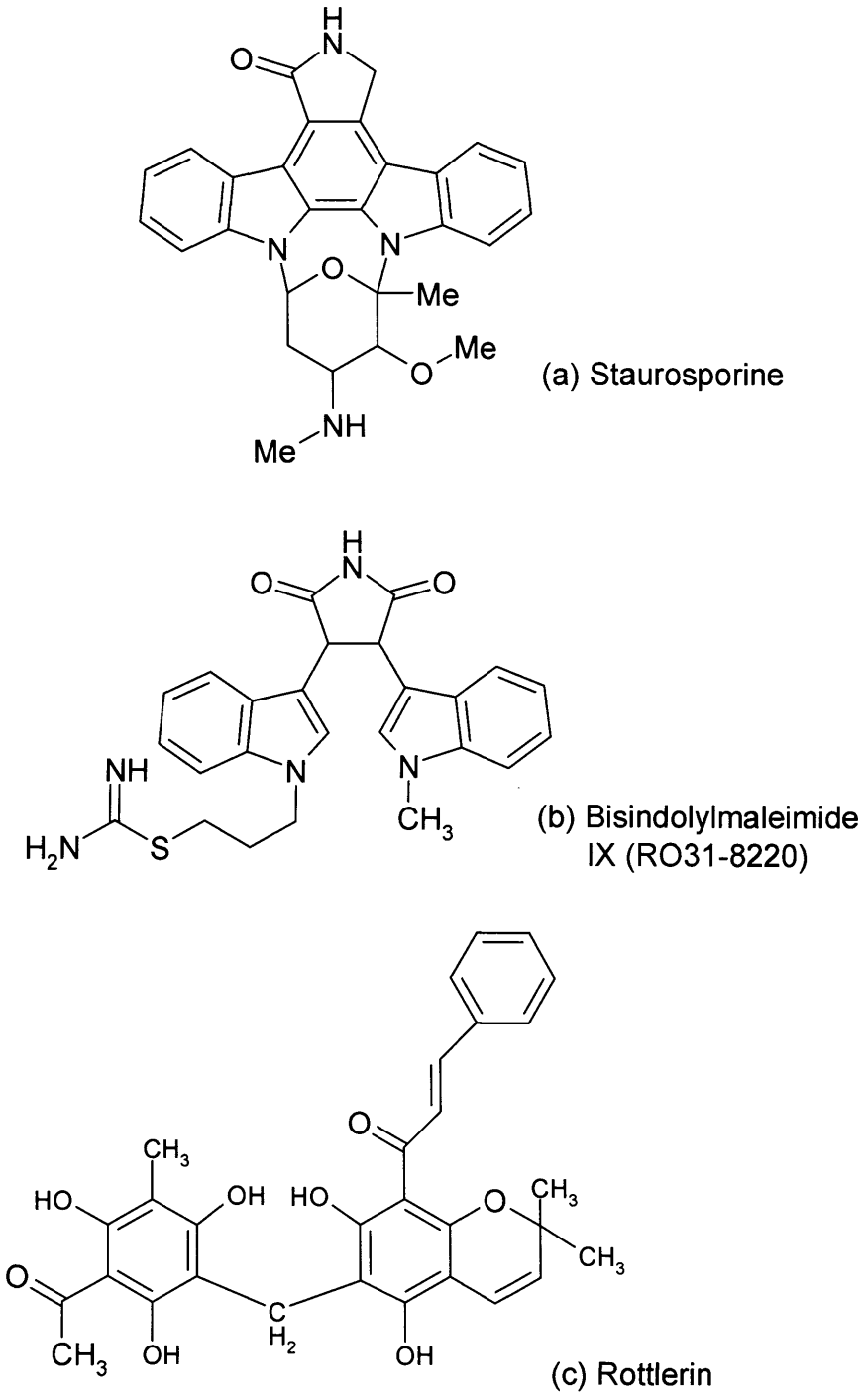


Figure 3.2. The structure of some commonly used PKC inhibtors

The diagrams above are structural representations of three reported PKC inhibiting compounds; (a) staurosporine, (b) one of its derivatives, bisindolylmaleimide IX (RO31-8220) and (c) Rottlerin.

3.2. RESULTS

3.2.1. Determination of relative protein levels of PKC-α and PKC-δ in the oestrogen receptor positive MCF-7 cell line compared to the oestrogen receptor negative MB-MDA-231 cell line

It has previously been shown in various breast cancer cell line models that a correlation exists between the relative PKC-α and PKC-δ isoform expression profile and a breast cancer cells oestrogen receptor status (Assender *et al.*, 1997; Shanmugan *et al.*, 2001; Tonetti *et al.*, 2003). To verify that we could detect this in our model cell line models, the PKC-α and δ isoform expression of the oestrogen receptor positive MCF-7 cell line was compared to the oestrogen receptor negative MB-MDA-231 cell line by Western analysis using isoform specific antibodies. In both cases the blotted membranes were additionally probed for β-actin as a control for overall protein levels and loading accuracy. (Figure 3.3). It can be seen that the relative expression of the two isoforms between the two cell lines concurs with the previously observed trends, with the MB-MDA-231 cell line expressing far greater PKC-α when compared to the MCF-7 cells whilst displaying relatively little PKC-δ. This verifies that our antibody regime is working effectively and that a cell lines PKC isoform expression profile may reflect its oestrogen receptor status.

3.2.2. Determination of relative protein levels of PKC-α and PKC-δ in the MCF-7 cell line compared to the TAM-R cell line.

As PKC-α and δ levels are altered in the MB-MDA-231 cell line which is oestrogen receptor negative and therefore displays *de novo* resistance to tamoxifen, we decided to investigate whether the expression of these isoforms was also altered in a breast cancer cell line that has acquired resistance to tamoxifen. Therefore we compared the protein levels of PKC-α and δ in the tamoxifen sensitive MCF-7 cell line compared to their tamoxifen resistant derivative (TAM-R) by western blotting using isoform specific antibodies with β-actin levels measured as a control of overall protein levels and loading accuracy. In each case the total levels of the PKC isoforms in the TAM-R cells were expressed as fold expression relative to that of the MCF-7 cell line ± SD

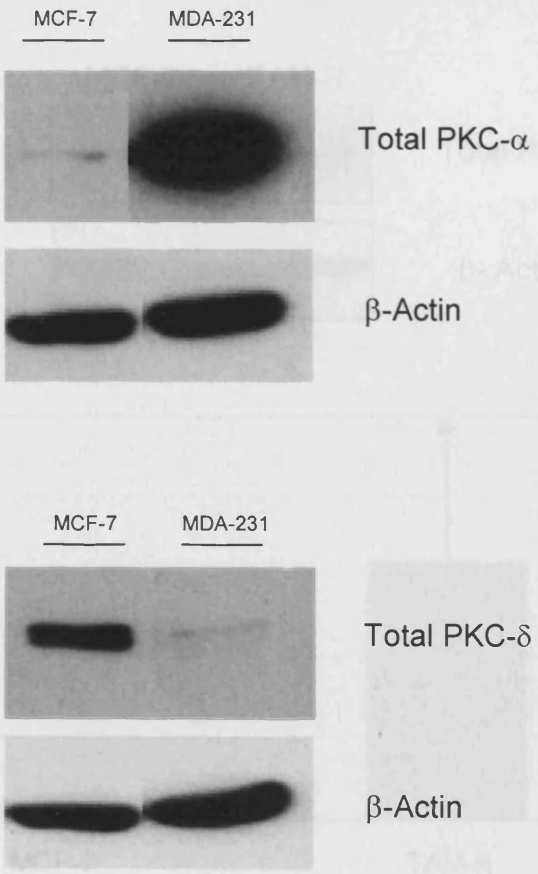


Figure 3.3. Relative PKC-α and PKC-δ protein expression between the ER positive MCF-7 breast cancer cell line and ER negative MB-MDA-231 breast cancer cell line.

Western analysis was carried out whole cell extracts taken from MCF-7 and MB-MDA-231 cells grown to 80% confluency in media containing 5% csFCS. The blotted membranes were probed with primary antibodies specific for either PKC-α or PKC-δ. In addition, the membranes were probed with a β-actin specific antibody as a loading and protein concentration control.

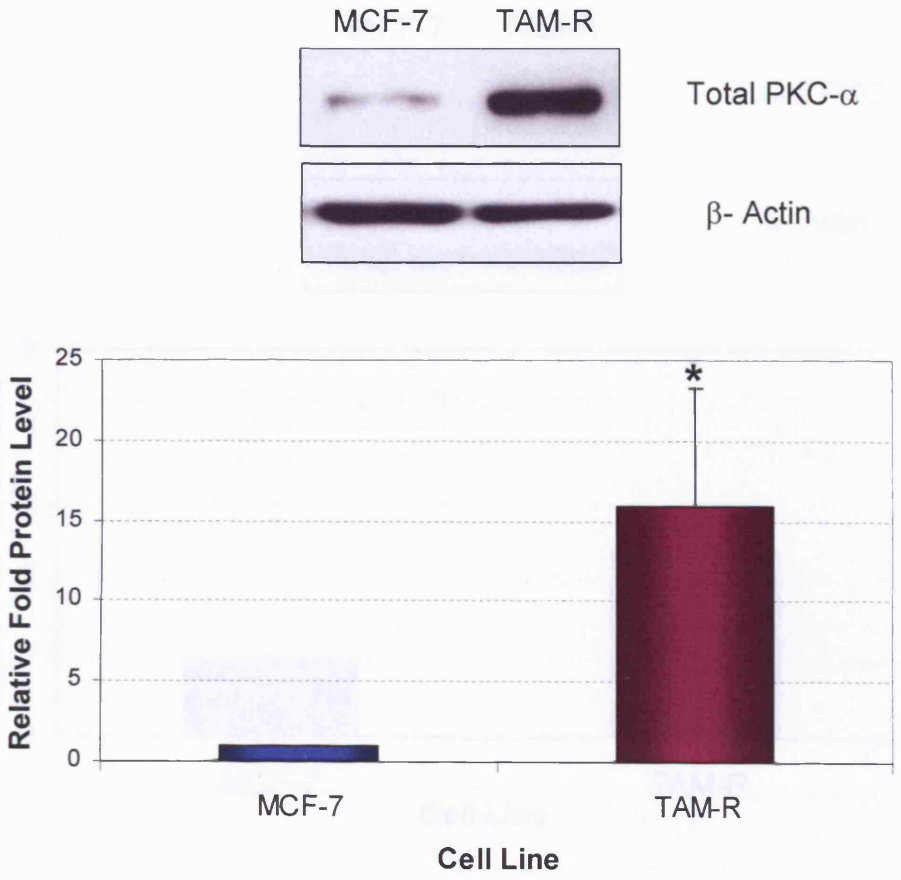


Figure 3.4. Relative PKC-α protein expression between the MCF-7 breast cancer cell line and the Tamoxifen resistant (TAM-R) cell line

Western analysis was carried out using whole cell extracts taken from MCF-7 and Tamoxifen resistant cells grown to 80 % confluency in media containing 5% csFCS. The blotted membranes were probed with primary antibodies specific for PKC-α. In addition, the membranes were probed with a β-actin specific antibody as a loading and protein concentration control. The graph shown illustrates the mean fold difference expressed as the fold increase in protein level detected in the TAM-R cells compared to the MCF-7 cells ± SD from 5 independent experiments. The data was analysed by paired t-test (*p=0.02).

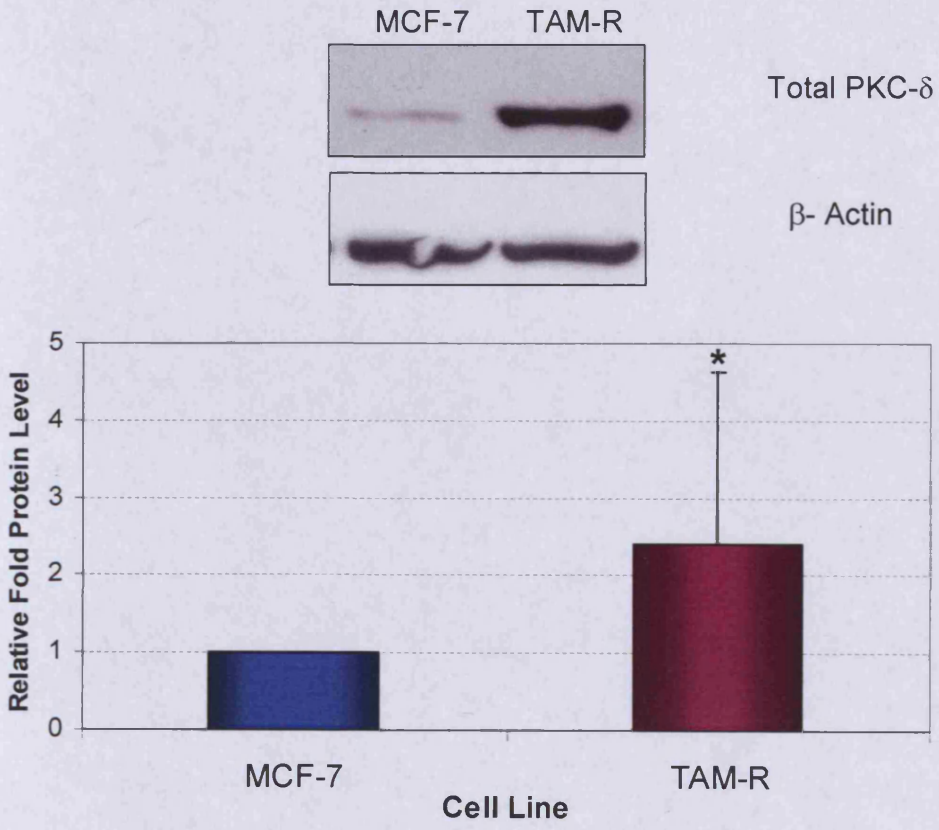


Figure 3.5. Relative PKC-δ protein expression between the MCF-7 breast cancer cell line and the Tamoxifen resistant (TAM-R) cell line

Western analysis was carried out using whole cell extracts taken from MCF-7 and Tamoxifen resistant cells grown to 80 % confluency in media containing 5% csFCS. The blotted membranes were probed with primary antibodies specific for PKC-α. In addition, the membranes were probed with a β-actin specific antibody as a loading and protein concentration control. The graph shown illustrates the mean fold difference expressed as the fold increase in protein level detected in the TAM-R cells compared to the MCF-7 cells ± SD from 6 independent experiments. The data was analysed by paired t-test (*p=0.03).

(Figures 3.4 and 3.5). As with the MB-MDA-231 cells, the TAM-R cell line displayed a large increase in total levels of PKC-α with a mean level of expression exceeding 15 fold those observed in the MCF-7 cell line. However, unlike the MB-MDA-231 cells, the TAM-R cell line didn't exhibit a reduced relative expression of PKC-δ but rather displayed a more that 2 fold increase in mean expression.

To visualise the PKC-α and δ levels and localisation in the whole cells and verify the increases in their expression already measured, the levels of total PKC-α and δ were measured in the MCF-7 and TAM-R cells by immunocytochemistry utilising the same isoform specific antibodies used in the Western analysis. It can be seen from Figure 3.6 that whilst some cytoplasmic staining of the PKC-α and δ isoforms is detectable in the MCF-7 cells there is a marked increase in the cytoplasmic staining of both isoforms in the TAM-R cells. Therefore the increase in relative expression of PKC-α and δ has been observed both in cell lysates and in the whole cell, by two separate techniques.

3.2.3. Determination of relative levels of PKC-α and PKC-δ mRNA expression in the MCF-7 cell line compared to the TAM-R cell line.

As we have established that the protein levels of PKC-α and PKC-δ are elevated in the TAM-R cells compared to the MCF-7 cells, we carried out RT-PCR analysis (section 2.5) on total RNA extracted from both cell lines to determine if the increase in protein is attributable to a concomitant increase in mRNA expression. Primers for PKC-δ were obtained from a published sequence (Assender *et al.*, 1994) and produced un-saturated bands after 30 cycles (Figure 2.2). Primers for PKC-α were designed using the Primer3 software package and also produced un-saturated bands after 30 cycles (Figure 2.3). These primers were used to measure the mRNA expression of PKC-α and δ in 3 individual sets of MCF-7 and TAM-R total RNA. Additionally primers for β-actin were used as a control (Figure 3.7). In each of the 3 sets there is an increase in the expression of mRNA for both isoforms in the TAM-R cells with the level of PKC-α mRNA nearly 3 fold higher and the level of PKC-δ over

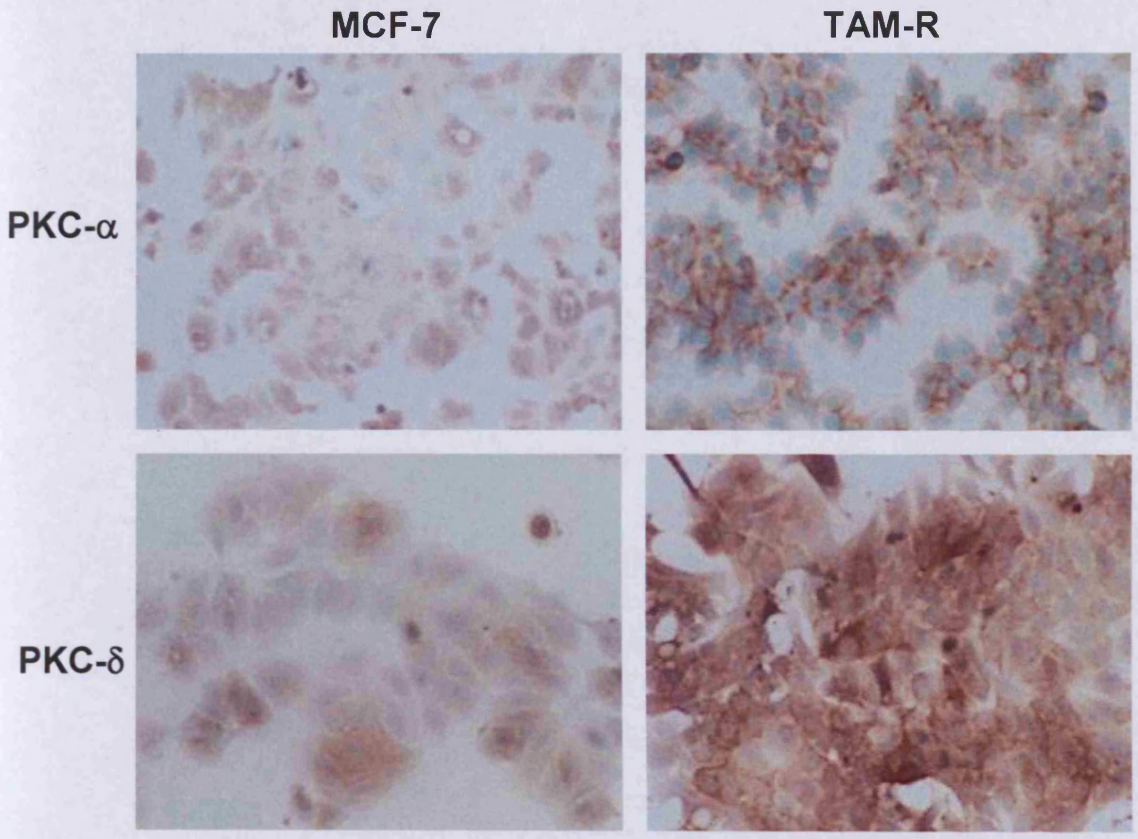
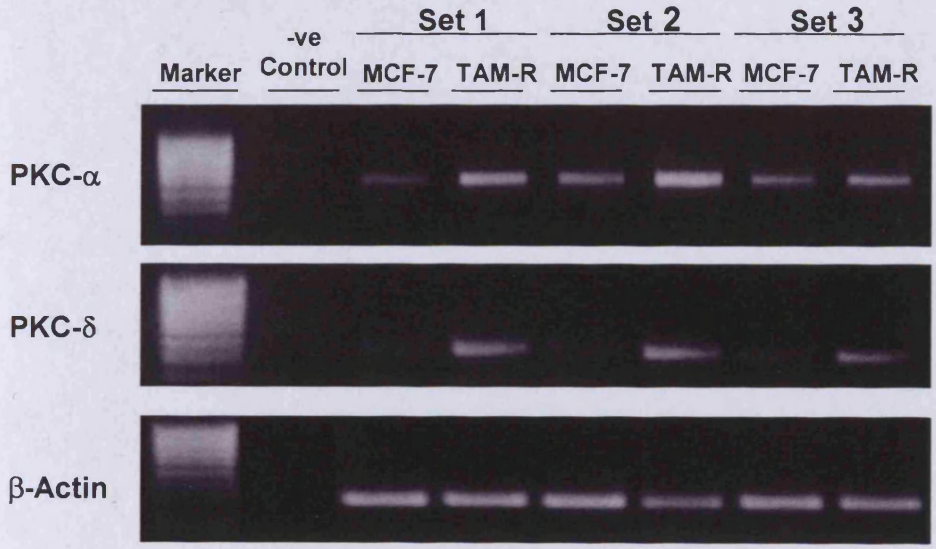


Figure 3.6. Immunocytochemical staining of PKC-α and PKC-δ levels in the MCF-7 and TAM-R cell lines.

MCF-7 and TAM-R cells were grown on coverslips in media containing 5% cs FCS to a confluency of 60-70%. The cells were then fixed and stained using antibodies specific to either PKC-α or PKC-δ as outlined in section 2.x (Materials and Methods) and then photographed x 400 magnification.

Figure 3.7. Comparative levels of PKC-α and PKC-δ mRNA expression in the MCF-7 and TAM-R cell line

MCF-7 and TAM-R cells were grown in media contain 5% charcoal stripped FCS to a confluency of around 70%. cDNA was prepared from the total cellular RNA of three separate sets of cells and subjected to RT-PCR using PKC-α, PKC-δ and β–Actin specific primers as outlined in section 2.5. The amplification products were then size fractionated on a 2% (w/v) agarose gel. The graphs represent the mRNA levels of PKC-α and –δ in the TAM-R cells expressed as a fold increase of levels expressed in the MCF-7 cell line relative to the β-Actin control ± SD from 3 independent experiments. The data was analysed by paired t-test (TAM-R PKC-α expression cf. MCF-7= 2.9 fold increase ± 1.33, p= 0.128; TAM-R PKC-δ expression cf. MCF-7= 5.6 fold increase ± 0.54, *p=0.01).


Set 1
Set 2
Set 3
Marker
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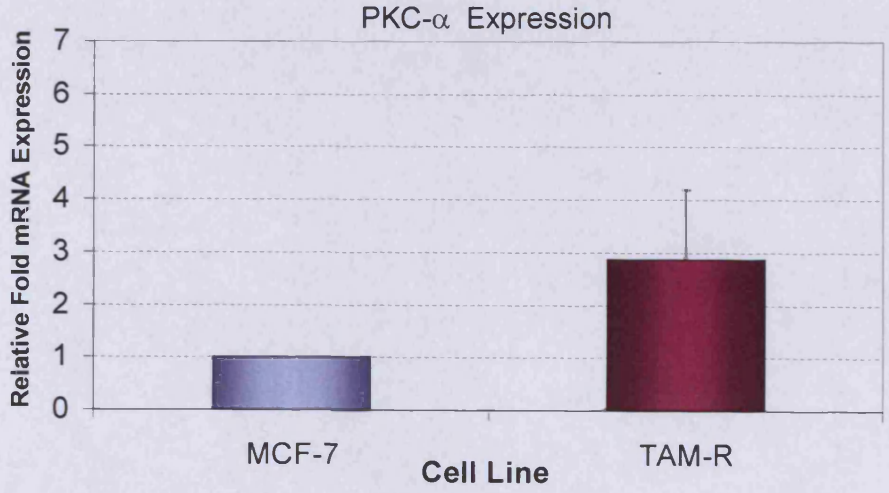
MCF-7
TAM-R
MCF-7
TAM-R
MCF-7
TAM-R
PKC-α
PKC-δ
β-Actin


PKC-α Expression
Relative Fold mRNA Expression
0
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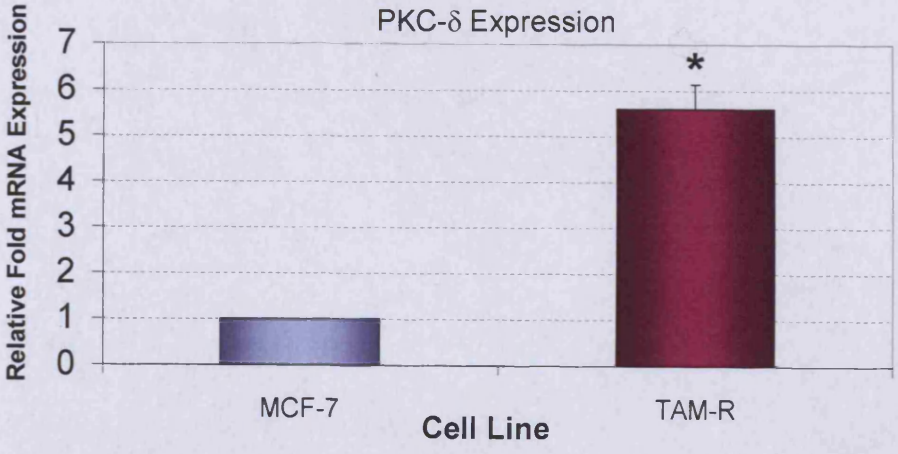
MCF-7
TAM-R
Cell Line


PKC-δ Expression
Relative Fold mRNA Expression
0
1
2
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7
*
MCF-7
TAM-R
Cell Line

5 fold higher compared to the MCF-7 cells. Whilst these increases don't mirror those seen at the protein level it does indicate a role for increased expression of both isoforms at the gene level in the increases observed at protein level, though there may be additional posttranslational factors that ultimately determine the endogenous protein levels of these PKC isoforms.

3.2.4. Determination of relative activation of PKC-α and PKC-δ in the MCF-7 cell line compared to the TAM-R cell line.

Western analysis was performed using phosphorylation site specific antibodies for residues that are associated with a catalytically competent and active enzyme to determine if the increased levels of PKC-α and δ within the TAM-R cells confers a greater level of isoform activity within the cell. Additionally, in each case the membranes were probed with β-actin as a control of protein concentration and loading accuracy. To measure activation of the PKC-α isoform, an antibody specific for a phosphorylated serine 657 residue on the hydrophobic C-terminal V5 sub domain domain was used. Phosphorylation of this residue has been shown to be crucial for the accumulation of phosphate at other sites on the enzyme and contributes to the maintenance of a phosphatase resistant phenotype (Bornancin and Parker, 1997). When compared to the MCF-7 cells, the TAM-R cells displayed a significant mean increase of over 4 fold the level of PKC-α phosphorylated at the serine 657 residue (Figure 3.8). This is indicative of a greater level of catalytically competent enzyme and therefore a greater level PKC-α activity.

To detect activation of PKC-δ two separate antibodies were used that are specific for different regions on the isoform. The first antibody was directed at a phosphorylated threonine 505 residue that resides in the activation loop of the catalytic domain of PKC-δ. Although PKC-δ threonine 505 phosphorylation has been shown not to be a prerequisite requirement for catalytic competency (Stempka *et al.*, 1997) phosphorylation at the activation loop is known to be crucial for full catalytic activity and membrane associated allosterically activated PKC-δ is increased by phosphorylation at this site (Steinberg, 2004). The second phosphorylated residue

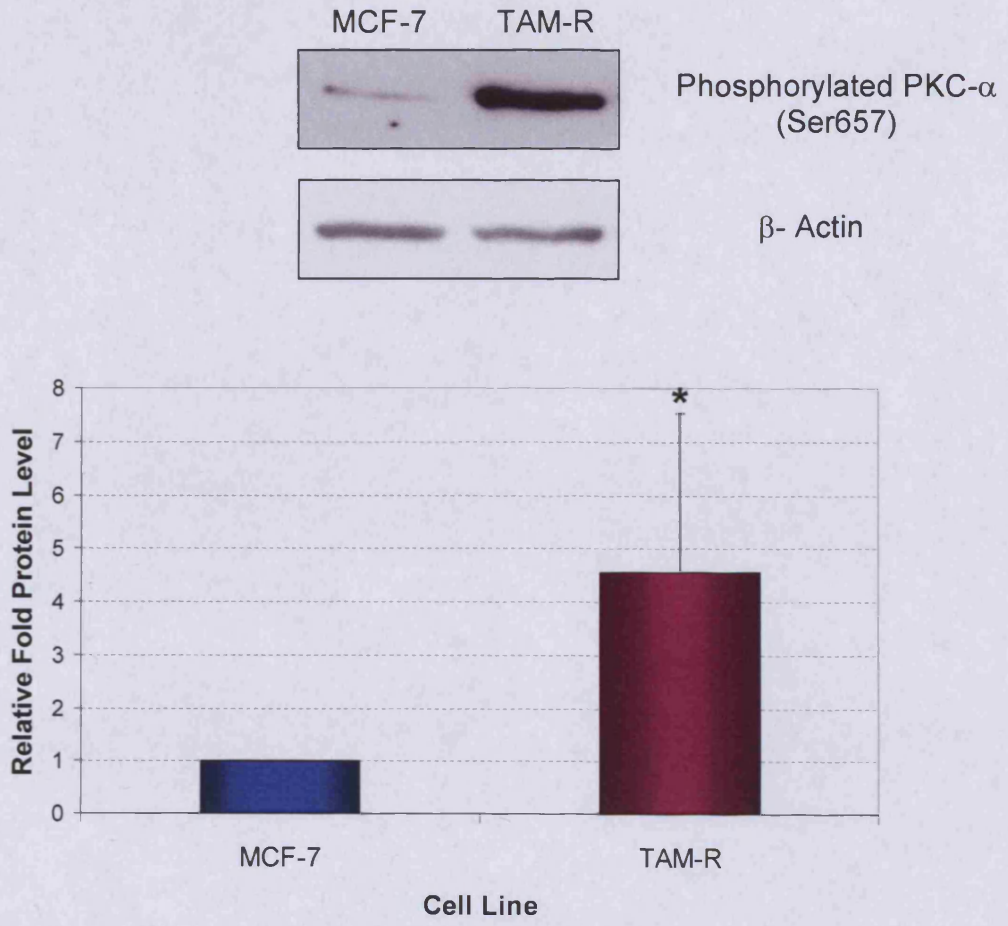


Figure 3.8. Relative expression of phosphorylated PKC-α (Serine657) between the MCF-7 breast cancer cell line and the Tamoxifen resistant (TAM-R) cell line

Western analysis was carried out using whole cell extracts taken from MCF-7 and Tamoxifen resistant cells grown to 80 % confluency in media containing 5% charcoal stripped sFCS. The blotted membranes were probed with primary antibody specific for PKC-α phosphorylated at the serine 657 residue. In addition, the membranes were probed with a β-actin specific antibody as a loading and protein concentration control. The graph shown illustrates the mean fold difference expressed as the fold increase in protein level detected in the TAM-R cells compared to the MCF-7 cells ± SD from 6 independent experiments. The data was analysed by paired t-test (*p=0.04).

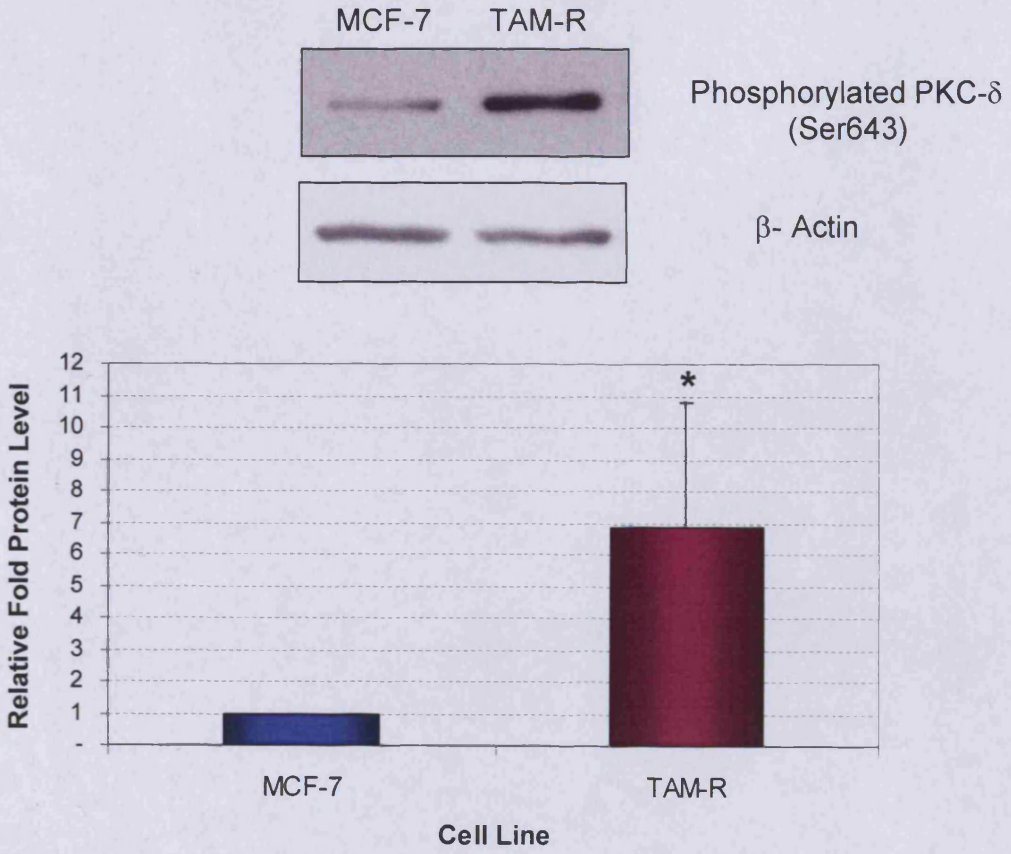


Figure 3.9. Relative expression of phosphorylated PKC-δ (Ser643) between the MCF-7 breast cancer cell line and the Tamoxifen resistant (TAM-R) cell line

Western analysis was carried out using whole cell extracts taken from MCF-7 and Tamoxifen resistant cells grown to 80 % confluency in media containing 5% charcoal stripped sFCS. The blotted membranes were probed with primary antibody specific for PKC-δ phosphorylated at the serine 643 residue. In addition, the membranes were probed with a β-actin specific antibody as a loading and protein concentration control. The graph shown illustrates the mean fold difference expressed as the fold increase in protein level detected in the TAM-R cells compared to the MCF-7 cells ± SD from 5 independent experiments. The data was analysed by paired t-test (*p=0.02).

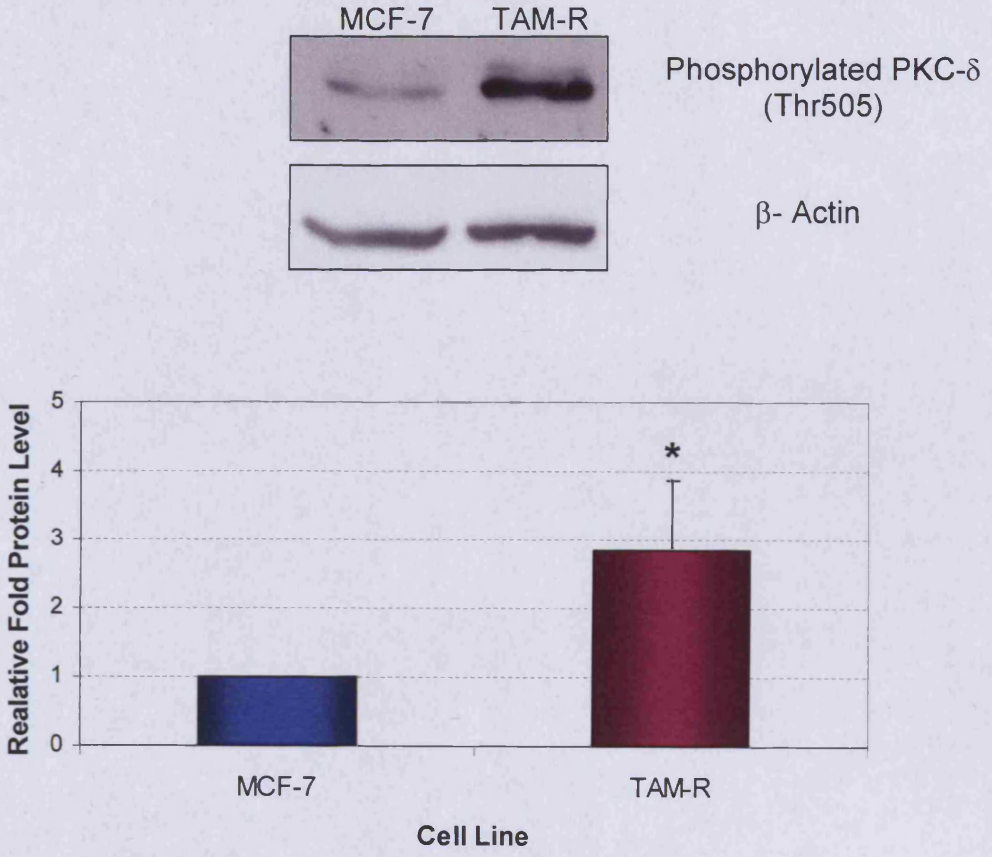


Figure 3.10. Relative expression of phosphorylated PKC-δ (Thr505) between the MCF-7 breast cancer cell line and the Tamoxifen resistant (TAM-R) cell line

Western analysis was carried out using whole cell extracts taken from MCF-7 and Tamoxifen resistant cells grown to 80 % confluency in media containing 5% charcoal stripped sFCS. The blotted membranes were probed with primary antibody specific for PKC-δ phosphorylated at the threonine 505 residue. In addition, the membranes were probed with a β-actin specific antibody as a loading and protein concentration control. The graph shown illustrates the mean fold difference expressed as the fold increase in protein level detected in the TAM-R cells compared to the MCF-7 cells ± SD from 5 independent experiments. The data was analysed by paired t-test (*p=0.04).

targeted the serine 643 residue which has been identified as an autophosphorylation site *in vitro* and on serine 643 containing PKC-δ peptides *in vivo* (Stempka *et al.*,1999). The importance of phosphorylation at this serine residue has been shown by the reduction of PKC-δ activity when it is substitutionally mutated (Li *et al*, 1997). The phosphorylated threonine 505 specific antibody showed a significant mean increase of nearly 4 fold in the TAM-R cell line cf. MCF-7 cells (Figure 3.9). This observed increase was corroborated by the phosphorylation specific serine 643 antibody. PKC-δ phosphorylation at this site displayed nearly a 7 fold mean increase in the TAM-R cell line compared to MCF-7 cells (Figure 3.10). Therefore, the TAM-R cell line shows increased levels of PKC-δ phosphorylation at both the threonine 505 and serine 643 residue indicating that there is an increased level of overall PKC-δ activation. As both phosphorylation specific PKC-δ antibodies detected comparable levels of activation across the cell lines the use of the threonine 643 was discontinued and the threonine 505 antibody used where required for all subsequent PKC-δ activation experiments. As there was little to choose between the two antibodies this decision was made on the grounds that it was in more common usage both within my group and published articles.

3.2.5. Effect of bisindolylmaleimide IX (RO31-8220) on growth in the MCF-7 and TAM-R cell line.

As described in section 3.1, bisindolylmaleimide IX (bis) is a PKC specific inhibitor with a reported slight selectivity for PKC-α (Wilkinson *et al.*, 1993). To verify that bis would be an effective inhibitor of PKC-α and/or PKC-δ in our cell models, MCF-7 and TAM-R cells were treated with 500nM bis for 15 minute, 1 hour, 6 hour, 24 hour and 48 hour time periods with untreated controls at each time point. The cells were then harvested and assayed for PKC-α and PKC-δ levels by Western analysis (section 2.3) using isoform specific antibodies. The bis concentration of 500nM utilised in these experiments was derived from growth studies of MCF-7 cells treated with bis at concentrations of 100nM, 500nM and 1μM. At 500nM, MCF-7 cell growth was inhibited by 77% after 14 days (control 406,390 ± 48,532 cf. bis 92,280 ± 7,974, n=3, p=<0.02; Assender *et al.*, unpublished data). This concentration was not cytotoxic as when the cells were replated in fresh bis free media they resumed normal

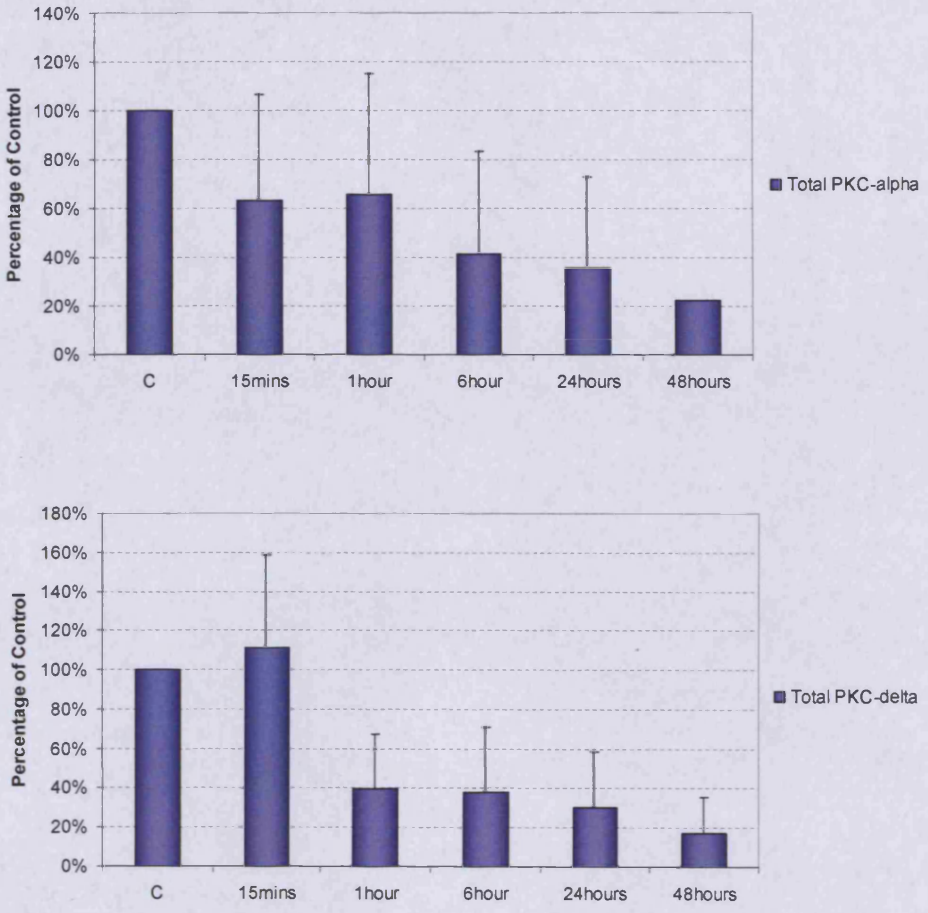


Figure 3.11. The effect of bisindolylmaleimide (RO31-8220) on PKC-α and PKC-δ levels in the MCF-7 cell line

MCF-7 cells were grown in media containing 5% S-FCS ± 500nM of bisindolylmaleimide for 15 minutes, 1 hour, 6 hours, 24 hours or 48 hours. Levels of PKC-α and PKC-δ were measured by Western analysis and expressed as a percentage of control levels at each of the time points. Results above are derived from three independent experiments ± SD.

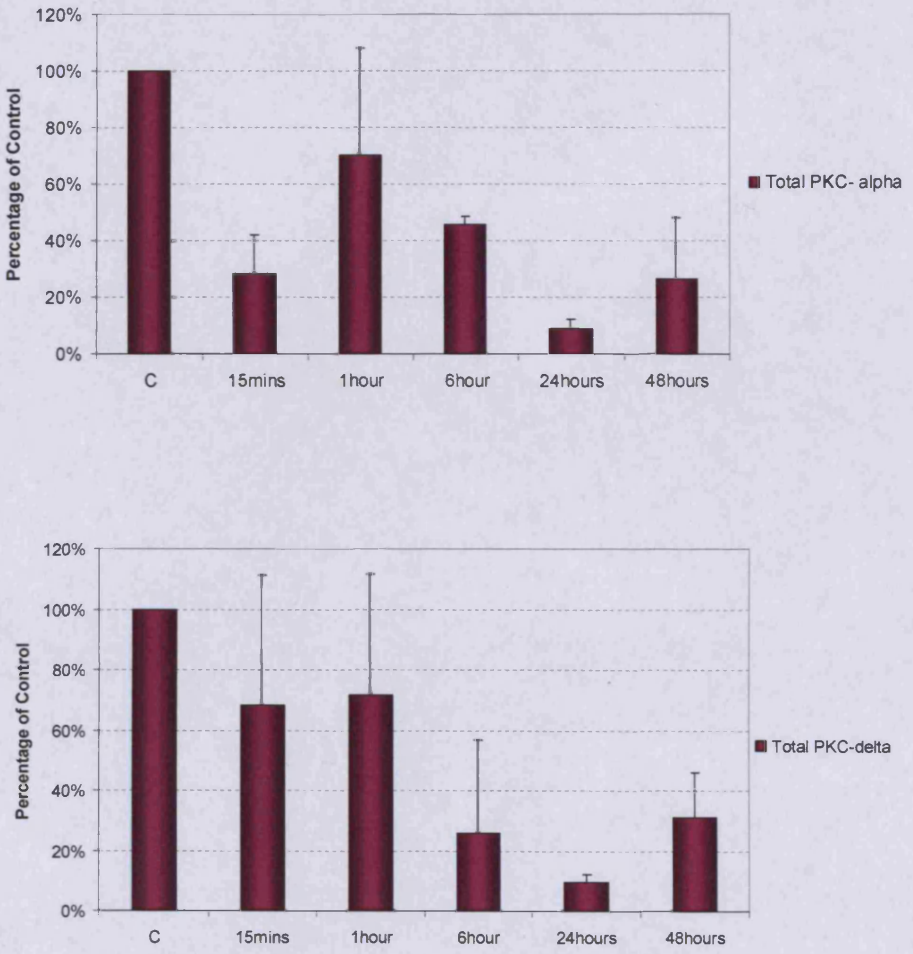


Figure 3.12. The effect of bisindolylmaleimide (RO31-8220) on PKC-α and PKC-δ levels in the TAM-R cell line

TAM-R cells were either untreated (control) or exposed to 500nM of bisindolylmaleimide for 15 minutes, 1 hour, 6 hours, 24 hours or 48 hours. Levels of PKC-α and PKC-δ were measured by Western analysis and expressed as a percentage of control levels. Results above are derived from three independent experiments ± SD.

growth. The concentrations of 0.1μM had little effect whilst 1μM completely inhibited growth.

The relative levels of PKC-α and PKC-δ in the bis treated MCF-7 and TAM-R cell lines are expressed graphically as a percentage of the control values ± SD (Figure 3.11). In the MCF-7 cell line, bis displayed variable PKC-α inhibition up to 24 hours but consistently produced around 80% inhibition after 48 hours. Whilst the inhibition of PKC-δ in the MCF-7 cells showed less variation, it too was inhibited around 80% after 48 hours. In the TAM-R cell line (Figure 3.12) both isoforms were nearly 90% inhibited after 24 hours. Interestingly, there was a slight decrease in the level of inhibition of both isoforms after 48 hours, to around 20-30% of control. However this was still a marked decrease compared to control and comparable to the inhibition seen after 24 hours in the MCF-7 cell line.

As we have shown that Bis can inhibit both PKC-α and δ it was used to assess the importance of these isoforms on cell growth in the MCF-7 and TAM-R cells. Both cell lines were grown in parallel over a 6 day period in media containing 5% csFCS with 500nM Bis or without Bis as a control. The cell numbers of both the cell lines ± 500nM Bis were counted by Coulter counting (section 2.2.6) at days 0, 4 and 6. The growth of the bis treated cells was then expressed graphically as the percentage cell number relative to untreated controls from 3 separate experiments ± SD (Figure 3.13). After 4 days growth in the presence of 500nM Bis there was significant growth inhibition of both cell lines. However, whilst MCF-7 cell growth was only inhibited by around 17% (±8) compared to control, the TAM-R cell line was inhibited by around 62% (±14). This divergence of growth inhibition is continued at day 6 where the mean cell number of Bis treated MCF-7 cells was 37% (±14) less than control whilst the bis treated TAM-R cells were inhibited by 71% (±8). These results indicate that although both cell lines are sensitive to the growth inhibitory effects of Bis, the TAM-R cells display a much greater sensitivity. However, as Bis is a relatively non-specific inhibitor we cannot determine for sure that it is the compounds effects on PKC-α and δ that are causative of the growth inhibition.

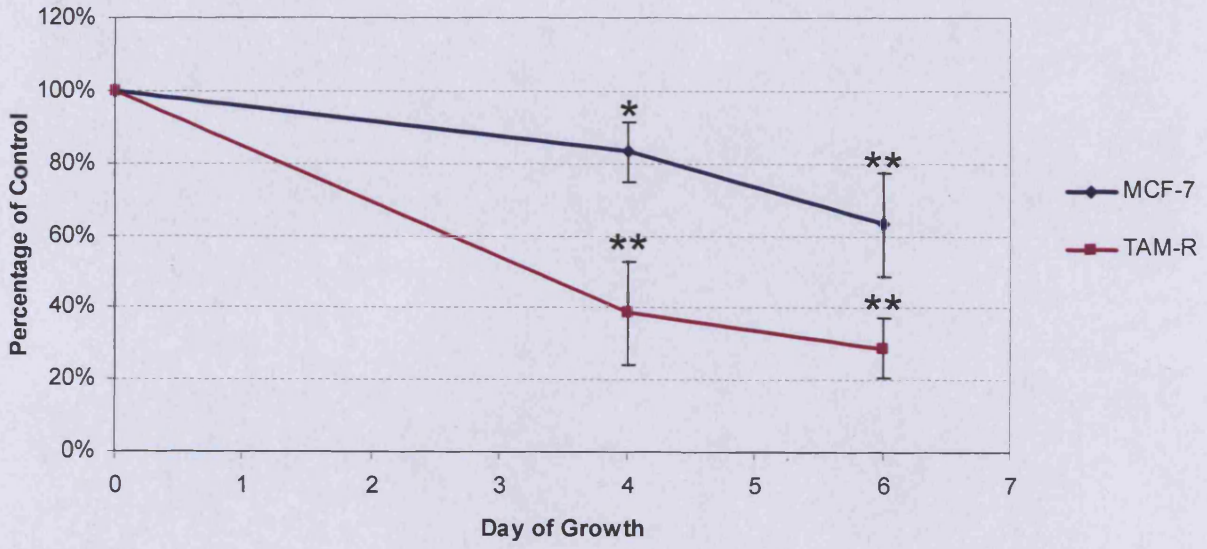


Figure 3.13. Effect of the PKC inhibitor Bisindolylmaleimide IX (RO31-8220) on the growth of the MCF-7 and TAM-R cell lines

MCF-7 and TAM-R cells were grown in media containing 5% charcoal stripped FCS ± 500nM of the PKC inhibitor Bisindolylmaleimide IX (RO31-8220) (bis). The cell number was then counted using a Coulter Counter at days 0, 4 and 6. The graph represents the cell count of the bis treated cells at each of the time points expressed as a percentage of the untreated cell number from 3 separate experiments ± SD. The data was analysed by paired t-test (*$p<0.05$; **$p<0.005$).

3.2.6. Effect of the isoform specific inhibitor Rottlerin on PKC-α and δ in the MCF-7 and TAM-R cell line.

As the retardation of growth caused by bis treatment of the MCF-7 and TAM-R cell lines could be attributed to its inhibitory effects on multiple PKC isoforms and other unrelated intracellular kinases, a more specific inhibitor was needed to isolate the individual PKC isoforms to study their relative importance in these cell models. The compound rottlerin has been reported to selectively inhibit PKC-δ at a concentration of 3-6μM, around 10 times lower than the concentration needed to inhibit any of the other PKC isoforms (Gschwendt *et al*, 1994). Therefore to confirm that rottlerin does selectively inhibit PKC-δ in this concentration range, the MCF-7 and TAM-R cells were grown to 50% confluency in medium containing 5% csFCS and treated with rottlerin at a concentration of 5μM for 15minute, 1 hour, 6 hour, 24 hour and 48 hour durations. Untreated control cells were grown in parallel with the rottlerin treated cells and harvested with them at each of the time points. The total levels of PKC-α and PKC-δ in the rottlerin treated samples and controls were measured by Western analysis using isoform specific antibodies. Unfortunately the MCF-7 cells treated with rottlerin for 48 hours detached from the plate prior to harvesting whilst the control plates at 48 hours remained healthy and attached. Therefore the effect of MCF-7 treatment with rottlerin for 48 hours could not be assessed. Each of the blotted membranes were also probed for β-actin as a control of protein concentration and loading accuracy. After 24 hours treatment with rottlerin, the MCF-7 cells did display some inhibition of PKC-δ (Figure 3.14). However at this time point there was also evidence of inhibition of the PKC-α isoform compared to the untreated control cells. Therefore, at a concentration stated in the literature to achieve selective inhibition of PKC-δ there is also inhibition of PKC-α after 24 hours in the MCF-7 cells.

As with the MCF-7 cells, the rottlerin treated TAM-R samples were run in parallel with untreated time point controls and levels of total PKC-α and δ measured by Western analysis along with β-actin as a protein concentration and loading control (Figure 3.15). Unlike the MCF-7 cells, the TAM-R cells displayed no appreciable reduction of either PKC-α or δ levels after 24 hours rottlerin treatment, though there

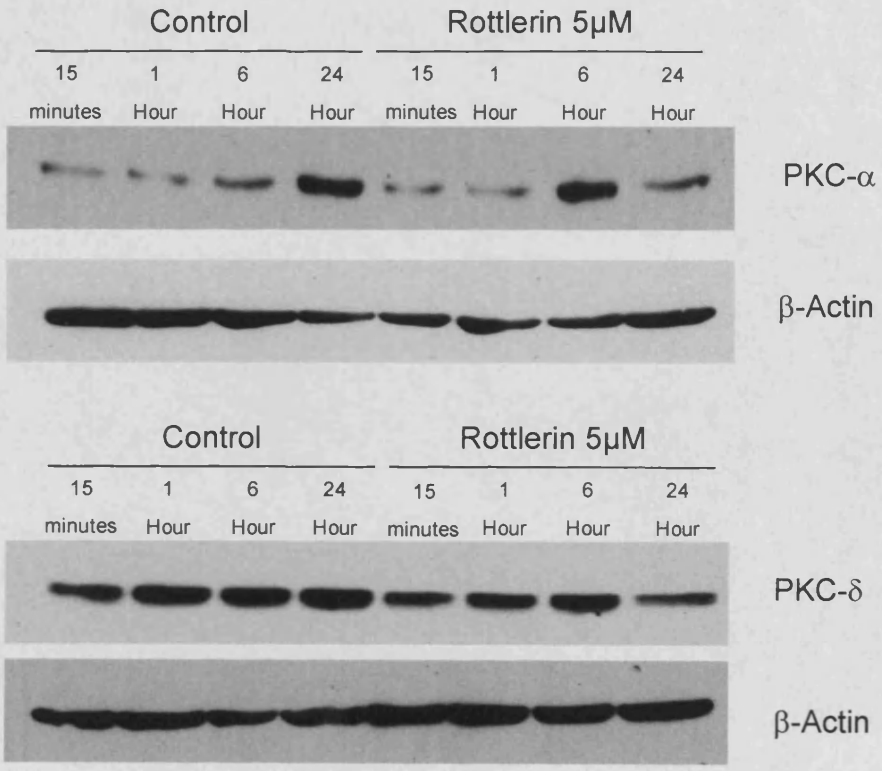


Figure 3.14. The effect of rottlerin (5μM) on the total levels of PKC-α and PKC-δ in the MCF-7 cell line

Western analysis was carried out using whole cell extracts taken from MCF-7 cells treated ± 5μM rottlerin for 15 minute, 1 hour, 6 hour 24 hour and 48 hour time points. The cells treated with rottlerin for 48 hours detached prior to harvesting and therefore could no be assessed. The blotted membranes were probed with primary antibodies specific for PKC-α or PKC-δ. In addition, the membranes were probed with a β-actin specific antibody as a control of protein concentration and loading accuracy.

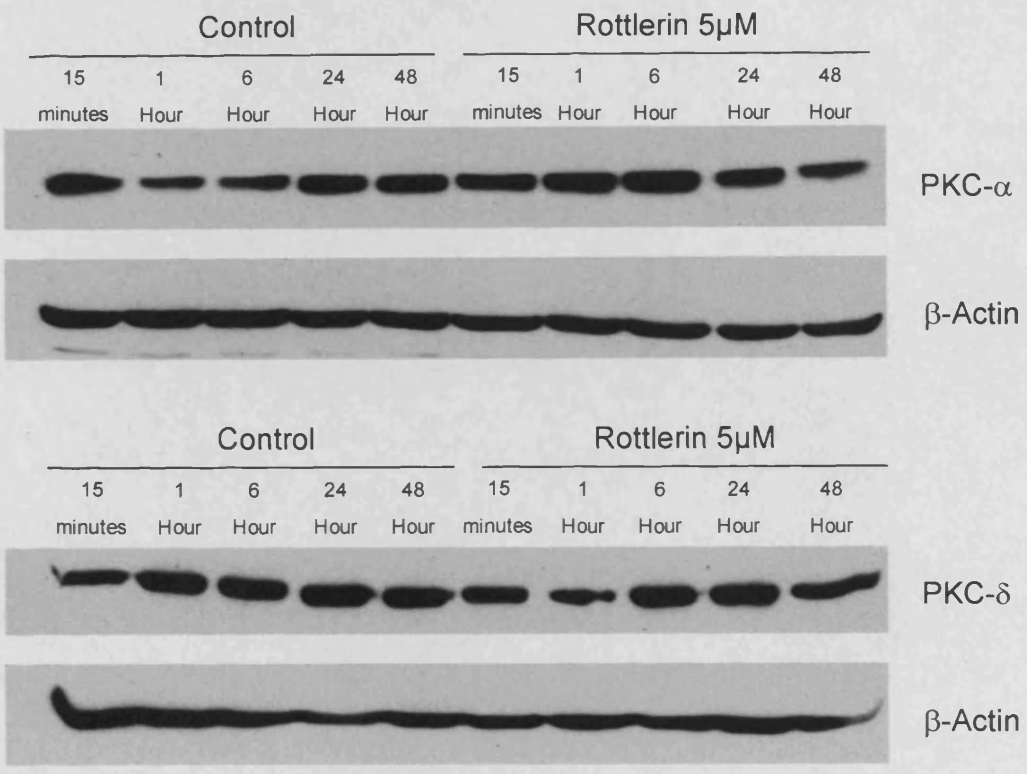


Figure 3.15. The effect of rottlerin (5μM) on the total levels of PKC-α and PKC-δ in the TAM-R cell line

Western analysis was carried out using whole cell extracts taken from MCF-7 cells treated ± 5μM rottlerin for 15 minute, 1 hour, 6 hour 24 hour and 48 hour time points. The blotted membranes were probed with primary antibodies specific for PKC-α or PKC-δ. In addition, the membranes were probed with a β-actin specific antibody as a control of protein concentration and loading accuracy.

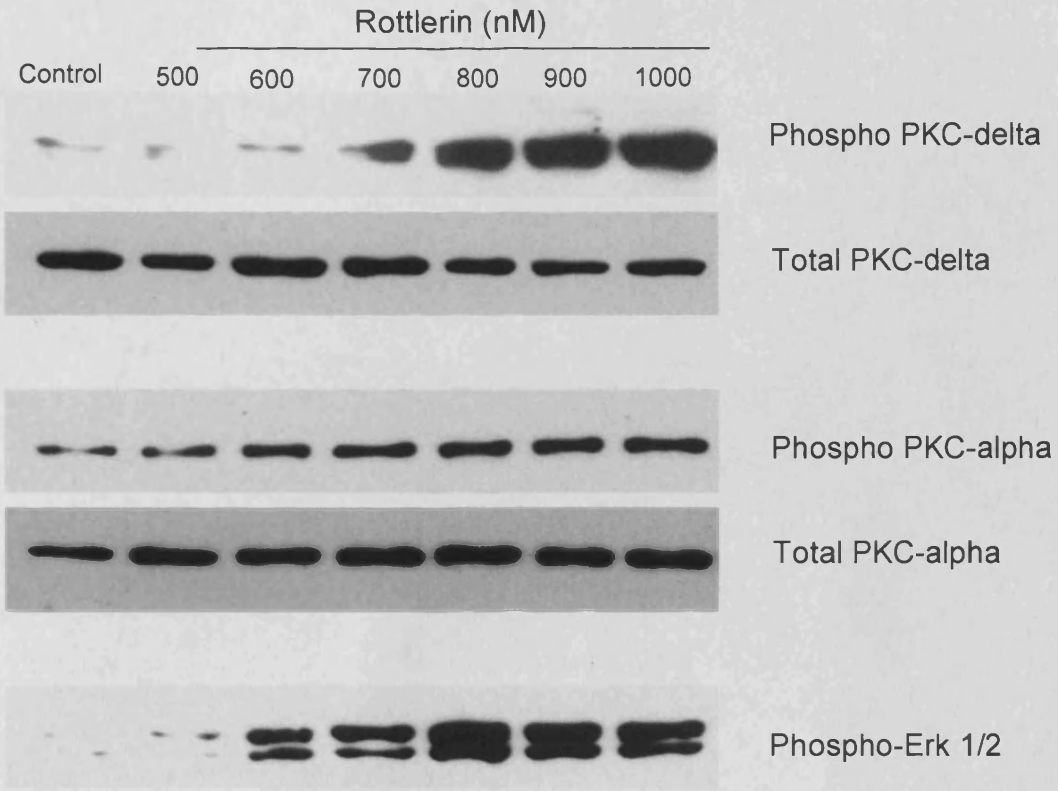


Figure 3.16. Dose response of rottlerin on levels of PKC-α and δ activation and expression after 1 hour treatment in the MCF-7 cell line

Western analysis was carried out using whole cell extracts taken from MCF-7 cells treated with rottlerin for 1 hour at concentrations ranging from 500nM to 1μM. The blotted membranes were probed with primary antibodies specific for total and phosphorylated levels of PKC-α or PKC-δ and phosphorylated levels of ERK 1/2. In addition, the membranes were probed with a β-actin specific antibody as a control of protein concentration and loading accuracy.

was evidence of a slight reduction in PKC-α after 48 hours. Therefore in our hands rottlerin displayed no effect, at the reportedly efficacious concentration of 5μM, on the level of PKC-δ after 48 hours in the TAM-R cells and, although it did reduce both PKC-δ and PKC-α levels in the MCF-7 cells after 24 hours very slightly, it also caused detachment in the MCF-7 cells after 48 hour treatment indicating that it wouldn't be suitable for long term experiments such as growth curves.

To determine if a lower concentration of rottlerin could confer selective inhibition of the PKC-δ isoform in the MCF-7 cells they were treated for 1 hour with rottlerin at concentrations ranging from 500nM-1μM (Figure 3.16) and the total and phosphorylated levels of PKC-α and δ measured by Western analysis using isoform and phosphorylation state specific antibodies. Additionally, the blotted membranes were probed for the phosphorylated form of the prominent kinase ERK 1/2 to assess the kinase specificity of the rottlerin inhibitor. Whilst at these concentrations the rottlerin had no effect on the PKC-α isoform or the total levels of PKC-α and δ it did have the surprising effect of causing a very large dose dependent increase in the level of the phosphorylated PKC-δ isoform. This dose dependent increase in PKC-δ activation was mirrored by the activation of ERK 1/2. Whilst it's possible that the increase in activated PKC-δ is causing downstream activation of the ERK 1/2 (Ueda *et al.*, 1996) the increase in activation of ERK 1/2 is evident at 500nM and markedly increased at 600nM, concentrations where there is no indication that there is an increase in PKC-δ activation.

As rottlerin failed to selectively inhibit PKC-δ over PKC-α in the MCF-7 cell line and failed to reduce the levels of either in the TAM-R cell line over 48 hours when used at the reportedly efficacious concentration of 5μM it was rejected as a possible tool in further studies. It may also be a cause for concern that when used for 1 hour at concentrations ranging from 500nM to 1μM the rottlerin displayed a dose dependent of increase in the levels of phosphorylated PKC-δ and ERK 1/2 which is a kinase that is almost ubiquitously involved in cell signalling pathways in normal and malignant cells.

3.3. DISCUSSION

Whilst PKC expression levels have been extensively studied in ER+ and ER- cell lines (e.g. Morse-Gaudio *et al.*, 1998; Shanmugan *et al.*, 2001), good models of acquired endocrine resistance have been lacking until recently. Having established that we can reproduce the results of other groups using ER+ and ER- cell line models, we therefore decided to study the acquired Tamoxifen resistance phenotype. It may have been expected that the PKC-α and δ expression profile of the TAM-R cells would mirror those of MB-MDA-231 cells as they are both utilising pathways that circumvent the antagonistic effects of Tamoxifen. However from Western analysis and immunocytochemical staining it can be seen that the expression levels of both the PKC-α and PKC-δ isoforms are significantly and consistently higher in the Tamoxifen resistant cell line compared to the MCF-7 cell line. This increase in PKC-α and δ levels has also been recently observed by Nabha *et al.* (2005) in two further anti-oestrogenic resistant cell lines; an ErbB2/ HER2 overexpressing MCF-7 cell line and a Faslodex resistant MCF-7 cell line, in addition to their Tamoxifen resistant MCF-7 cell line. The observed increase in both PKC-α and PKC-δ expression appears to be due in part to the increase in mRNA expression observed though alterations in turnover of the endogenous protein or stabilisation could be a factor (Figure 3.7). Interestingly our parallel studies on clinical samples demonstrate that the PKC-α+/δ+ phenotype is associated with poor prognosis both in terms of survival and duration of endocrine response (Assender *et al.* (2005) in prep). Thus our cell line model of acquired endocrine resistance does in fact appear to reflect the clinical scenario well.

The increases in PKC-α and δ expression between the MCF-7 and the TAM-R cell lines are paralleled by concomitant increases in the levels of the phosphorylated isoforms. The increase in PKC-α phosphorylated at the serine 657 residue and PKC-δ phosphorylated at both the threonine 505 and serine 643 residues indicate that the TAM-R cell line also has a greater level of activated PKC-α and δ compared to the MCF-7 cell line.

As these increased levels of PKC could be an artefact of across the board upregulation of growth factor mediated molecules we wished to determine whether the increases in

the total and activated PKC expression parallels a greater reliance and employment of the isoforms in the TAM-R cells. A growth study was therefore performed to observe how inhibiting the isoforms would effect the growth of the cells compared to the MCF-7 cell line. The inhibitor bisindolylmaleimide IX (bis) was shown to cause a comparable reduction in both PKC-α and δ activity in both cell lines at a concentration of 500nM after 24-48 hours and was therefore determined to be an appropriate compound for this experiment. Whilst treating both the cells lines with bis caused a significant retardation of growth compared to analogous untreated control cells, the effect on the TAM-R cells was far greater than in the MCF-7 cell line. This implies that the TAM-R cells rely far more on PKC-α and/or PKC-δ for growth than in the MCF-7 cell line.

Our experiments demonstrated a lack of specificity of bis towards the PKC-α and δ, as have many others, and Bis is known to also lack specificity between PKC-α and the βI/II, γ, and ε isoforms (Way *et al.*, 2000). Additionally it has been shown to be a *in vitro* inhibitor of several unrelated protein kinases including glycogen synthase kinase-3 (GSK-3) (Hers *et al.*, 1999), MAPK activating protein kinase-1β (Rsk-1), and p70S6 kinase with a similar potency to that of the PKC isoforms (Alessi, 1997). It has even been shown to stimulate the expression of c-Jun and the activation of Jun N-terminal kinase (JNK) independently of its effects on PKC (Beltman *et al.*, 1996). Consequently any effects observed following treatment of the model cell lines with bis may not even be wholly attributable to the isolated inhibition of the PKC-α and δ isoforms, or even the pan inhibition of PKC, but rather the varying degrees of inhibition imposed on several functional kinases.

Therefore to specifically target individual PKC isoforms an alternative inhibitor had to be sought. The compound rottlerin has been utilised in many studies as an inhibitor with a reported specificity for PKC-δ (IC_{50} value in the range of 3-6μM)(Gschwendt *et al.*, 1994). To verify this efficacy we treated the MCF-7 and the TAM-R cells at time points up to 48 hours with 5μM rottlerin and measured the effect on PKC-α and δ levels by Western analysis. Growing cells in rottlerin for 48 hours however appeared to be cytotoxic suggesting that rottlerin would be an unsuitable compound to use in long term time courses and experiments such as growth studies. Although 24

hour treatment with rottlerin did appear to slightly decrease PKC-δ levels in MCF-7 cells, it produced a comparable decrease in PKC-α levels. This rottlerin induced reduction of PKC-α levels was also observed after 48 hours in the TAM-R cell line but there was no concomitant effect on PKC-δ. Furthermore whilst lower concentrations of rottlerin were without effect on PKC-α they had the surprising effect of causing a dose dependent activation of PKC-δ and, even more worryingly, ERK 1/2, a kinase implicated in almost all cellular pathways. Although there are reports that ERK 1/2 can be activated downstream of PKC-δ (Ueda *et al.*, 1996), the ERK 1/2 activation was evident at concentrations below the threshold where PKC-δ activation was observed. These observations make us doubt the suitability of rottlerin as a PKC inhibitor. Interestingly, general concern surrounding the use of rottlerin as a PKC-δ specific inhibitor have been echoed in several recent publications. Whilst it was known from the first reports of its PKC-δ inhibitory activity that it also a weak inhibitor of calmodulin dependent kinase (CAM kinase III), protein kinase A (PKA) and casein kinase II (Gschwendt *et al.*, 1994) it has since been discovered to be a potent inhibitor of other protein kinases such as PRAK (IC50=1.9μM) and MAPKAP-K2 (IC_{50}=5.4μM) (Soltoff, 2001). Whereas this cross reactivity could be explained by common folding motifs or the conserved structure of the kinase domains across these enzymes, it has also been shown that rottlerin can inhibit the activity of enzymes unrelated to the kinase family. The enzymes β-lactamase, chymotrypsin and malate dehydrogenase share no obvious similarity with kinases or each other yet are all inhibited by rottlerin at IC_{50} values lower than those stated for PKC-δ (IC_{50}= 1.2μM, 2.5μM and 0.7μM respectively) (McGovern and Shoichet, 2003). These non-specific effects are coupled with the fact that rottlerin was a very poor inhibitor of PKC-δ and α in both the MCF-7 and TAM-R cell lines at 5μM. This lack of efficacy has also been reported by Davies and colleagues who were unable to elicit an inhibitory response of either isoform with rottlerin at a concentration of 20μM (Davies *et al.*, 2000). This discrepancy between rottlerins previously reported effects and our observations are probably due to the method of assay. The original studies on rottlerin were carried out on isolated PKC-δ using an in vitro system. Our studies are however done in whole cells grown in the compound. Other studies agree that rottlerin is a poor inhibitor of PKC-δ within whole cell extracts, perhaps due to difficulty crossing

the cell membrane or being metabolised within in the cell. It has even been suggested that PKC-δ can actually be activated by rottlerin under some conditions (Soltoff, 2001). Our studies concur with this, showing a dose dependent activation of PKC-δ in MCF-7 cells treated with rottlerin at concentrations between 500nM and 1μM. It has now been reported that rottlerin's inhibition of PKC-δ comes indirectly through the reduction of intracellular ATP by the direct uncoupling of mitochondria (Soltoff, 2001). Even with this evidence, the growing wave of literature questioning the usefulness of rottlerin, and the fact that some suppliers are discontinuing its sale as a PKC inhibitor (www.lclabs.com) has not prevented the continued use of rottlerin to supposedly demonstrate the involvement of PKC-δ in signalling pathways (e.g. Zhang *et al.*, 2005; De Servi *et al.*, 2005; Nabha *et al.*, 2005). However, our observations and the mounting wealth of literature discrediting its use, make us conclude that the only sensible way to elucidate the role of the individual PKC isoforms in the acquisition and maintenance of the tamoxifen resistant phenotype will be through the employment of molecular biology techniques. To this end we decided to investigate two possible molecular approaches to ablate PKC isoform function. These are the deletion of specific PKC isoform expression by the technique of RNA interference and the use of adenovirally delivered, kinase deficient PKC dominant negatives.

CHAPTER 4.

STUDY INTO PKC-α AND PKC-δ DOWNREGULATION BY RNA INTERFERENCE

4.1. INTRODUCTION

One of the most effective ways of elucidating the functional properties of a component in a biological system is to ablate it or suppress its function. The problems inherent with such an endeavor include not only efficacy but also specificity. These criteria may yet be answered by a technique that is being hailed by the scientific community almost as much as it's being marketed by the scientific companies, RNA interference. The term RNA interference was coined following a serendipitous discovery that the injection of both sense and anti-sense strands of RNA into the nematode *Caernohabditis elegans* gave a roughly tenfold greater effect on gene expression silencing than the application of either of the strands alone (Fire *et al.*, 1998). However, this was probably not the first time that the effect had been documented. It now appears that RNAi was first discovered under the moniker of post-transcriptional gene silencing (PTGS) during experiments to deepen the purple colour of petunias by the introduction of transgenes (Hamilton and Baulcombe, 1999). It was observed that when the introduced transgene shared sequence homology with the endogenous gene in the petunia then it caused the inhibition of that gene's expression. Similar phenomena have since been observed in invertebrates such as *C. elegans* and *Drosophila melanogaster*, in plants such as *Arabidopsis thaliana* and the fungi like *Neurospora Crassa* (Hutvagner and Zamore, 2002).

RNAi post-transcriptionally silences gene expression in an ATP dependent manner. When a double stranded RNA is introduced into a cell it is processed, independently of it's target mRNA, to dsRNA segments of between 21 and 23 nucleotides in length by an RNase-III-like dsRNA-specific ribonuclease called Dicer (Zamore *et al.*, 2000). These short lengths of cleaved RNA are then integrated into a multiprotein effector complex termed the RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000). The double stranded RNAs are then unwound by the RISC and the single stranded RNA fragments are utilised as guide sequences for the recognition of endogenous complementary mRNA within the cell (Nykamen *et al.*, 2001). The target mRNA is then cleaved by an endoribonuclease across the centre of the guide sequence and the subsequent fragments degraded by exoribonucleases (Hammond *et al.*, 2000) (Figure 4.1).

Initial attempts to recreate RNAi in mammalian cells proved unsuccessful. This is due to the activation of the dsRNA dependent protein kinase, PKR. When dsRNA of a greater size than 30 nucleotides is introduced into a mammalian cell it causes the autophosphorylation and subsequent activation of PKR which ultimately leads to the inhibition of translation (Clemens, 1997). This problem was overcome by the discovery that the introduction of synthetic strands of dsRNA that were between 21 and 22 nucleotides long with overhanging 3' ends, thereby mimicking the structure of an RNase III cleavage product, could also cause RNAi whilst circumventing the PKR response. These shorter strands of dsRNA were termed small interfering RNAs (siRNAs) (Elbashir *et al.*, 2001).

The ubiquity of the RNAi phenomenon in eukaryotes marks it out as an ancient cellular process that may even predate the divergence of plants and animals. RNAi appears to function as an immune response against disruption of the genome by endogenous RNAs, such as transposons, or exogenous RNA such, as that introduced by viruses (Elbashir *et al.*, 2001). There is also growing evidence that it may have epigenetic effects such as silencing by chromatin remodelling (Stevenson and Jarvis, 2003). RNAi is now being touted as a solution to problems as diverse as silencing oncogenes (Agami, 2002) to creating a more flavoursome, caffeine free coffee (Ogita *et al.*, 2003).

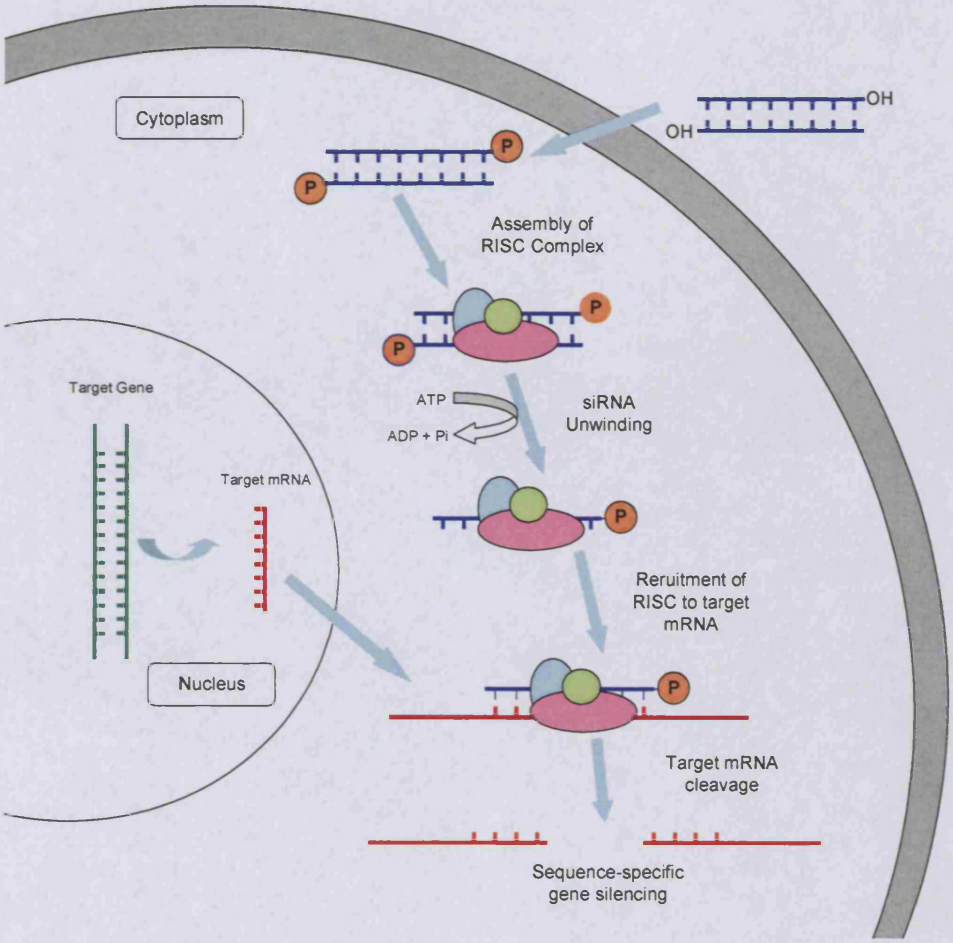


Figure 4.1. Gene Silencing by RNA interference using siRNA

Artificial small interfering (siRNAs) introduced into the cell cytoplasm are phosphorylated on their 5' ends by cellular kinases. The siRNAs assemble with cellular proteins to form a RNA-induced silencing complex (RISC) that includes a helicase that unwinds the double stranded RNA and a ribonuclease that cleaves the target sequences. The RISC is directed by the antisense strand of the unwound siRNA to the target mRNA through sequence complementarity and the target mRNA is cleaved (Figure adapted from Stevenson, 2003).

4.2. RESULTS

4.2.1. Transfection of a β-Galactosidase expressing plasmid into the MCF-7 and TAM-R cell to assess the transfection efficiency of different reagents.

MCF-7 and TAM-R cells were transfected with a β-Galactosidase expressing vector using Lipofectamine 2000, Lipofectin and GeneJuice (section 2.6.4) to determine which of these transfection reagents could deliver the plasmid most effectively. The GeneJuice transfection was additionally carried out in both serum free media and W+5% to determine which of these conditions were more favourable to the cells. The cells were fixed and stained using the chromogenic substrate X-Gal (section 2.6.3) and the percentage of cells in the overall population displaying the characteristic dark blue staining were visually counted by an independent observer. The mean percentages of stained MCF-7 and TAM-R cells from triplicate wells are displayed below in Table 4.1. and photographs of the MCF-7 and TAM-R cells shown in Figure 4.2 and 4.3 respectively. In each case the cells were tested for passive uptake by adding the β- galactosidase plasmid in DCCM with 1% DMSO, as a control.

Table 4.1. Transfection of MCF-7 and TAM-R cell lines with a β-Galactodidase expressing vector using different transfection reagents.

The efficiency of transfection is expressed as the percentage of cells displaying blue staining ± SD from triplicate wells.

	Transfection Efficiency (% ± SD)	
Transfection Reagent	**MCF-7**	**TAM-R**
Lipofectamine 2000	31% ± 13	37% ± 16
Lipofectin	30% ± 16	22% ± 6
GeneJuice (serum free)	31% ± 9	30% ± 11
GeneJuice (with serum)	34% ± 7	31% ± 11

The cells treated with the plasmid and DMSO displayed no negative effects on the cells and staining of <1%. The MCF-7 cells uptake with each of the transfection reagents was around 30% with the presence of serum having little effect on the effectiveness of GeneJuice. The presence or absence of serum also had no effect on the GeneJuice's effectiveness in the TAM-R cells. Lipofectamine 2000 had the best

transfection efficiency in the TAM-R cells with around 37% uptake and very pronounced staining, however it also caused a high degree of cell loss and the remaining cells looked unhealthy. This was also true when used in the MCF-7 cell line. If the Lipofectamine 2000 does indeed have cytoxic effects on the cell it may make the cell membrane more permeable to the uptake of the β-Galactosidase vector and therefore account for the increased staining seen in the cells. Lipofectin also displayed cytoxic effects on both cell lines and with a transfection efficiency of only around 22% in the TAM-R cell lines was ruled out for use in future experiments. The GeneJuice displayed no negative effects on the cells attachment or appearance in either cell line.

4.2.2. Transfection with varying amount of Lipofectamine 2000 in an attempt to reduce its cytotoxic effects.

The ratio of Lipofectamine 2000 relative to the amount of β-Galactosidase vector used in the above experiment (section 4.2.1) was 3:1 (v/w). To determine whether reducing the amount of the reagent added to the cells could lessen the cytotoxic effects, TAM-R cells were transfected with various ratios of Lipofectamine 2000 to DNA, e.g. 0.5:1, 1:1, 1:2 and 1:3 (v/w). Also the cells were grown for 48 hours prior to transfection to allow the cells to reach a higher confulency (Approx 80-90%) as recommended by the manufacturer's protocol. The cells were then transfected with the β-Galactosidase plasmid using the same basic method outlined in section 2.6.4.1, and 24 hours after transfection, stained and fixed (section 2.6.3). Photographs of this staining are shown in Figure 4.4. The amount of cell staining was proportional to the reagent:DNA ratio with the least staining seen in the cells treated with the reagent:DNA ratio (0.5:1) and 3:1 producing the highest degree of cell staining of around 40%, comparable to the degree of transfection observed previously. Importantly in each of these conditions the degree of cell loss was markedly lower than before. This is probably attributable to the higher confluency of the cells upon transfection.

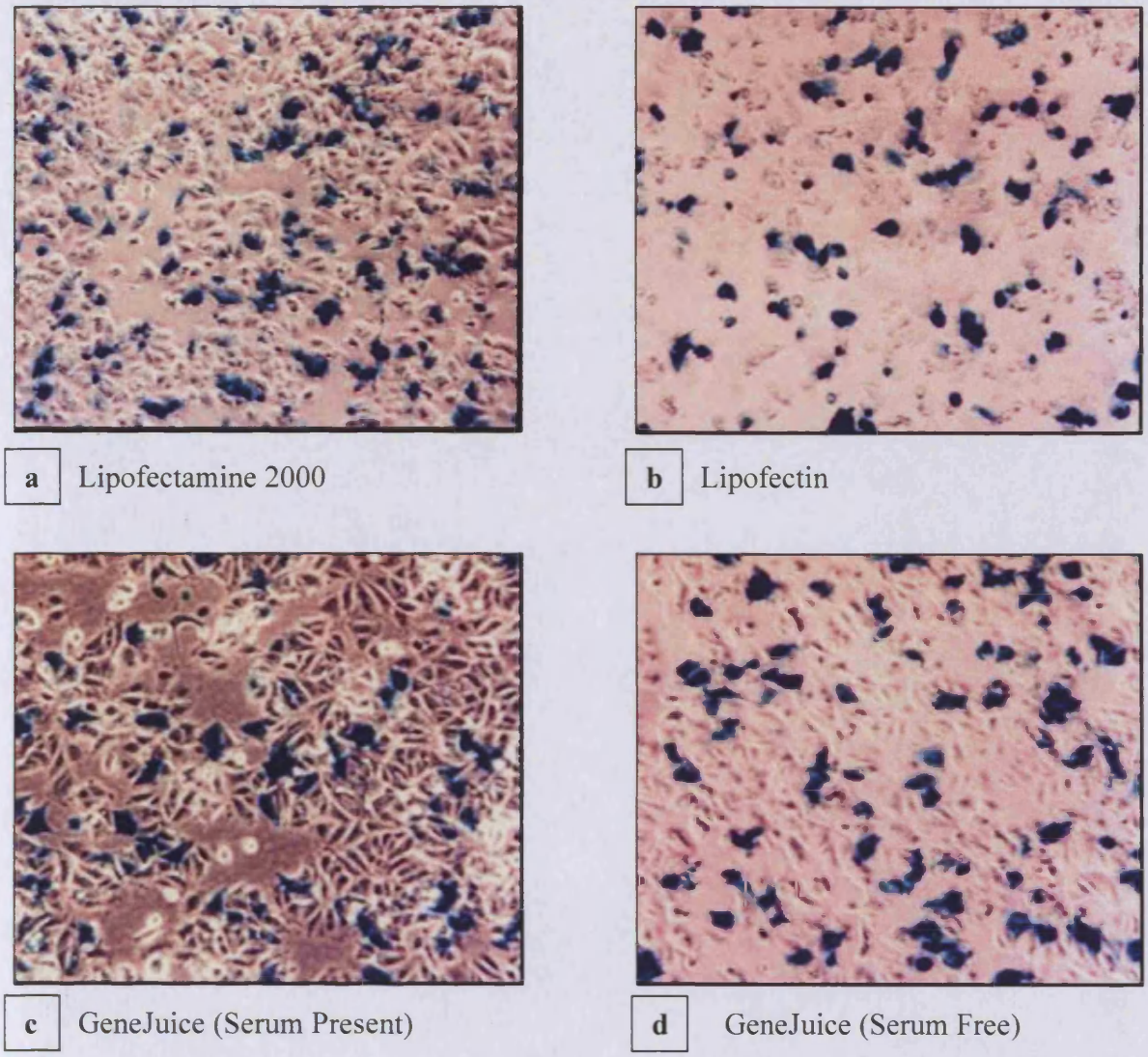


Figure 4.2. Photographs of MCF-7 cells transfected with a β-Galactosidase expressing plasmid using different transfection reagents.

MCF-7 cells were transfected with a β-Galactosidase expressing plasmid using either (a) Lipofectamine2000, (b) Lipofectin, (c) GeneJuice with serum present in the media during transfection, or (d) GeneJuice in serum free media during transfection. After 24 hours the cells were fixed and stained using the chromogenic substrate X-Gal causing cells expressing β-Galactosidase to become dark blue in colour. Images shown at x 400 magnification.

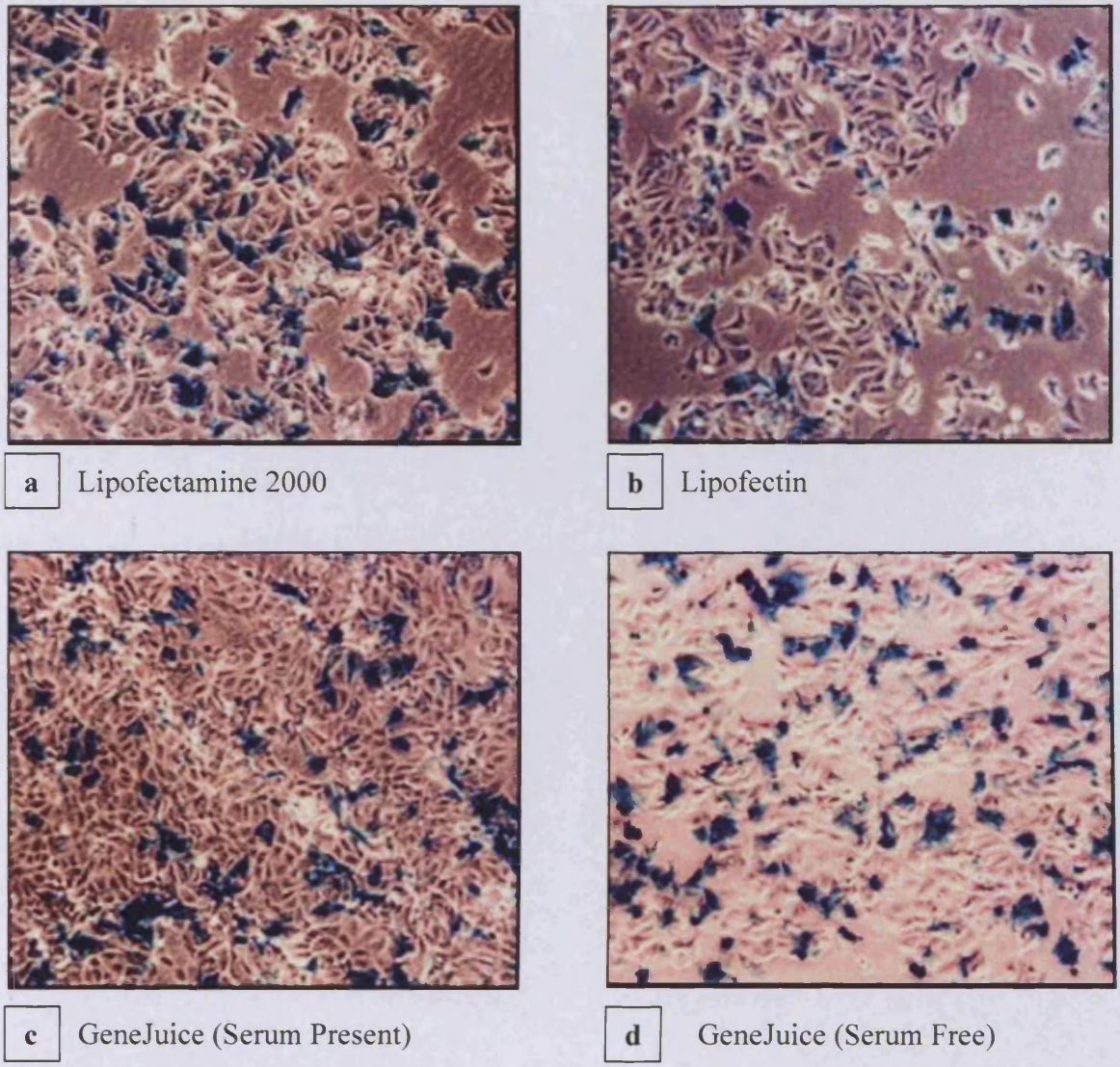


Figure 4.3. Transfection of TAM-R cells with a β-Galactosidase expressing plasmid using different transfection reagents.

TAM-R cells were transfected with a β-Galactosidase expressing plasmid using either (a) Lipofectamine2000, (b) Lipofectin, (c) GeneJuice with serum present in the media during transfection, or (d) GeneJuice in serum free media during transfection. After 24 hours the cells were fixed and stained using the chromogenic substrate X-Gal causing cells expressing β-Galactosidase to become dark blue in colour. Images shown at x 400 magnification.

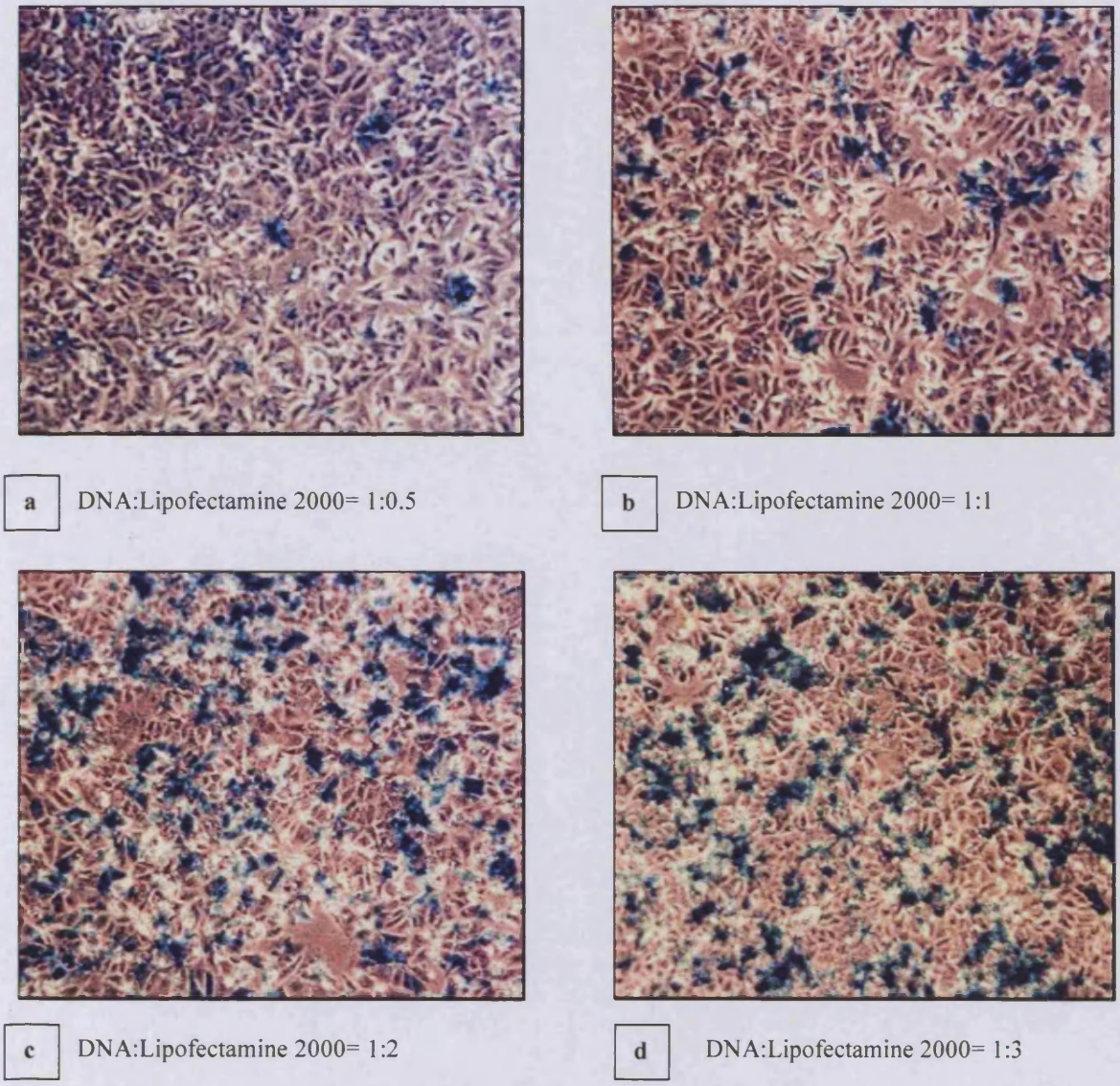


Figure 4.4. Transfection of TAM-R cells with a β-Galactosidase expressing plasmid using different ratios of plasmid to lipofectamine 2000 transfection reagent.

A β-Galactosidase expressing plasmid was transfected into TAM-R cells using plasmid to lipofectamine 2000 ratios of 1:0.5, 1:1, 1:2 and 1:3 (v/w) to determine if reducing the amount of Lipofectamine 2000 reduces its toxic effects on the cells. The cells were fixed and stained with the chromogenic substrate X-Gal, causing the cells that express β-Galactosidase to become dark blue in colour. Images shown at x 400 magnification.

4.2.3. Transfection of the TAM-R cell line with GAPDH specific siRNA

Although Lipofectamine 2000 provided the highest level of gene delivery the necessity for a high confluency of cells to minimise the cytotoxic effects of the reagent may preclude it from use in longer term experiments such as growth studies and extended treatment regimes. Therefore the use of GeneJuice in the presence of serum was not discounted at this time as it provided similar transfection efficiencies to Lipofectamine 2000 (Table 4.1) without displaying any cytotoxic effects on the cells. With the advent of siRNA technology the manufacturers of Lipofectamine 2000 (Invitrogen) and GeneJuice (Novagen) have produced analogous reagents specifically tailored to the delivery of oligonucleotides such as siRNAs. These are called Oligofectamine (Invitrogen) and RiboJuice (Novagen). To assess which of these four reagents could most effectively deliver siRNA into the cell and produce the knockout of a targeted protein, TAM-R cells were transfected with GAPDH specific siRNA duplexes using either Lipofectamine 2000 (section 2.6.5.1), GeneJuice (section 2.6.5.2), Oligofectamine (section 2.6.5.3) or Ribojuice (section 2.6.5.4).

The TAM-R cells were also transfected with a non-specific scrambled siRNA using the same reagents as a specificity control for each condition. The cells were harvested 48 hours post transfection and assayed for GAPDH levels by Western analysis. Figure 4.5 shows the level of GAPDH in the cells transfected with the GAPDH selective siRNA relative to the GAPDH levels in cells transfected with a scrambled siRNA control. The cells transfected with the GAPDH specific siRNA using Lipofectamine 2000 and Oligofectamine displayed reductions in GAPDH protein levels of 12% and 32% respectively. However the cells transfected with GAPDH using Ribojuice had no effect compared to control and the cells transfected with Genejuice actually displayed an increase in GAPDH level.

As transfection with Oligofectamine displayed the most effective knockdown, the reproducibility of this effect was investigated by repeating the experiment two further times. Again, the knockdown of GAPDH protein was measured in cells transfected with GAPDH specific siRNA using Oligofectamine and compared to the level in cells transfected with a scrambled siRNA control. Figure 4.6 displays the mean level of

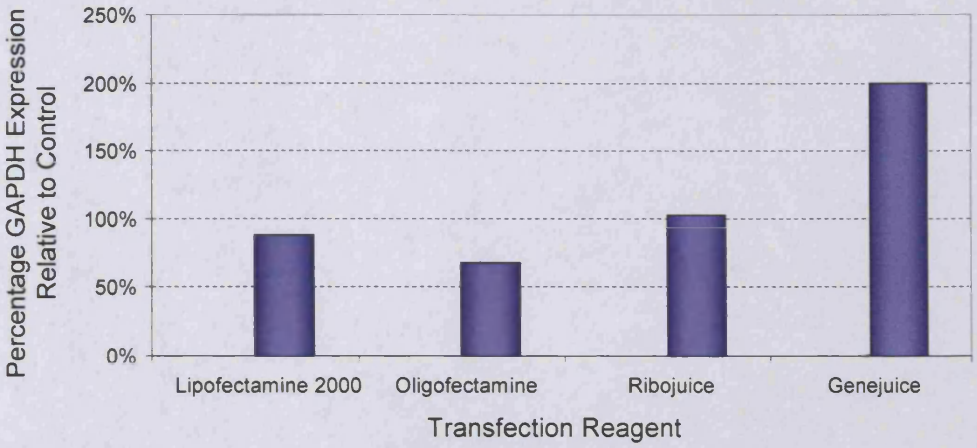


Figure 4.5. Transfection of TAM-R cells with GAPDH specific siRNA using different transfection reagents.

TAM-R cells were transfected with either a GAPDH specific siRNA or a scrambled control siRNA using the transfection reagents Lipofectamine 2000, Oligofectamine, Ribojuice and Genejuce. The cells were harvested 48 hours post transfection and assayed for GAPDH levels by Western analysis. The graph represents the percentage GAPDH expression in the cells transfected with GAPDH specific siRNA relative to the cells transfected with the scrambled control.

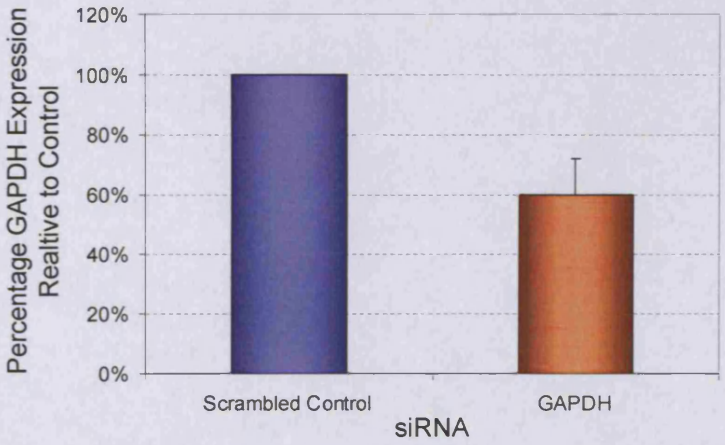


Figure 4.6. Transfection of TAM-R cells with GAPDH specific siRNA using the Oligofectamine transfection reagent.

TAM-R cells were transfected with either a GAPDH specific siRNA or a scrambled control siRNA using the transfection reagent Oligofectamine. The cells were harvested 48 hours post transfection and assayed for GAPDH levels by Western analysis. The graph represents the percentage GAPDH expression in the cells transfected with GAPDH specific siRNA relative to that in cells transfected with the scrambled control ± SD. N=3 separate experiments.

GAPDH in the TAM-R cells transfected with the GAPDH specific siRNA relative to TAM-R cells transfected with the scrambled siRNA control ± SD, as measured by Western analysis (section 2.3.3) from all 3 experiments. The results confirm that transfection with siRNA using Oligofectamine can elicit approximately a 40% knockdown of the GAPDH protein after 48 hours.

4.2.4. Transfection of the MCF-7 cell line with PKC-α and PKC-δ specific siRNA

To determine if PKC-α and δ expression could be ablated by the technique of RNAi effectively in our cells, PKC-α and δ specific siRNA oligonucleotides were transfected into the MCF-7 cell line using either Lipofectamine 2000 (section 2.6.5.2) or Oligofectamine (section 2.6.5.3). The PKC-α specific siRNA was designed, synthesised and validated by Qiagen. The PKC-δ specific siRNA was obtained from a published oligonucleotide sequence by Yoshida *et al.* (2003) and synthesised by Qiagen. The protein levels of PKC-α and PKC-δ after transfecting with their specific siRNAs were assayed by Western analysis (section 2.3.3), and expressed in Figure 4.7 as a percentage of the isoform levels in the control cells transfected with scrambled control siRNA. Transfecting the PKC-α specific siRNA into the MCF-7 cell line with Lipofectamine 2000 or Oligofectamine induced a 10% and 28% reduction in PKC-α protein level respectively. Transfecting the MCF-7 cell line with the PKC-δ specific siRNA using the same reagents caused a 56% and 63% reduction in PKC-δ protein level respectively.

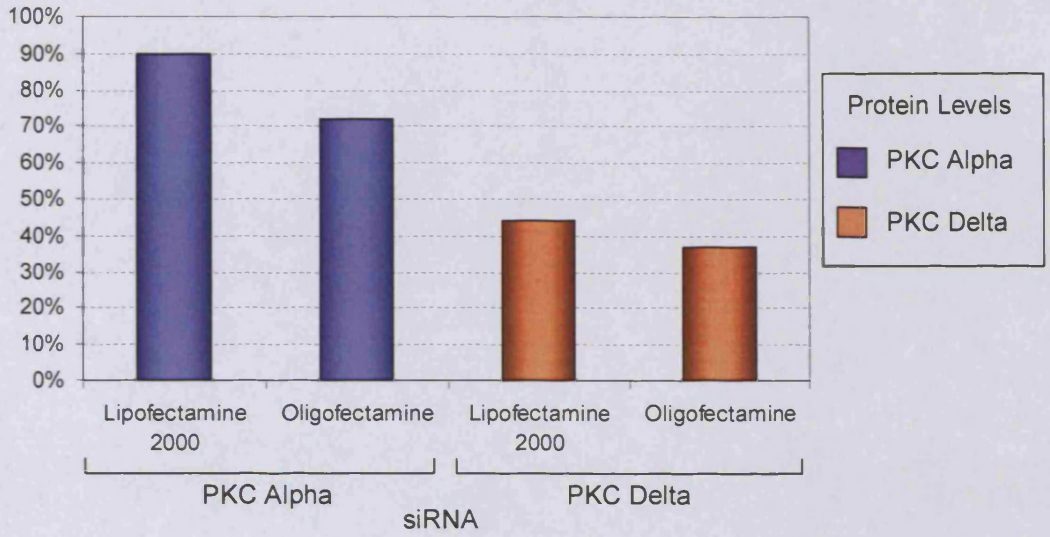


Figure 4.7. Reduction of PKC-α and δ protein levels in the MCF-7 cell line by transfection with PKC-α and δ specific siRNA oligonucleotides

MCF-7 cells were transfected with either PKC-α or PKC-δ specific siRNAs or a scrambled control siRNA using the Lipofectamine 2000 or Oligofectamine transfection reagents. The cells were harvested 48 hours post transfection and assayed for total levels of their targeted PKC isoforms by Western analysis. The graph represents the percentage PKC isoform expression in the cells transfected with the specific siRNAs relative to the cells transfected with the scrambled control.

4.2. DISCUSSION

Sequence specific gene silencing through the use of siRNA is now a frontline technique in many molecular biology studies and has been used to target and cleave the mRNA of a multitude of disease related genes in mammalian cell lines (Dykxhoorn *et al.*, 2003). However, the efficacy with which siRNAs can execute their function is dependent on the ability to fascilitate their introduction into the cell. The most common method of siRNA delivery into mammalian model cell lines is through the use of lipid/polymer based transfection reagents (Brazas and Hagstrom, 2005).

We utilised several such lipid/polymer transfection reagents to introduce a β-galactosidase expressing vector into the MCF-7 and TAM-R cell lines. Whilst this plasmid DNA is far larger than siRNA it was used as an easily detectable method of assessing how permissive our model cell lines would be to transfection and the relative toxicities of the transfection reagents used. All of the transfection reagents tested achieved less than 40% transfection efficiency with the β-gal plasmid. The least effective was Lipofectin which was discounted from future use as it only transfected an average of 22% of the TAM-R cells and displayed cytotoxicity in both cell lines. Whilst the Lipofectamine 2000 displayed the highest transfection efficiency in the TAM-R cell line, it too caused a high degree of cell detachment and left the remaining cells with an unhealthy appearance. Although these cytotoxic effects were not evident when the cells were transfected with Lipofectamine 2000 at high confluencies, the lack of growing space within the dish precludes its use in extended experiments such as long term treatments and growth studies.

Despite these limitations Lipofectamine 2000's effectiveness at transfecting GAPDH specific siRNA into TAM-R cells was tested, along with that of GeneJuice, RiboJuice and Oligofectamine by measuring the knockdown of GAPDH protein. Although siRNAs bring about their effect through the degradation of the target mRNA, we measured changes in protein level rather than mRNA level because it is reduction in protein that will lead to physiological effects. Due to the transient nature of the transfection and the half-life of the protein, the mRNA degradation may not equate to

a significant reduction in target protein and therefore no phenotypical effect, giving a false impression of the efficacy of the system.

Transfection of GAPDH siRNA using either Oligofectamine or Lipofectamine 2000 resulted a reduction in GAPDH protein levels, with Oligofectamines status as a tailored oligonucleotide delivery reagent seemingly justified by its superior and reproducible GAPDH knockdown of around 40%. Although RiboJuice is tailored for the delivery of siRNA oligonucleotides, its utilisation to transfect TAM-R cells with GAPDH siRNA resulted in no detectable protein knockdown and its analogous, DNA transfecting stablemate GeneJuice actually induced an apparent increase in GAPDH protein. This aberrant effect with GeneJuice seems unlikely to be directly caused by the transfection reagent and requires further investigation.

As Lipofectamine 2000 and Oligofectamine were both able to deliver GAPDH siRNA, they were utilised to transfect PKC-α and PKC-δ specific siRNAs into the MCF-7 cells. Transfection of the PKC specific siRNAs with Oligofectamine was more effective at reducing protein levels than Lipofectamine 2000 in both cases. This concurs with the earlier observation that GAPDH transfections were also most effectively performed with Oligofectamine. The PKC-δ siRNA was much more effective at knocking down its target protein than the PKC-α siRNA, with a reduction of over 60% compared to less than 30%. This difference in protein knockdown could be due to differences in the effectiveness of the actual siRNA oligonucleotides because whilst the PKC-δ siRNA was derived from a published siRNA sequence that has been shown to be effective (Yoshida *et al.*, 2003), the PKC-α comes directly from Qiagen and has only been validated by them in-house. Alternatively the rate of PKC-α turnover may be slower than that of PKC-δ in the MCF-7 cell line and therefore the knockdown of target mRNA would take longer to impart a reduction in protein level. Interestingly it is the more abundantly expressed PKC-δ that is readily knocked down whilst the PKC-α levels, which were modestly expressed, were less amenable to knockdown. In this context it would have been very interesting to repeat this experiment with TAM-R cells (which express both high PKC-α and PKC-δ) and MB-MDA-231 (which express high PKC-α but low PKC-δ) to see whether when the α

isoform is more suseptible to breakdown when more abundantly expressed. Unfortunately time constraints did not permit this.

In actively dividing cells the duration of silencing is directly proportional to the rate at which the siRNA is diluted below the level necessary to maintain the inhibition of gene expression and is therefore dependent on the rate of cell growth (Dykxhoorn *et al.*, 2003). Therefore whilst reductions in protein levels of the PKC isoforms were detectable the effect would be out grown rapidly and therefore rules out siRNA induced knockdown for growth studies, especially with the PKC-α siRNA where the knockdown is already very poor at 48 hours. To overcome the transient nature of transfecting chemically synthesised siRNAs into mammalian cells, our cell lines could have been transfected with DNA vectors which express substrates that could be converted into specific siRNAs within the cell. For example, one recently developed technique utilises RNA polymerase III to produce short hairpin (sh) RNA that can be converted into siRNAs within the cell by Dicer. Although silencing via this plasmid based strategy is not as immediate as siRNA transfection, due to the requirement for transcription and Dicer processing, cell lines can be generated that stably express the plasmid and therefore produce siRNA over successive generations of cells. These stably expressing clones can then be screened and propagated to derive a homogenous population of cells with the target gene effectively silenced (Shi *et al.*, 2003).

In recent years, the halcyon notion that siRNAs function in a completely blinkered manner, only targeting their intended gene, has also been thrown into doubt. When RNAi was originally attempted in mammalian cells, the 500 base pair RNA oligonucleotides that were efficacious in the invertebrate models elicited a non-specific interferon response that resulted in transcriptional shut down of the cell (Stark *et al.*, 1998). It was believed that the use of siRNAs of around 22 nucleotides circumvented this. However it has since been shown that the interferon response can be activated by siRNAs whether chemically synthesised or transcribed from vectors (Sledz *et al.*, 2003; Bridge *et al.*, 2003). As attempts are being made to circumvent these problems, as our knowledge of the mechanisms increases so does our knowledge of its limitations, bringing into doubt its utility as a therapeutic strategy.

Although it was interesting to investigate an siRNA approach, within the time constraints of this project we were unable to overcome two major hurdles. First, the transfection efficiencies achieved were too low to be likely to yield sufficient protein knockdown to viably ablate an enzyme from a signalling pathway. Second, in order to transfect the cells whilst maintaining their viability we had to plate the cells at densities that excluded to possibility of long term growth studies. For these reasons we decided to concentrate our efforts on other techniques which can yield higher transfection efficiencies at lower plating densities.

Despite the limitations, this study demonstrated that PKC isoform specific knockdown could be achieved via an siRNA approach in breast cancer cells. This was also recently achieved by Nabha *et al.* (2005) who demonstrated that a PKC-δ specific siRNA could knock out PKC-δ protein expression after 72 hours. They also showed that this knockdown was able to inhibit oestrogen induced cell proliferation. However, whilst their technique of measuring cell proliferation using [^{3}H] thymidine incorporation overcomes the transient nature of the system, it is only a temporary measure of the inhibition of DNA synthesis.

CHAPTER 5.

PKC-α AND PKC-δ MODULATION BY ADENOVIRAL GENE TRANSFER

5.1. INTRODUCTION

The transfer of genetic information into mammalian cells, whether it be plasmid DNA, anti-sense oligonucleotides or siRNAs, has traditionally been impeded by the inefficiencies of the techniques facilitating their introduction into the target cell. Whilst scientists have endeavoured for decades to produce viable transfection and gene transfer techniques, there is a population that have quietly spent millions of years perfecting the transfer of genetic information into mammalian cells, a process that is vital to their propagation and survival, the virus.

Scientists have identified that since viruses exist by virtue of their ability to efficiently transfer genetic information into a wide range of cell types, this property could be subverted so that rather than executing the introduction their own intrinsic viral genome, the virus actually facilitates the transfer of tailor made genetic information designed to alter the expression or activation of a desired protein in a target cell.

The best-studied and most extensively used viral vector for gene transfer is the adenovirus (Becker *et al.*1994) Adenoviruses were first isolated from human adenoidal tissue in 1953 (Rowe *et al.*, 1953) during attempts to establish tissue culture lines from tonsils and adenoidal tissue removed from children. The concept of using them as delivery vectors did not arrive till the 1960s when it was observed during studies by the military to produce a vaccine against acute respiratory disease (ARD), that foreign DNA could integrate and be expressed in the adenoviral genome (Roy-Chowdhury and Horwitz, 2001). Subsequent studies on adenovirus and their infected cells have lead to several important discoveries including mRNA splicing, the existence of introns and capped polyadenylated mRNAs (Shenk, 2001). The extensive knowledge of their biological and genetic characteristics has lead to them being the most commonly used viral vectors. There have currently been 51 serotype strains of adenovirus identified most of which are associated with benign respiratory tract infections, gastrointestinal infections in infants or conjunctivitis. These serotypes are classified into 6 subgroups (A-F). The most commonly studied and best understood of these are types 2 and 5 which belong to subgroup C (Mizuguchi *et al.*, 2001).

Adenoviruses are non-enveloped icosahedral DNA viruses with a diameter of 70-100nm that multiply in the nuclei of infected host cells. Each virion contains a DNA core comprising a linear double stranded genome of approximately 36kb that encodes over 70 gene products (13% of mass) encapsulated by a protein shell (87% mass) consisting of 252 subunits called capsomeres (Shenk, 2001). The viral genome contains five early transcription units (E1A, E1B, E2, E3, E4) two early delayed (intermediate) transcription units (pIX and IVa2) and 5 late units (L1-L5), which mostly encode structural proteins for the capsid and the internal core (Mizuguchi *et al.*, 2001). The E1A gene is the first transcription unit to be expressed shortly after infection and is essential to the activation of other promoters and expression of subsequent transcription units of the viral genome (Shenk, 2001). In first generation adenoviral vectors the E1 (E1A and E1B) gene is deleted to accommodate a further 3.2kb of inserted DNA. As this deletion creates replication deficient virus, the function of the E1 region of the gene must be provided in *trans* to facilitate propagation. This is achieved by growing the E1-deleted recombinant virus in Ad5 transformed human embryonic kidney (HEK) 293 cells that retain the E1A and E1B regions of the adenoviral genome (Graham *et al.*,1977). The E3 is frequently also deleted to provide a further 3.1kb of packageable space for foreign genes. This is viable since the region encodes products that modulate the response of the host to infection defence mechanisms, which are not required for viral replication in vitro. In total, E1/E3-deleted adenoviral vectors allow the packaging of approximately 8.1-8.2kb of foreign DNA (Mizuguchi *et al.*, 2001) with the added benefit of creating a replication deficient virus which is therefore biologically safer from the perspective of laboratory use.

The introduction of foreign genetic information into a cell using adenoviral vectors could have several useful applications in the treatment of disease. These include the delivery of a functioning gene to a cell *in vivo* to treat a disease caused by the congenital absence or mutation of a gene encoding a vital protein. The introduction of genes *in vivo* could also be used in the treatment of cancer via the delivery of genes that control cell growth or apoptosis to kill tumour cells or retard their growth. Another possible technique is to deliver epitopes or antigens of other infectious agents for the purpose of immunisation (Horwitz, 2002). Whilst these applications are

potentially very powerful they all have obstacles to overcome before they become clinically viable.

The intrinsic problems of *in vivo* therapy with adenoviral vectors do not however exclude them from functioning as very useful tools for *in vitro* analysis of cellular function in model cell lines. Adenoviruses that overexpress dominant negative (DN) mutants of PKC-α and PKC-δ were provided as a kind gift from Prof David Knight (URCN, Bristol University, UK). These dominant negatives are mutants possessing a single point mutation at a residue within the catalytic kinase domain crucial for ATP binding. Substitutional mutations were made by replacing the lysine at postion 369 with an arginine in the PKC-α DN whilst in the PKC-δ mutant the analogous lysine at postion 376 is replaced with an alanine (Figure 5.1). When overexpressed within the cell, these DNs can disrupt the activity of the endogenously expressed PKCs in an isoform specific manner (Ohno *et al.*, 1990; Hirai *et al.*, 1994). Additionally Dr. Knight furnished us with adenoviruses that overexpress the wild type (WT) PKC-α and PKC-δ isoforms. Therefore we have the potential tools to examine the role of PKC-α and δ expression *in vitro* in an isoform specific manner through their overexpression in the tamoxifen sensitive phenotype and ablation in the Tamoxifen resistant derivatives.

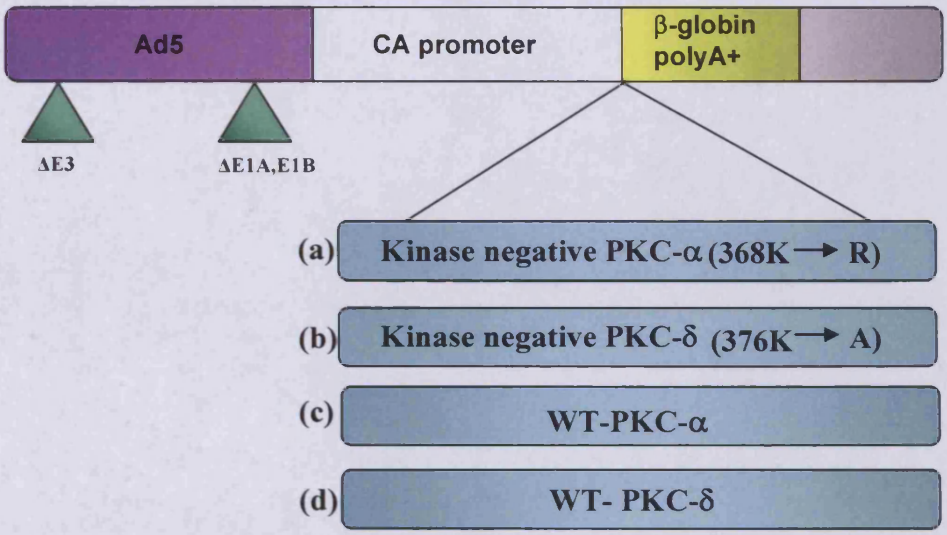


Figure 5.1. Dominant negative and wild type overexpressing PKC-α and δ vectors

The activity of PKC-α and PKC-δ was modulated using adenoviral vectors that overexpressing either wild type (WT) PKC-α or PKC-δ, or dominant negative (DN) PKC-α or δ. The dominant negative PKC isoforms differ from the wild type by single point mutations in the kinase domain that renders the isoform kinase deficient. In the case of the (a) DN PKC-α this mutation is at residue 368 where a lysine is substituted for an arginine and in the case of the (b) PKC-δ DN the lysine at residue 376 is substituted for alanine.

5.2. RESULTS

5.2.1. Visualisation of adenoviral infection efficiency in MCF-7 and TAM-R cell lines using a β-galactosidase expressing adenovirus.

To assess the infection efficiency of the adenoviral vector delivery system, and to identify a suitable MOI to utilise in further experiments, the MCF-7 and TAM-R cell lines were infected with an adenovirus containing a β-galactosidase (β-gal) expressing vector at MOI of 20, 50, 100 and 200 (section 2.7.6). Table 5.1 displays the mean percentage of cells containing blue staining from the triplicates of each condition. The wells that were not infected with the adenovirus possessed a negligible number of cells with any blue staining (<1%) in either cell line, as can be seen in Figure 5.2. This indicates that any blue staining seen in the adenovirally infected cells is therefore fully attributable to its β-gal expressing plasmid. The infected MCF-7 cells displayed staining ranging from 37% (± 10) at MOI 20 to 78% (± 2.5) at MOI 200 and the infected TAM-R cells displayed staining ranging from 41% (± 5) at MOI 20 to 75% (± 6) at MOI 200. Whilst the cells infected with MOI 200 had the greatest overall percentage of stained cells, the numbers of both MCF-7 and TAM-R cells were visibly lower after fixation than those infected at lower MOI. This could be attributed to decreased growth rates or a greater fragility of the cells at this MOI causing cell loss during the washing and fixing process. If the cells are indeed damaged in any way by infection at MOI 200 then the higher degree of staining could also be attributable to lowered cell membrane integrity allowing greater susceptibility to the adenoviral infection. The cells infected at MOI below 200 showed no visible decrease in cell number or integrity.

Table 5.1. Percentage of cells (± SD) with blue staining after infection with β-Galactosidase (β-Gal)

MCF-7 and TAM-R cells were infected with a β-Gal expressing adenovirus at different MOIs and stained through the addition of the chromogenic substrate X-Gal. Percentage staining was scored blind by an independent assessor familiar with the technique.

	Percentage β-Gal Staining	
	Cell Line	
MOI	**MCF-7**	**TAM-R**
0	<1%	<1%
20	37% ± 10%	41% ± 5%
50	72% ± 6%	69% ± 5%
100	63% ± 6%	69% ± 10%
200	78% ± 3%	75% ± 6%

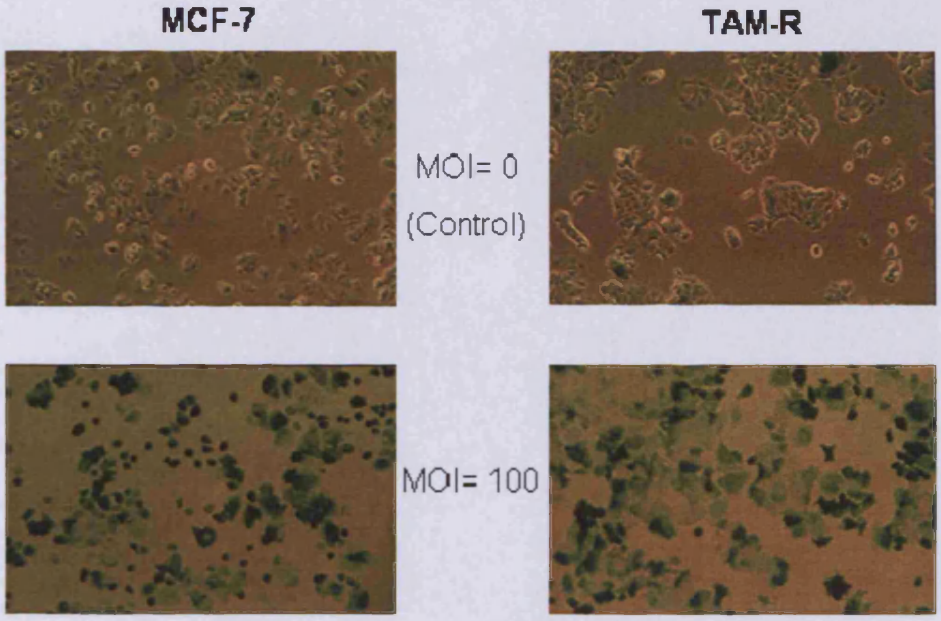


Figure 5.2. Photographs of MCF-7 and TAM-R cells stained with the chromogenic substrate X-Gal after infection at MOI 100 with a β-Galactosidase expressing vector compared with uninfected cells.

MCF-7 and TAM-R cells were grown for 48 hours in 12 well plates and infected with a β-Galactosidase expressing vector at a MOI of 100 or left uninfected. After 16 hours the media was changed and cells allowed to grow for a further 24 hours. After this time the cells were treated with the chromogenic substrate X-Gal. The cells stained blue are those that contain β-Galactosidase. The cells were fixed and photographed at magnification 400x.

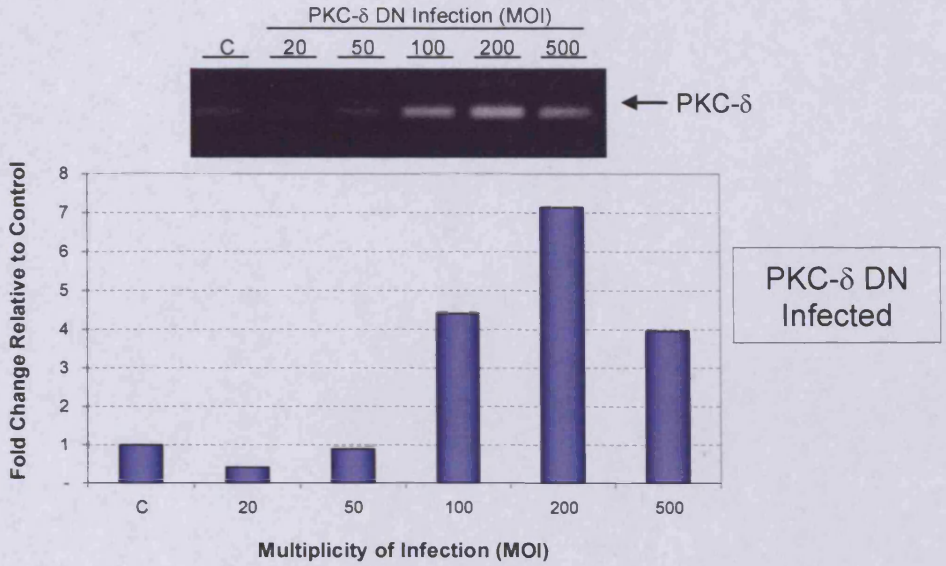
5.2.2. Establishment of suitable multiplicity of infection (MOI) for the PKC-δ overexpressing (WT) and dominant negative (DN) expressing adenoviral vectors.

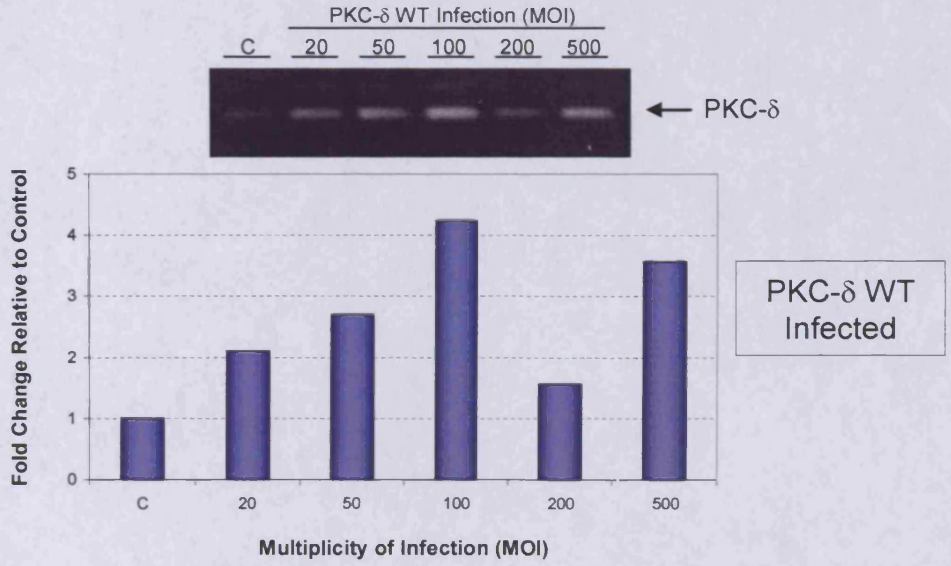
To establish a suitable MOI for the PKC-δ WT and DN expressing adenoviruses, the MCF-7 cell line was infected at a series of different MOI and the expression of PKC-δ measured by semi-quantitative RT-PCR (section 2.7.5). This technology was suitable for the measurement of PKC-δ WT and DN expression within the cells due to the fact that, apart from a single point mutation present in the dominant negative PKC-δ, they share sequence homology with the cells endogenous PKC. Therefore our PKC-δ specific primers can measure the increases in PKC-δ mRNA as an indication of the adenovirally infected vectors exogenous PKC-δ expression. Photographs of the bands produced are shown in Figure 5.3. with a graph of their relative levels compared to control.

The MCF-7 cells infected with PKC-δ DN displayed little change in PKC-δ mRNA levels until they were infected at a MOI of 100 or 200, which caused increases of over 4 fold and 7 fold respectively. There was also a 4 fold increase in PKC-δ levels relative to control in the cells infected with MOI 500 and though at this level of infection there was a large degree of cell loss indicating that the magnitude of infection was having an adverse effect on the cells. Unlike the PKC-δ DN infected cells, the cells infected with the PKC-δ WT displayed increases in PKC-δ expression at MOI 20 and 50 but showed the greatest increase of over 4 fold at MOI 100. As with the infection with PKC-δ DN at MOI 500, infection at this level with the WT adenovirus caused a high level of cell loss with signs of cell damage and detachment. As infection with both the β-gal expressing adenovirus at MOI 200 (described in section 5.2.1) and the PKC-δ WT and DN viruses at MOI 500 caused a degree of cell damage and detachment, subsequent experiments will be carried out at MOI 100. This MOI displayed no apparent affect on cell number or viability whilst inducing a four

Figure 5.3. Determination of a suitable multiplicity of infection (MOI) for the PKC-δ overexpressing (WT) and PKC-δ dominant negative (DN) adenovirus

MCF-7 cells, seeded into 24 well plates and grown for 48 hours in media containing 5% charcoal stripped FCS, were infected with adenovirus containing either PKC-δ WT or DN expressing plasmids at MOI of 20, 50, 100, 200 or 500. Uninfected cells were used as a control. After 16 hours the media was changed and the cells allowed to grow for a further 48 hours. After this time, cDNA was prepared from the total cellular RNA of three separate sets of cells and subjected to RT-PCR using PKC-δ and β–Actin specific primers as outlined in section 2.5. The amplification products were then size fractionated on a 2% (w/v) agarose gel. The graphs represent the fold increase of mRNA levels of PKC-δ relative to control.


PKC-δ DN Infection (MOI)
C
20
50
100
200
500
PKC-δ
Fold Change Relative to Control
8
7
6
5
4
3
2
1
-
C
20
50
100
200
500
Multiplicity of Infection (MOI)
PKC-δ DN Infected


PKC-δ WT Infection (MOI)
C
20
50
100
200
500
PKC-δ
Fold Change Relative to Control
5
4
3
2
1
0
C
20
50
100
200
500
Multiplicity of Infection (MOI)
PKC-δ WT Infected

fold increase in PKC-δ mRNA over the endogenous levels when infected with the exogenously expressed PKC-δ WT or DN.

5.2.3. Effect of adenoviral infection at MOI 100 on the growth of the MCF-7 and TAM-R cell line.

To assess whether infection with the adenoviral system would have any non-specific effects on growth in the MCF-7 and TAM-R cell lines, they were infected with an empty adenovirus that contained no transferable genetic material at MOI 100 (section 2.7.4), and the growth compared to uninfected cells after four days by Coulter counting (section 2.2.6). Figure 5.4 represents the mean percentage cell number of the infected cells (±SEM) after 4 days growth relative that of the uninfected cells. The values are derived from the mean of 12 counts from 4 separate experiments in the MCF-7 cells and the mean of 6 counts, from 2 separate experiments, in the TAM-R cells. It can be seen that infection with the empty adenovirus at MOI 100 has no significant effect on growth after four days in either the MCF-7 or the TAM-R cell lines. Therefore any growth effects observed after the cell lines are infected with the PKC-δ/α WT or DN adenoviruses are not attributable to non-specific growth effects caused by the infection. This also confirms that MOI 100 would be a non detrimental level of infection for subsequent experiments in both cell lines.

5.2.4. Western analysis of immunoprecipitated PKC-α and δ from TAM-R cells adenovirally infected with either PKC-α or -δ expressing (WT) or dominant negative (DN) vectors.

Before analysing the adenovirally infected cells by immunoprecipitation (IP) the optimum volume of PKC-δ and PKC-α primary antibody necessary for the procedure was established and how selectively the IP could isolate the individual isoforms investigated (section 2.3.5). It can be seen in Figure 5.5 that samples immunoprecipitated with PKC-α contained no detectable PKC-δ when probed by Western analysis with the PKC-δ isoform specific antibody. This isoform specificity was also seen with the sample immunoprecipitated with the PKC-δ antibody displaying no detectable PKC-α. Therefore the antibodies have been shown to be

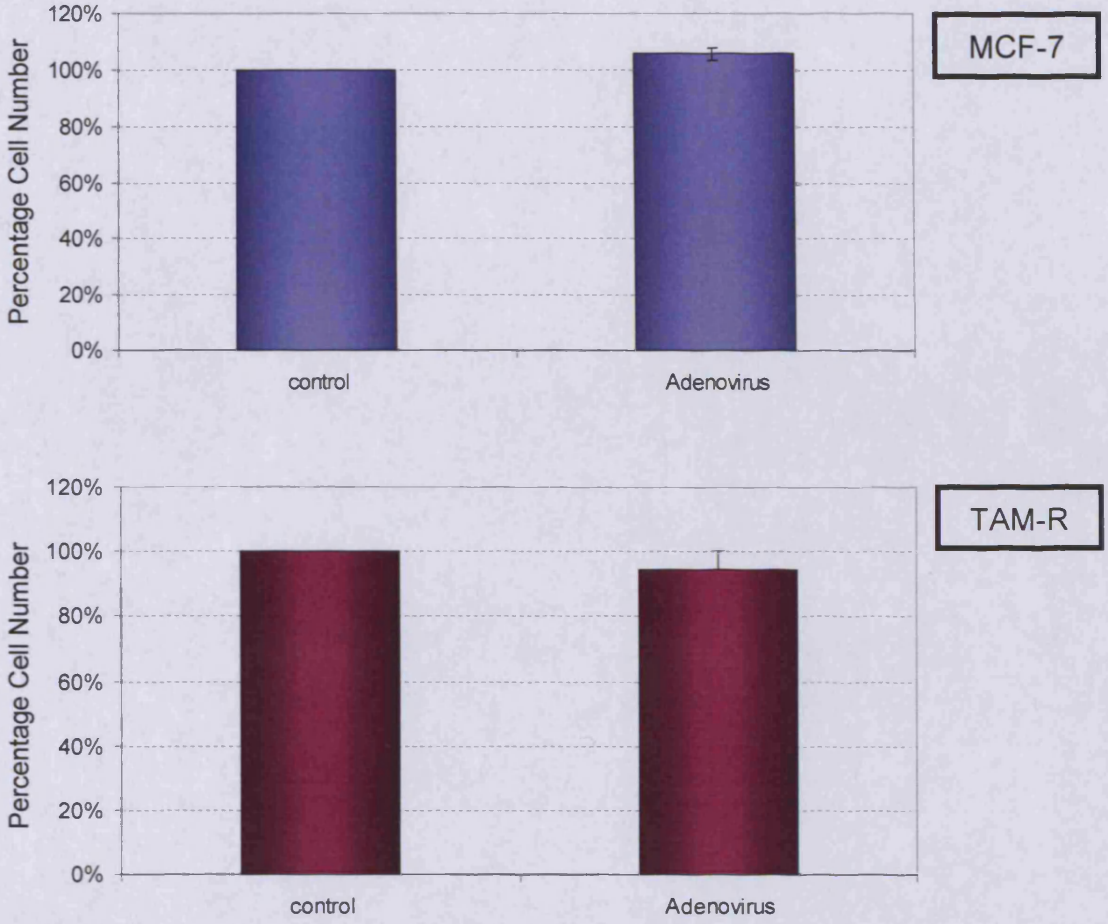


Figure 5.4. Effect of adenoviral infection at MOI 100 on the growth of the MCF-7 and TAM-R cell lines 4 days post infection.

MCF7 and TAM-R cells were grown for 48 hours in 24 well plates. The cells were then either infected with an empty adenovirus or left uninfected to assess the non-specific effects of adenoviral infection on growth. After 16 hours the media was changed and the cells grown for a further four days after which time the cells were counted by Coulter counting as outlined in section x. The MCF-7 graph represents the mean cell numbers of 12 counts taken from 4 separate experiments ± SEM. The TAM-R graph represents the mean cell numbers of 6 counts from 2 separate experiments ± SEM.

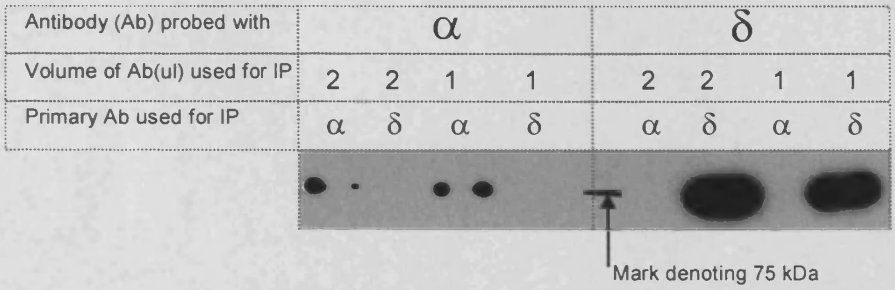


Figure 5.5. Western analysis of immunoprecipitated PKC-α and δ from TAM-R cells probed with the corresponding antibodies.

Total PKC-α or δ was immunoprecipitated (IP) from TAM-R cells using either 1μl or 2μl of primary antibody as described in section 2.3.5. The IPs were then probed for both PKC-α and δ using the same primary

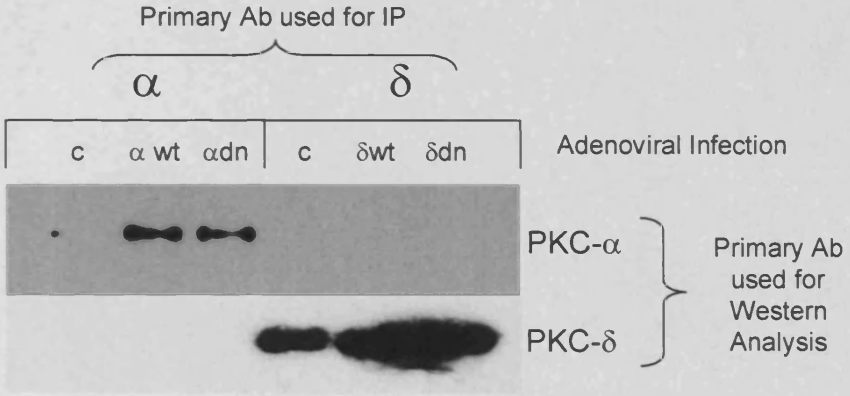


Figure 5.6. Western analysis to confirm adenoviral expression of PKC-α and PKC-δ wild type (WT) and dominant negative (DN) plasmids.

Total PKC-α or δ expression in Tam-R cells infected with PKC-α WT, PKC-α DN, PKC-δ WT or PKC-δ DN at a MOI of 100 for 48 hours prior to harvesting (as outlined in section 2.7.4) and immunoprecipitated (as

specific for their individual isoform. Performing the IP with either 1μl or 2μl of primary antibody made little difference with regards the amount of PKC obtained so subsequent IP will be carried out with 1μl of antibody.

The isoform specific antibodies can IP the PKC WT and DN proteins along with the endogenous PKC as they share amino acid sequence homology apart from a single amino acid substitution outside the immunogenic regions in the case of the DN PKCs. This homology in the immunogenic region means that we are also able to detect the expression of the WT and DN vectors as an increase in the PKC isoforms over the control levels of expression. It can be seen from Figure 5.6 that the cells infected with the exogenously expressing PKC vectors (section 2.7.4) displayed an isoform specific increase in protein expression compared to the controls, indicating that the vectors are expressing PKC proteins analogous to the endogenous isoforms. Furthermore the levels are around 15-20 fold higher with the PKC-α expressing plasmids and around 2 fold higher with the PKC-δ expressing plasmids than endogenous control levels. Whilst we have shown that the adenoviruses are capable of delivering the PKC expressing vectors and that they are expressed within the cell we still do not now if they are functionally active in the case of the WT or kinase deficient in the case of the DN.

5.2.5. Verification of the kinase deficient phenotype of the PKC-α and δ dominant negative expressing adenoviruses.

Tumour promoting phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) are known to induce translocation to the cell membrane and activation of PKC (Lu *et al.*, 1997). However with prolonged treatment, TPA causes depletion of PKC due to an increased rate of proteolytic degradation (Young *et al.*, 1987). However this turnover is dependent on the PKCs initial activation. This has been shown previously in a PKC-α DN which contains a point mutation in the ATP binding site when rendered unable to autophosphorylate and therefore insensitive to proteolytic degradation by prolonged treatment with TPA (Ohno *et al.*, 1990). As the PKC-δ DN shares a homologous mutation in the ATP binding site and is also unable to autophosphorylate (Li *et al.*, 1995) it should also possess an insensitivity to

downregulation by TPA. Therefore to verify that our PKC-α and δ DN are unable to autophosphorylate, we decided to treat them with TPA to see if they were insensitive to its downregulatory effects.

To first confirm that the endogenous PKC-α and δ can be degraded by prolonged treatment with TPA in both cell lines, and to establish a suitable time point for treatment, we treated the MCF-7 and TAM-R cells either with or without 500nM TPA for 15 minutes, 1 hour, 6 hours and 24 hours. The cells were harvested and analysed by Western analysis with PKC-α and PKC-δ specific antibodies (section 2.3) and the bands produced analysed by densitometry. Figure 5.7 and Figure 5.8 illustrate the mean percentages of PKC-α or δ levels after TPA treatment relative to control for 3 separate experiments ± SD. It can be seen from these figures that after 24 hours, almost all PKC-α and δ has been degraded in the TPA treated samples relative to control in both cell lines. Therefore 24 hour treatment with 500nM TPA are appropriate conditions to test the DN PKCs functionality as they should cause the degradation of the WT and endogenous PKC but not the DN.

Thus TAM-R cells were either infected with PKC-α WT or DN, or PKC-δ WT or DN at MOI 100, or left uninfected as controls. The cells were then allowed to grow for 16 hours, the media changed for media containing 500nM TPA or left untreated as controls. After 24 hours the cells were harvested and assayed by Western analysis (section 2.3.3) with PKC-α and δ specific antibodies. Figure 5.9 shows that the TPA treatment was effective in inducing proteolytic degradation of endogenous PKC-α in control and PKC-α overexpressing cells. Moreover the level of PKC-α in the DN infected cells remained at a level comparable to the non-TPA treated control cells indicating that they were indeed insensitive to TPA induced degradation. The cells infected with the PKC-δ DN also retained greater levels of PKC-δ after TPA treatment than the uninfected control cells and the WT infected cells. As the expressed PKC-α and PKC-δ DNs were insensitive to degradation by TPA this confirms that they were unable to autophosphorylate and therefore truly kinase deficient.

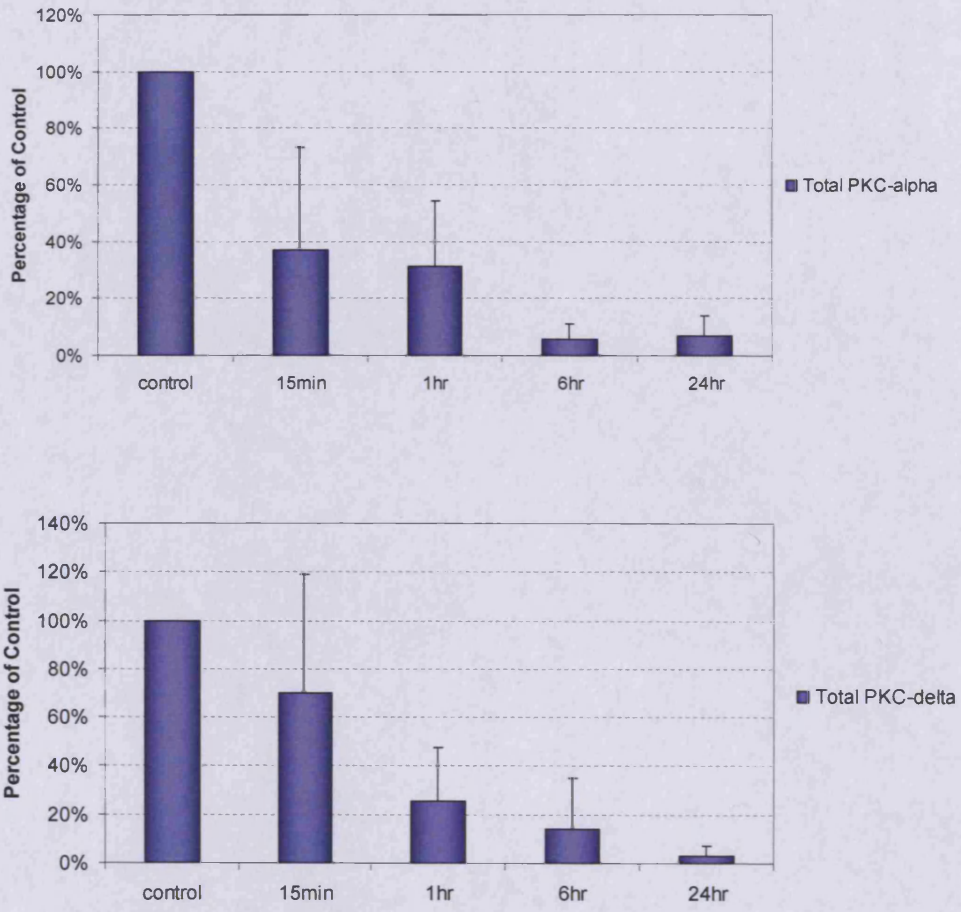


Figure 5.7. The effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on PKC-α and PKC-δ expression in the MCF-7 cell line

MCF-7 cells were grown in media containing 5% charcoal stripped FCS ± 500nM TPA for 15 minutes, 1 hour, 6 hours or 24 hours. Proteolytic degradation of PKC-α and PKC-δ was measured by Western analysis and expressed as a percentage of control levels at each of the time points ± SD (n=3 independent experiments).

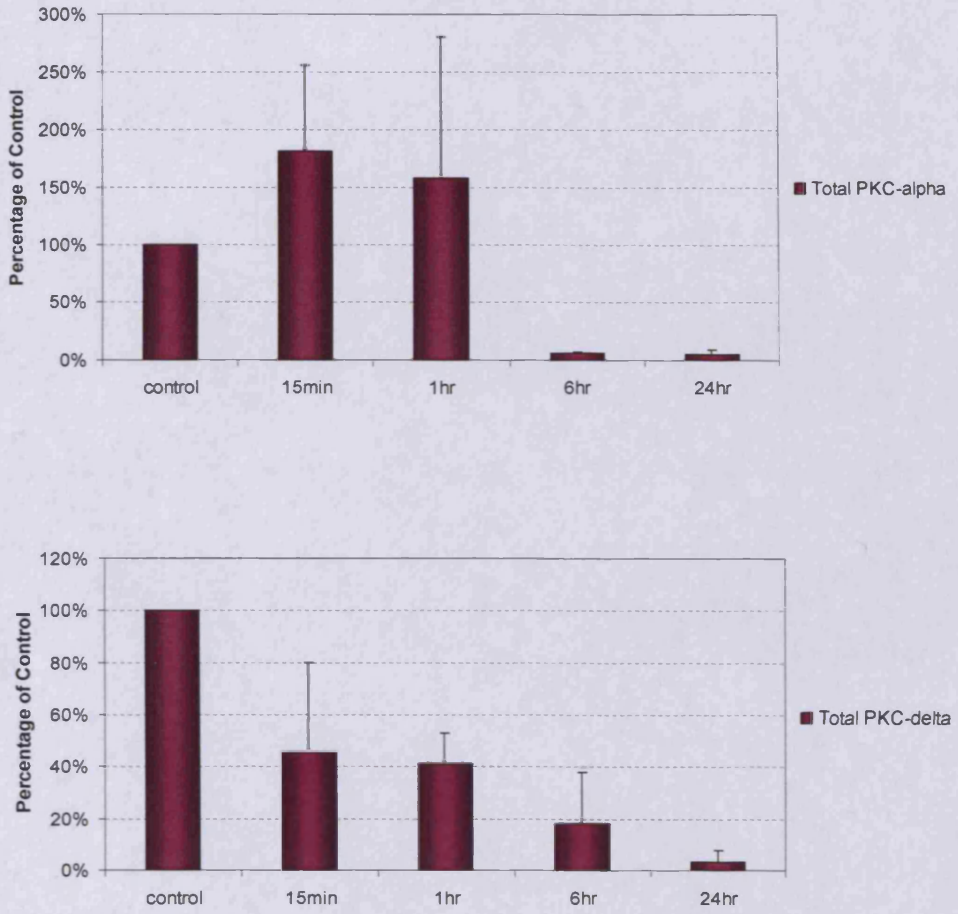


Figure 5.8. The effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on PKC-α and PKC-δ levels in the TAM-R cell line

TAM-R cells were grown in media containing 5% charcoal stripped FCS ± 500nM TPA for 15 minutes, 1 hour, 6 hours or 24 hours. Proteolytic degradation of PKC-α and PKC-δ was measured by Western analysis and expressed as a percentage of control levels at each of the time points ± SD (n= three independent experiments).

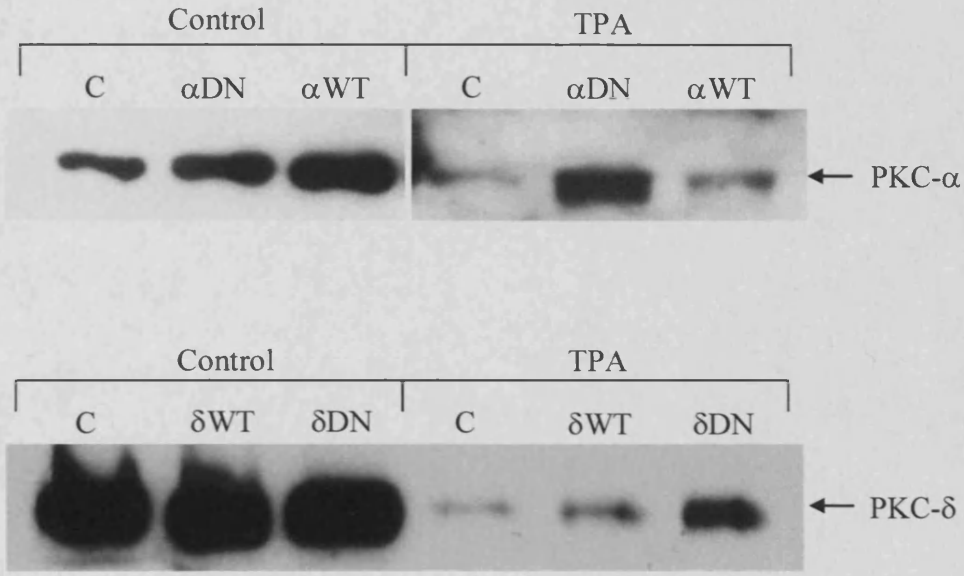


Figure 5.9. Prolonged TPA treatment does not lead to proteolytic degradation of PKC in DN expressing cells

Tam-R cells infected at MOI 100 with PKC-α DN, PKC-α WT, PKC-δ DN or PKC-δ WT expressing adenoviral vectors and grown for 48hour, were treated ± 500nM TPA for 24 hours. The cells were lysed as outlined in section 2.3.1 and expression of PKC-α and PKC-δ assessed by Western analysis using isoform specific antibodies.

5.2.6 In vitro kinase assay of PKC-δ activity in TAM-R cells infected with either PKC-δ DN or WT expressing adenoviral vector

To demonstrate conclusively that the PKC-δ WT and PKC-δ DN constructs are functionally active or kinase deficient respectively, an *in vitro* kinase assay was performed (section 2.7.7) to measure changes in PKC-δ activity in TAM-R cells infected with the adenoviruses (section 2.7.4). Figure 5.10 shows the mean percentage P^{32} incorporation relative to control from 3 independent experiments. It can be seen that there is an inhibition of PKC-δ activity in the cells infected with the PKC-δ DN virus relative to control and conversely an increase in PKC-δ activity in the cells infected with the PKC-δ WT expressor. These results however are not statistically significant and the effects not of the magnitude one would expect. Unfortunately time constraints meant that the *in vitro* kinase assay was not fully optimised and I would expect that further repeats of the experiment would yield more conclusive results.

5.2.7. The effect of dominant negative PKC-α and PKC-δ on MCF-7 and TAM-R cell growth.

In Chapter 3 we demonstrated that the PKC inhibitor bisindolylmaleimide IX (bis) inhibited growth of the MCF-7 cell line and, to a much greater extent, the TAM-R cell line (Figure 3.12). This implied that TAM-R cells have an enhanced reliance on PKC for serum induced growth. However as discussed previously (section 3.3), bis cannot discern between the different PKC isoforms and has been reported to have effects on non-related kinases (Hers *et al.*, 1999). We therefore wanted to determine the relative importance of PKC-α and δ in the MCF-7 and TAM-R cell lines. To this end we adenovirally infected both cell lines with dominant negative expressing vectors of these isoforms (section 2.7.4) with their growth measured by Coulter counting at days 4 and 8 post infection (section 2.2.6).

From Figure 5.11 it can be seen that infecting the MCF-7 cells with PKC-α or δ DN has very little effect on growth at day 4 and only a slight inhibitory effect at day 8. The effect of PKC-α and δ DN on MCF-7 growth was repeated in a further 5

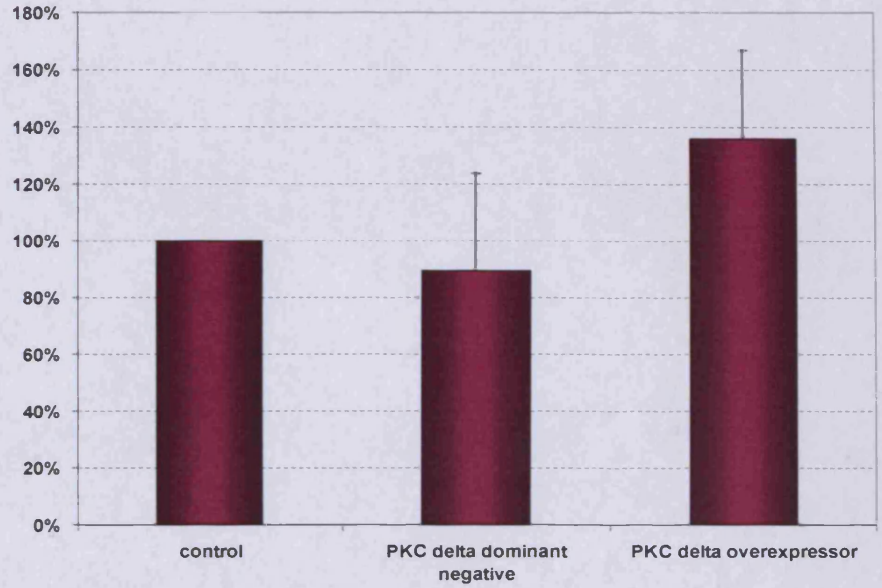


Figure 5.10. In-vitro kinase assay of PKC-δ activity in TAM-R cells infected with either a PKC-δ expressing (WT) or PKC-δ dominant negative (DN) expressing adenoviral vector

TAM-R cells were grown for 24 hours prior to infection at MOI 100 with either a PKC-δ expressing (WT) or PKC-δ dominant negative (DN) expressing adenoviral vector. After 24 hours the cells were lysed and the activity of PKC-δ assessed by *in vitro* kinase assay as outline in section 2.7.7. The graph represents the percentage pmol P^{32} incorporation relative to control ± SD (n= 3 independent experiments).

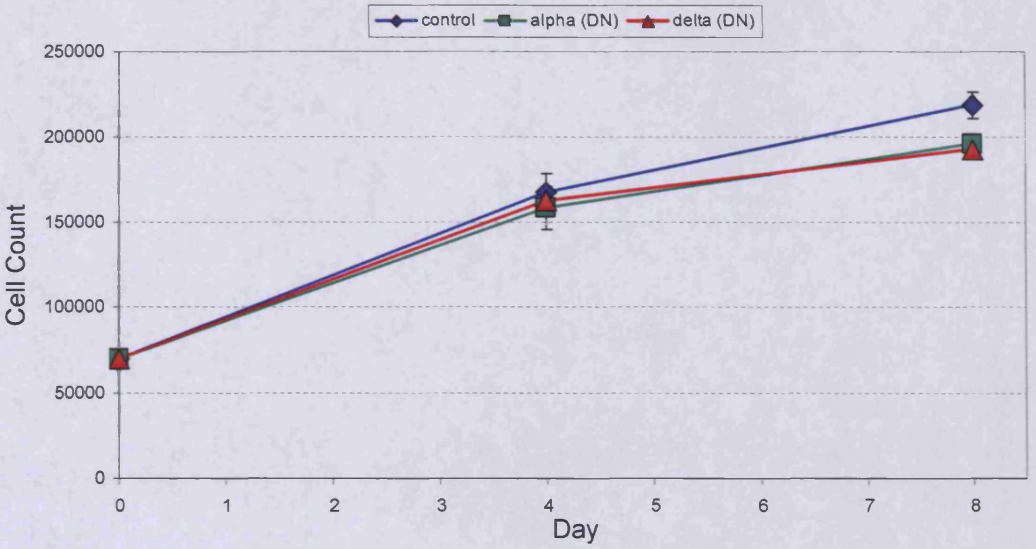


Figure 5.11 Growth curve of MCF-7 cells adenovirally infected with either PKC-α or PKC-δ dominant negative (DN) expressing plasmid at MOI 100

MCF-7 cells were infected with either PKC-α or PKC-δ dominant negative (DN) expressing plasmid at an MOI of 100 for 16 hours and the media changed. The cells were counted by Coulter counting (section 2.2.6) on the day of infection (day 0), day 4 and day 8. The graph is representative of 3 pairs of counts ± SEM.

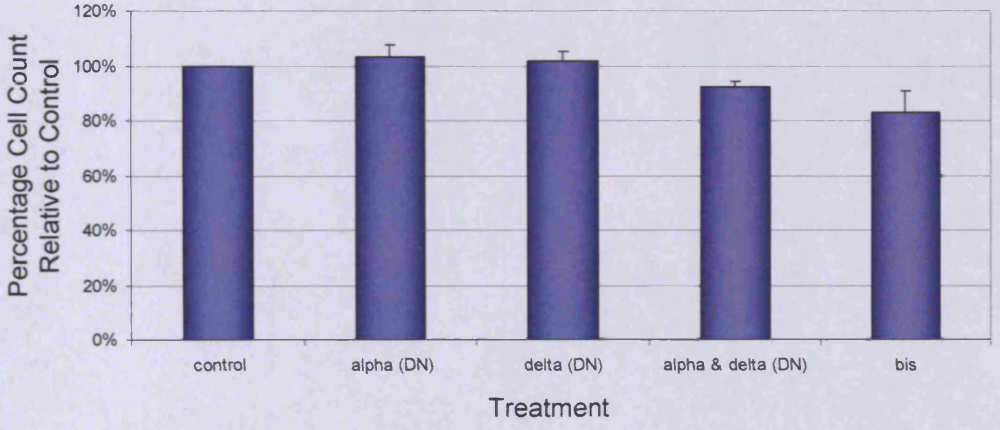


Figure 5.12 Growth study of MCF-7 cells adenovirally infected with PKC-α and/or PKC-δ dominant negative (DN) expressing plasmids

MCF-7 cells were infected with PKC-α and/or PKC-δ dominant negative (DN) expressing plasmid at an MOI of 100. Control cells were grown with empty virus or 500nM bis and grown for 4 days post treatment. The cells were counted by Coulter counting (section 2.2.6) on the day of treatment (day 0) and on day 4 post treatment. The graph shows 15 pairs of counts from 5 independent experiments ± SEM.

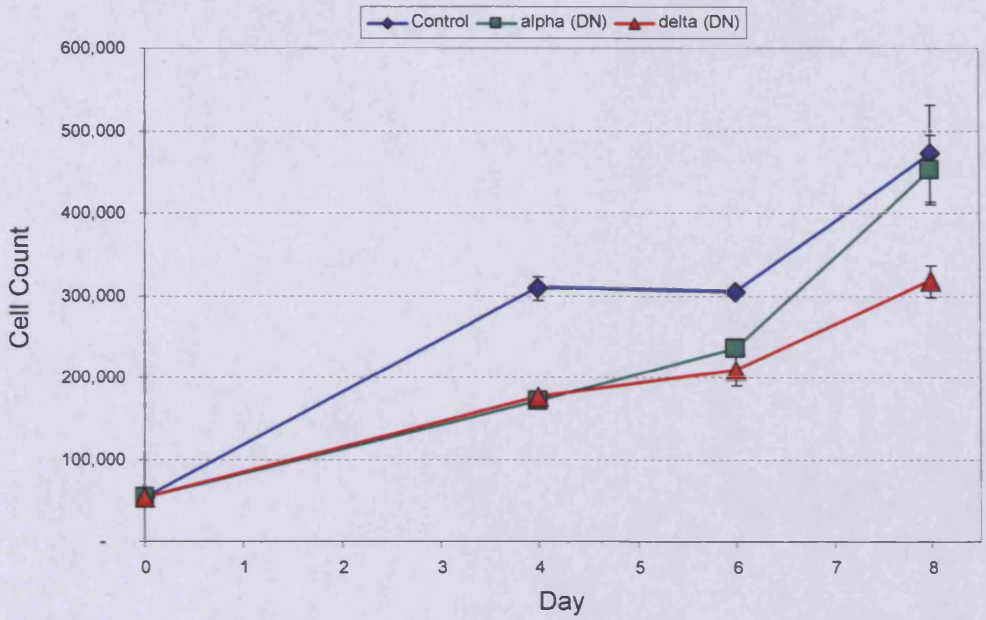


Figure 5.13 Growth curve of TAM-R cells adenovirally infected with either PKC-α or PKC-δ dominant negative (DN) expressing plasmid

TAM-R cells were infected with either PKC-α or PKC-δ dominant negative (DN) expressing plasmid at an MOI of 100 for 16 hours and the media changed. The cells were counted by Coulter counting (section 2.2.6) on the day of infection (day 0), day 4 and day 8. Each point represents 3 pairs of counts ± SEM.

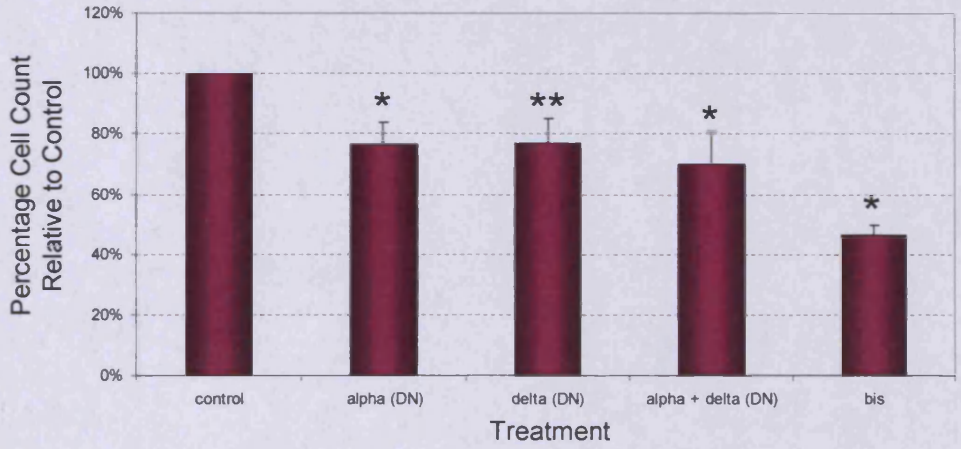


Figure 5.14 Growth study of TAM-R cells adenovirally infected with PKC-α and/or PKC-δ dominant negative (DN) expressing plasmids

TAM-R cells were infected with PKC-α and/or PKC-δ dominant negative (DN) expressing plasmid at an MOI of 100. Control cells were grown with empty virus or 500nM bis and grown for 4 days post treatment. The cells were counted by Coulter counting (section 2.2.6) on the day of treatment (day 0) and on day 4 post treatment. The graph shows 12 pairs of counts from 4 independent experiments ± SEM. (*$p<0.05$, **$p<0.005$ compared to control)

separate experiments at the day 4 timepoint, along with the effect of infecting with both PKC-α and δ dominant negatives together, and compared to treating with 500nM bis. The results of this experiment are shown in Figure 5.12, with cell number after four days growth expressed as a percentage relative to control cells (±SEM). It can be seen that infection separately with the PKC-α and δ DN viruses produced no effect on growth in the MCF-7 cell line relative to the control cells. Infecting with the viruses in combination produced a slight growth inhibitory effect, although this could be due to non-specific effects caused by the increased infection level with a total MOI of 200 (due to two viruses at MOI 100 each). The bis control produced a growth inhibitory effect of around 18% after four days treatment, an inhibition comparable with that seen in Chapter 3 (Figure 3.12).

When TAM-R cells were examined in a similar way, except that the cells were counted at day 6 in addition to day 0, 4 and 8, the PKC-α and δ adenoviruses were seen to have a much more significant effect on growth compared to the control cells, particularly at day 4 (Figure 5.13). The growth inhibitory effects were still marked at day 6 with both PKC DNs, but by day 8 only the PKC-δ DN displayed continued growth inhibition. The short duration of response could be due to the DN effect being out grown with time as infection with the replication deficient adenovirus is transient and the cells were only infected once at day 0. The effect of PKC-α and δ DN on the TAM-R growth was examined in a further 4 separate experiments at the day 4 time point, and again the combined effect of infection with both PKC-α and δ dominant negatives assessed and compared to the growth inhibitory effects of 500nM bis. Compared to the situation seen in the MCF-7 cells, the infection of the TAM-R cells with PKC-α and PKC-δ DNs produced a significant inhibition of growth of over 20% compared to control after 4 days. Interestingly, infecting with both adenoviruses together produces a slight additive effect although again this could be attributed to the increase in MOI. As would be expected, the bis had a far greater effect on growth in the TAM-R cells as observed previously in Chapter 3 (Figure 3.12) causing over 50% growth inhibition compared to controls. These results show that PKC-α and δ are more important for growth in the TAM-R cell line than in the MCF-7 cell line. Although the growth inhibition seen with the DNs was smaller than with the chemical inhibitor bis, this could be accounted for by the non-specific effects of bis, or due to

the possible scope for further optimisation of the DN conditions. Importantly however, we present here clear evidence of a specific role for PKC-α and δ in TAM-R growth which can truly be said to be specific for the isoform concerned.

5.2.8. The effect of dominant negative PKC-δ on ERK 1/2 activation in the TAM-R cell line.

It has been previously shown by Ueda *et al.* (1996) using constitutively active mutants, that PKC-δ, but not PKC-α or ε, can activate the mitogen activating protein kinase ERK 1/2. It has also been shown in the MCF-7 cell line that PKC-δ is upstream of the ras/raf/MEK pathway which leads to ERK 1/2 activation (Keshamouni *et al.*, 2002). As our TAM-R cell model has been shown to possess greatly increased levels of activated ERK 1/2 compared to the MCF-7 cell line (Nicholson *et al.*, 2004), we decided to investigate whether PKC-δ was also upstream of ERK 1/2 in these cells by infecting them with the PKC-δ DN adenovirus and, 72 hours post infection, harvesting the cells and assaying them for ERK 1/2 activation by Western analysis (section 2.3.3). Figure 5.15 represents the percentage ERK 1/2 phosphorylation relative to uninfected control cells ±SD from 3 separate experiments. Infecting the TAM-R cells with the PKC-δ DN caused an inhibition of ERK 1/2 phosphorylation of around 60%. Although the inhibition is not statistically significant ($p=0.31$), this inhibitory effect was seen to varying degrees in all 3 of the separate experiments. This indicates a role for PKC-δ in serum induced ERK 1/2 phosphorylation in the TAM-R cell line

5.2.9. The effect of IGF-I, IGF-II, EGF and HRG-β1 on PKC-α and δ activation in the MCF-7 and TAM-R cell lines

It has now been widely documented that breast cancers can be initiated and propagated through the inappropriate activation and utilisation of a multitude of growth factor signalling pathways. This deregulation can be the manifestation of enhanced growth factor ligand production and/or the up-regulation of their target receptors. Among the receptors and ligands implicated in breast cancer, IGF-I and II acting through the IGF-I receptor (IGF-IR) (Jones *et al.*, 2004)), and EGF (Nicholson

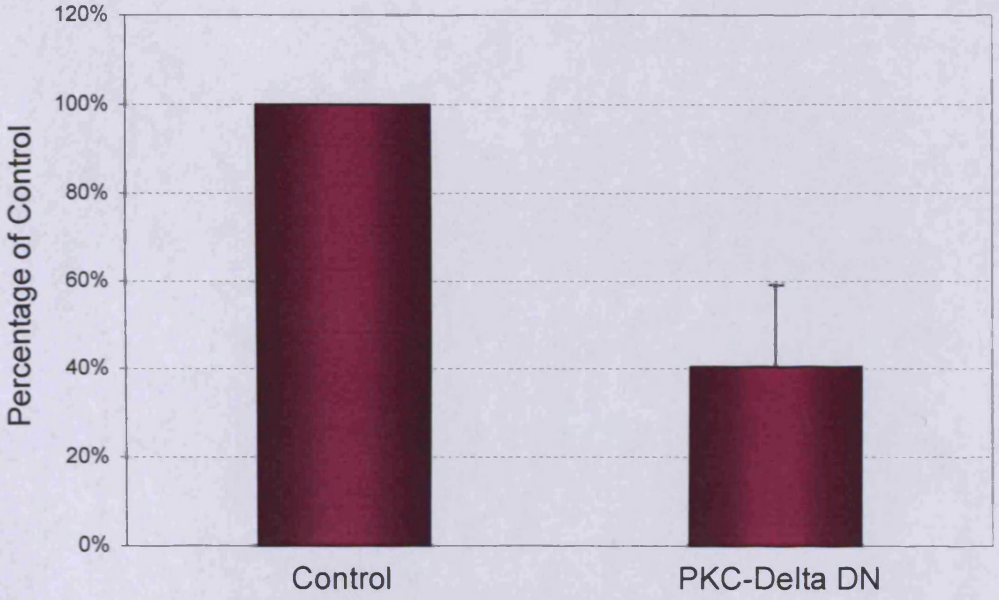


Figure 5.15. Relative levels of phosphorylated ERK 1/2 in TAM-R cells infected with a PKC-δ dominant negative (DN) expressing vector.

TAM-R cells were grown for 48 hours in 5% csFCS prior to adenoviral infection at MOI 100 with a PKC-δ expressing vector. After 16 hours the media was changed and the cells allowed to grow for a further 48 hours. The cells were then harvested and analysed by Western analysis as outlined in section 2.3 using antibodies specific for phosphorylated ERK 1/2. The graph represents the mean level of phosphorylated ERK 1/2 relative to control from 3 separate experiments.

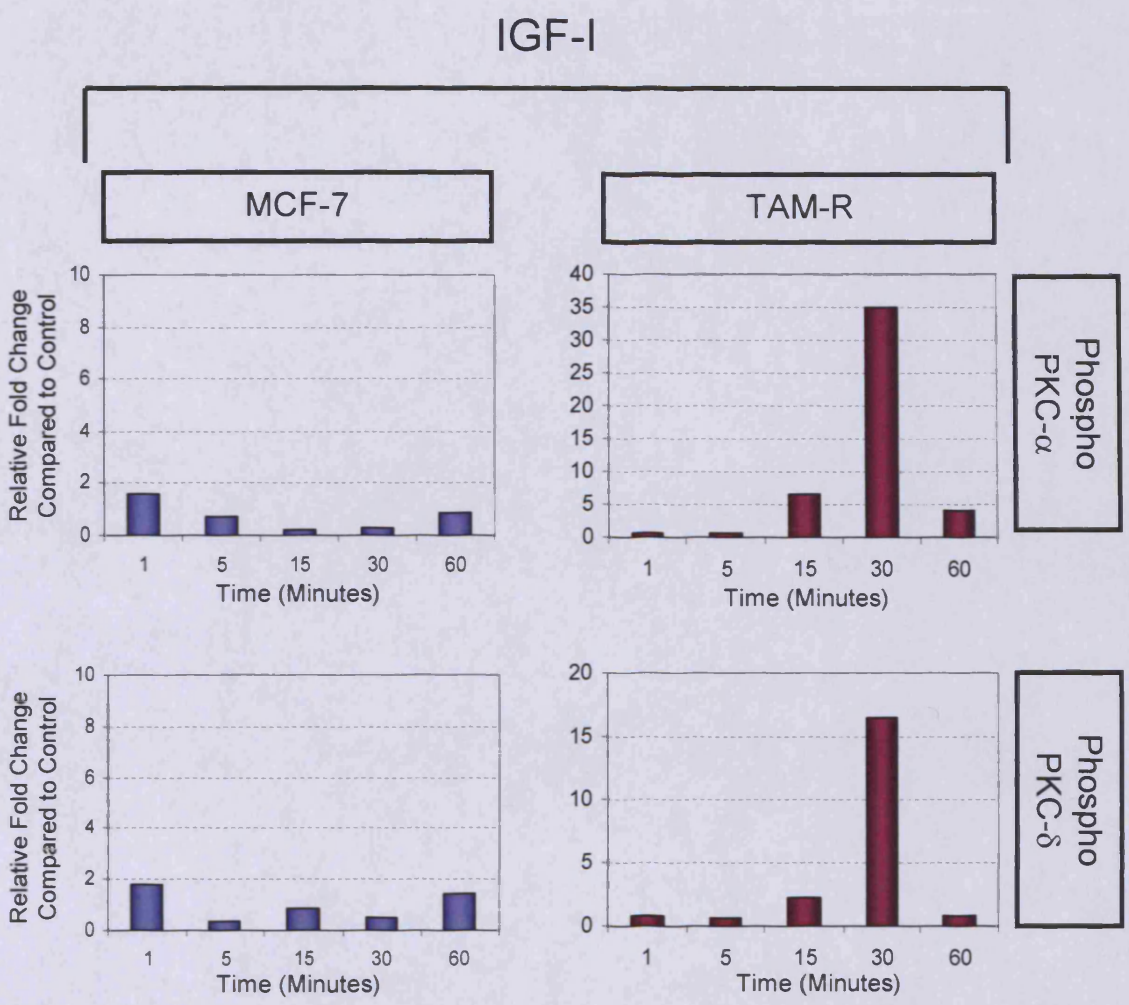


Figure 5.16. PKC-α and PKC-δ phosphorylation after short term treatment of MCF-7 and TAM-R cells with IGF-I

MCF-7 and TAM-R cells were treated for between 1 and 60 minutes with IGF-I (10ng/ml). At each time point the cells were harvested along with untreated control cells. The above graphs represent the level of phosphorylated PKC-α and PKC-δ in the treated cells relative to the untreated cells as determined by Western analysis (section 2.3).

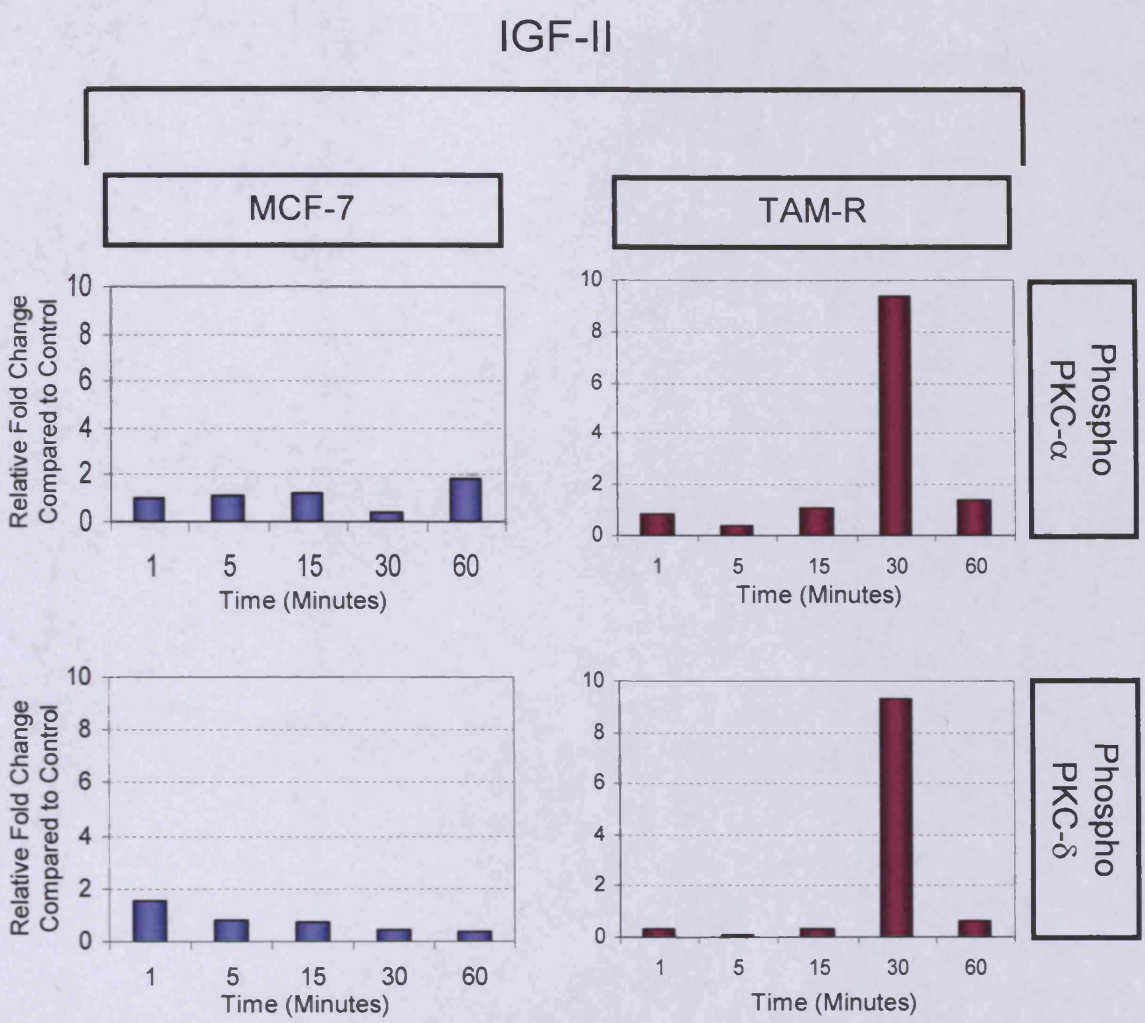


Figure 5.17. PKC-α and PKC-δ phosphorylation after short term treatment of MCF-7 and TAM-R cells with IGF-II

MCF-7 and TAM-R cells were treated for between 1 and 60 minutes with IGF-II (10ng/ml). At each time point the cells were harvested along with untreated control cells. The above graphs represent the level of phosphorylated PKC-α and PKC-δ in the treated cells relative to the untreated cells as determined by Western analysis (section 2.3).

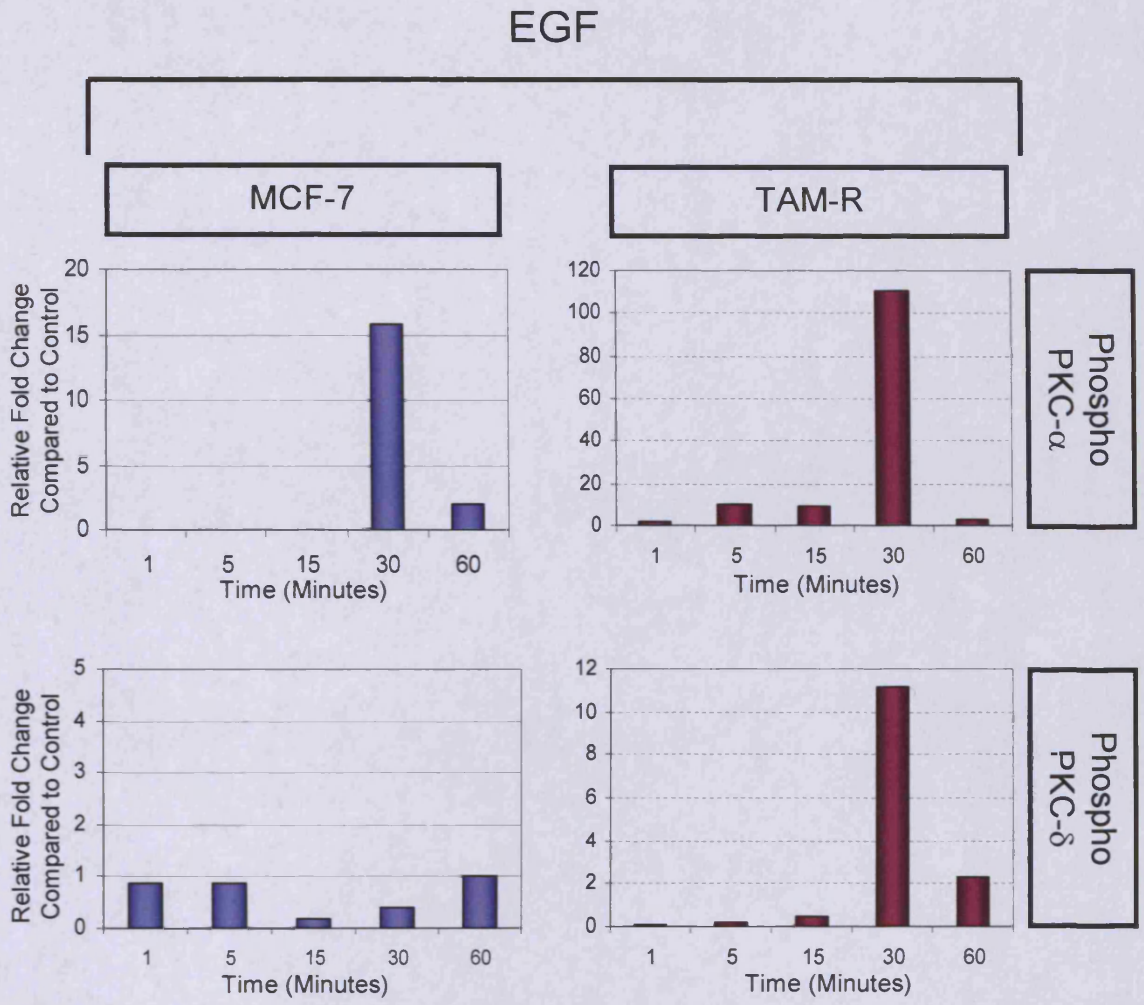


Figure 5.18. PKC-α and PKC-δ phosphorylation after short term treatment of MCF-7 and TAM-R cells with EGF

MCF-7 and TAM-R cells were treated for between 1 and 60 minutes with EGF (10ng/ml). At each time point the cells were harvested along with untreated control cells. The above graphs represent the level of phosphorylated PKC-α and PKC-δ in the treated cells relative to the untreated cells as determined by Western analysis (section 2.3).

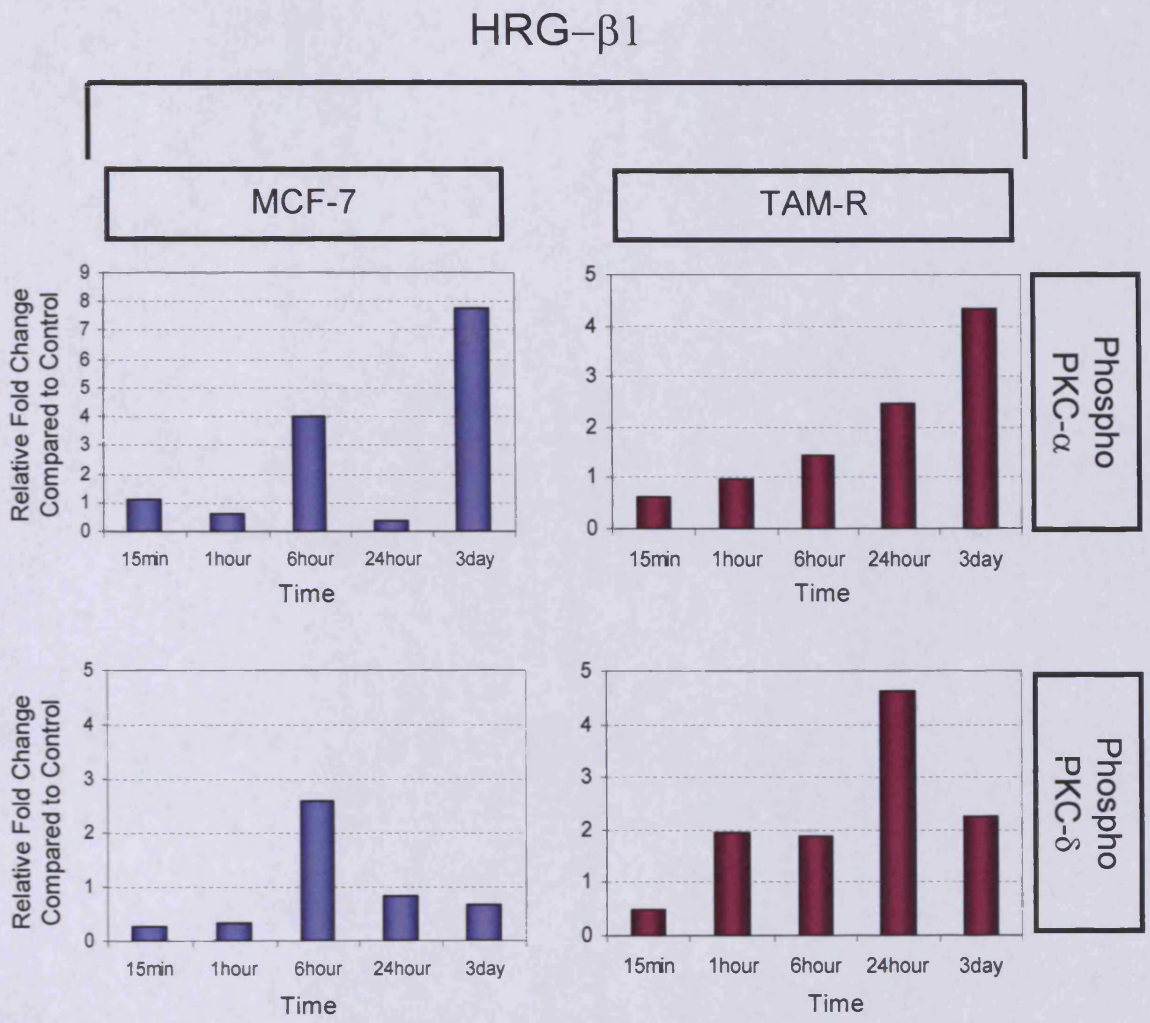


Figure 5.19. PKC-α and PKC-δ phosphorylation after short term treatment of MCF-7 and TAM-R cells with HRG-β1

MCF-7 and TAM-R cells were treated for between 15 minutes and 3 days with HRG-β1 (10ng/ml). At each time point the cells were harvested along with untreated control cells. The above graphs represent the level of phosphorylated PKC-α and PKC-δ in the treated cells relative to the untreated cells as determined by Western analysis (section 2.3).

et al., 2001) and the heregulins (Tang *et al.*, 1996) acting through the Erb/Her family of receptors, have been further implicated as signalling strategies through which breast cancer cells can circumvent the effects of antihormone therapy. To ascertain whether the signalling pathways that originate from these receptor-ligands interactions employ the activation of PKC-α or δ in the MCF-7 and TAM-R cell lines, they were treated with IGF-I, IGF-II or EGF for 1, 5, 15, 30 and 60 minute durations and assayed for levels of phosphorylated PKC-α or δ by Western analysis (section 2.3.3). The levels of phosphorylated PKC-α or δ are expressed as fold change relative to the untreated controls at each time point (Figures 5.16, 5.17 and 5.18). The MCF-7 cells treated with either IGF-I or II displayed no increase in the phosphorylation of PKC-α or PKC-δ. The TAM-R cells, however, displayed a large transient increase in both PKC-α and PKC-δ phosphorylation when treated with IGF-I or II for 30 minutes. This increase in PKC-α and δ phosphorylation also occurs in the TAM-R cells treated with EGF for 30 minutes. In addition there was an increase in PKC-α phosphorylation in the MCF-7 cell line after 30 minutes treatment with EGF, though without the concomitant increase in PKC-δ phosphorylation observed in the TAM-R cell line. The MCF-7 and TAM-R cell lines were also treated with HRG-β1 at 15 minute, 1 hour, 6 hour, 24 hour and 3 day time periods and the levels of activated PKC-α and δ assayed by Western analysis. The levels of phosphorylated PKC-α or δ are expressed in Figure 5.19 as fold change relative to the untreated controls at each time point. The MCF-7 cells displayed an increase in PKC-α and δ phosphorylation after 6 hours treatment with an increase in PKC-α phosphorylation also evident after 3 days continuous HRG-β1 treatment. The TAM-R cells displayed a gradual increase in PKC-α phosphorylation over the three day treatment whilst PKC-δ phosphorylation is at its greatest after 24 hours treatment.

5.2.10. The effect of inhibiting PKC-α and δ on IGF-I, IGF-II, EGF, HRG-β1 and oestrogen stimulated growth in the TAM-R cell line

As we have shown that each of the growth factors tested in section 5.2.9 can induce the phosphorylation of PKC-α and δ in the TAM-R cell line we decided to investigate whether inhibiting these isoforms impinges on each of the ligands growth stimulatory

Table 5.2. Summary table of the effect of PKC-α DN, PKC-δ DN and bisindolylmaleimide IX (500nM) on the growth of TAM-R cells treated with IGF-I, IGF-II, EGF, HRG-β1 (10ng/ml) or Oestrogen (E2) (10^{-9}M) for four days.

The growth is expressed as the mean percentage number of cells, relative to control, from 12 pairs of counts from 4 separate experiments ± SEM. The significance was analysed by paired t-test on actual cell numbers using SPSS software.

Conditions				
Treatment	**Inhibitor**	**% growth relative to control**	**± SEM**	**Significance (p=)**
Control	Control	100	-	-
	PKC-α DN	65	10	0.05
	PKC-δ DN	78	6	0.03
	Bis	53	9	0.02
IGF-I	Control	142	8	0.02
	PKC-α DN	95	9	0.03
	PKC-δ DN	109	7	0.06
	Bis	65	7	0.02
IGF-II	Control	217	28	0.04
	PKC-α DN	93	9	0.05
	PKC-δ DN	107	9	0.06
	Bis	71	7	0.04
EGF	Control	220	33	0.05
	PKC-α DN	101	7	0.05
	PKC-δ DN	146	18	0.06
	Bis	97	8	0.04
HRG-β1	Control	155	20	0.05
	PKC-α DN	116	5	0.02
	PKC-δ DN	119	4	0.01
	Bis	101	5	0.03
E2	Control	207	34	0.04
	PKC-α DN	135	4	0.02
	PKC-δ DN	135	5	0.00
	Bis	97	4	0.03

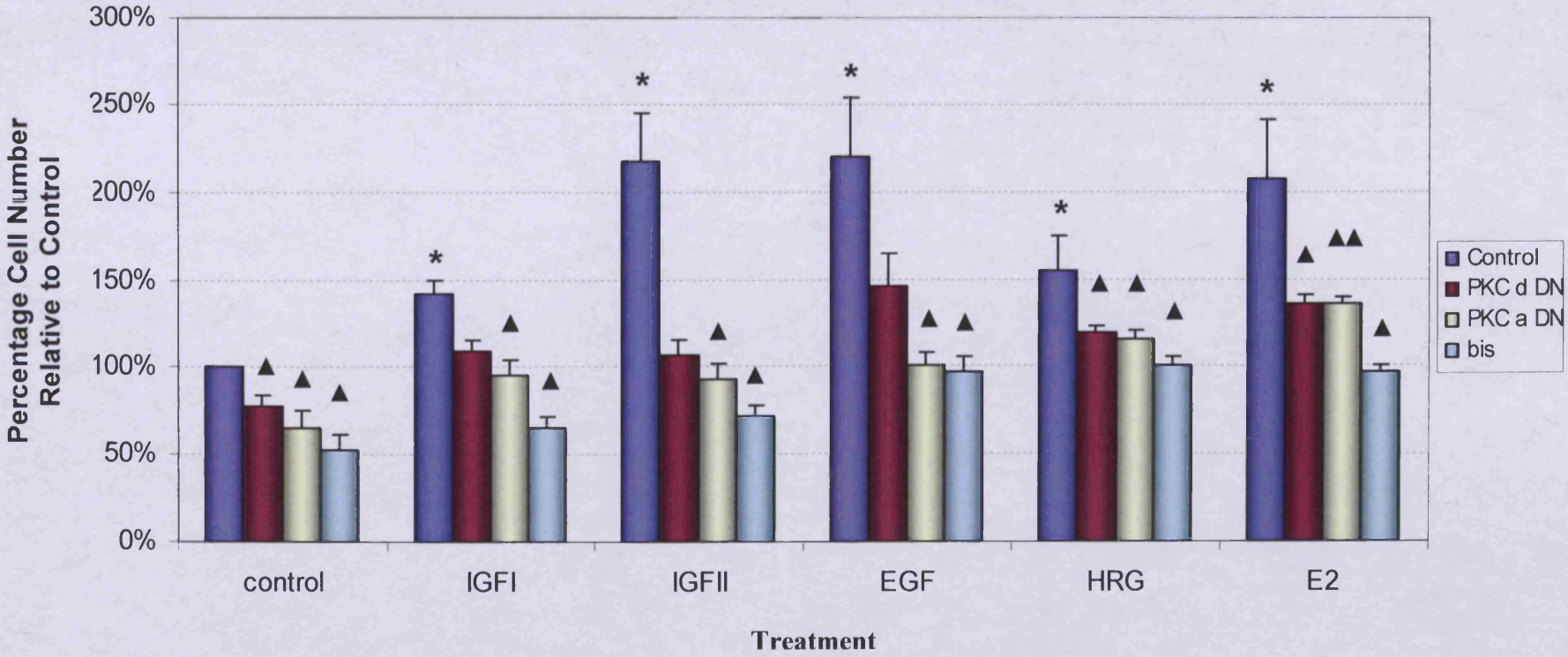


Figure 5.20. Graph showing the effect of infecting TAM-R cells with PKC-α DN or δ DN vectors or bisindolylmaleimide on growth factor stimulated growth after four days

TAM-R cells were adenovirally infected at MOI 100 with PKC-α DN or PKC-δ DN vectors or bisondolylmaleimideIX (bis) (500nM). After 16 hours the media was changed and the cells treated with either IGF-I, IGF-II, EGF, HRG-β1 (all 10ng/ml) or oestrodiol (E2) (10^{-9}M) for 4 days. After this time the cells were counted and expressed as the mean percentage number of cells relative to the uninfected, untreated controls. The above graph is representative of 12 pairs of counts from 4 separate experiments ± SEM. Statistical significance was calculated from the cell numbers by paired t-test using the SPSS software package (Significance between uninfected growth factor treated cells and uninfected, untreated control: *= 0.05, significance between infected, growth factor treated sample and uninfected control samples treated with same growth factor: ▲= 0.05, ▲▲= 0.005).

effects. In addition, we investigated whether oestrogen could elicit a growth stimulatory effect on the TAM-R cells, and if this effect could also be inhibited by PKC-α and δ. TAM-R cells were adenovirally infected with PKC-α or PKC-δ DNs (section 2.7.4) or treated with 500nM bis 16 hours prior to treatment with IGF-I, IGF-II, EGF, HRG-β1 or oestrogen for four days, after which time the cell growth was assessed by Coulter counting (section 2.2.6). Table 5.2 summarises the results from 4 separate experiments as percentage cell number relative to untreated control ± SEM. These values are also represented graphically in Figure 5.20. All the growth factors tested increased levels of TAM-R cell growth after four days. Treatment with IGF-I and HRG-β1 induced growth around 150% that of control whilst growth with IGF-II and EGF treatment was over 200% that of control. Interestingly oestrogen induced an increase in growth of around 200% even though the TAM-R cells home medium contains 10^{-7}M tamoxifen. Inhibiting the untreated cells with PKC-α DN, PKC-δ DN and bis caused growth inhibition after 4 days at levels comparable with those seen in Figure 5.14. Treatment with the PKC-α dominant negative significantly inhibited the growth stimulatory effects of each of the growth factor ligands to levels comparable with the untreated control. There is also a marked inhibition of growth when the cells were infected with the PKC-δ DN. The inhibition of growth factor induced growth stimulation is even more marked with the bis compound than with the DNs, though as previously discussed (section 3.3) this is most likely due to its inhibition of several related and non-related kinases. The treatment of the TAM-R cells with PKC-α DN, PKC-δ DN or bis inhibited oestrogen stimulating growth, highlighting a possible role for PKC-α and δ in oestrogen stimulated growth of the TAM-R cell line.

5.2.11. The effect of oestrogen receptor modulation on PKC-α and δ phosphorylation in the MCF-7 and TAM-R cell lines

As we have shown that inhibition of PKC-α and δ inhibits oestrogen induced growth of the TAM-R cell line we decided to investigate the effect of modulating the oestrogen receptor on the activation of PKC-α and δ. We treated the MCF-7 cell line with the tamoxifen, Fulvestrant or oestrogen for 7 days. Treatment with tamoxifen

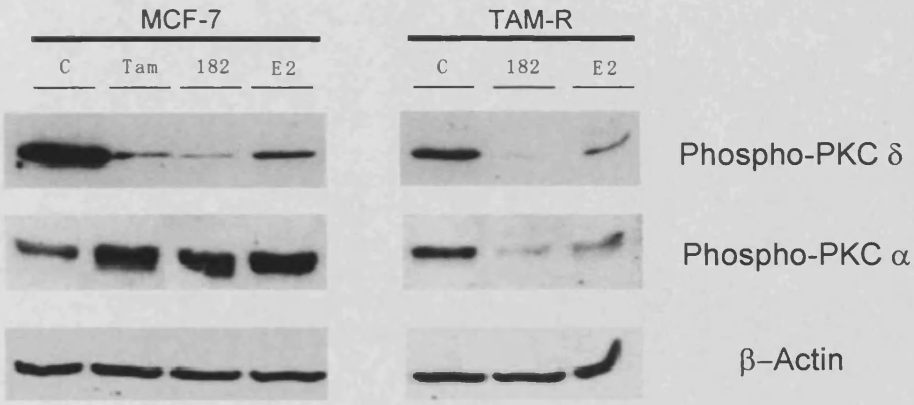


Figure 5.21. The effect of oestrogen receptor modulation on the activation of PKC-α and PKC-δ.

MCF-7 cells were treated for 7 days with either tamoxifen (10^{-7}M), Fulvestrant (182) (10^{-7}M), or oestrogen (E_2) (10^{-9}M) with control cells left untreated. TAM-R cells were treated for 7 days with either 182 (10^{-7}M) or E_2 (10^{-9}M). The cells were then harvested and analysed for levels of phosphorylated PKC-α or PKC-δ by Western analysis (section 2.3). The blotted membranes were also probed for β-actin as a loading and protein concentration control.

inhibits ER signalling through the ligand dependent trans-activational function (AF-2) (MacGregor and Jordan, 1998), Fulvestrant is a pure anti-oestrogen (Howell *et al.*, 2004) and prolonged treatment with oestrogen is known to cause the downregulation of the ER (Borras *et al.*, 1994). We also treated the TAM-R cell line with fulvestrant and oestrogen but not additional tamoxifen as it is already supplemented in their home media. After 7 days treatment, the cells were harvested and assayed by Western analysis for levels of activated PKC-α and δ using phosphorylation specific antibodies. The blotted membranes were also probed for β-Actin as a protein concentration and loading control. The MCF-7 cells treated with tamoxifen, fulvestrant and oestrogen all showed reduced activation of PKC-δ with a concomitant increase in PKC-α phosphorylation. This indicates that signalling through the oestrogen receptor affects pathways leading to the phosphorylation of PKC-δ. It also appears that the downregulation of the ER leads to signalling pathways that involve the phosphorylation of PKC-α. Treatment of the TAM-R cells with fulvestrant and prolonged oestrogen treatment also lead to decreased levels of PKC-δ, however instead of PKC-α being activated it was also downregulated. This implies that even in the presence of tamoxifen, the oestrogen receptor can still signal through pathways that involve PKC-α and δ.

5.2.12. The effect of upregulating PKC-α and δ on IGF-I, IGF-II, EGF, HRG-β1 and oestrogen stimulated growth in the MCF-7 cell line

We have shown that inhibiting PKC-α and δ causes a reduction in growth stimulation by growth factors and oestradiol in the TAM-R cell line. We decided to determine whether overexpressing these isoforms would have the reverse effect in the MCF-7 cell line and cause an increase in growth upon stimulation with IGF-I, IGF-II, EGF, HRG-β1 or oestrogen. Therefore, we infected the MCF-7 cell line with the PKC-α and δ WT overexpressing adenoviruses, changed the media after 16 hours and treated the cells for four days with each of the aforementioned ligands. The cell growth was then assessed by Coulter counting (section 2.2.6). The mean percentage cell count from 5 separate experiments are expressed relative to untreated control ± SEM in Table 5.3 and displayed graphically in Figure 5.22.

Table 5.3. Summary table of the effect of PKC-α WT and PKC-δ WT overexpressors on the growth of TAM-R cells treated with IGF-I, IGF-II, EGF, HRG-β1 (10ng/ml) or Oestrogen (E_2) (10^{-9}M) for four days.

Growth expressed as the mean number of cells expressed as a percentage relative to control for 15 pairs of counts from 5 separate experiments ± SEM. The data was analysed statistically by paired t-test on actual cell numbers using the SPSS software.

Conditions				
Treatment	**Overexpressor**	**% growth relative to control**	**± SEM**	**Significance (p=)**
Control	Control	100	-	-
	PKC-α WT	116	5	0.021
	PKC-δ WT	129	11	0.023
IGF-I	Control	139	7	0.000
	PKC-α WT	152	8	0.083
	PKC-δ WT	162	14	0.035
IGF-II	Control	149	10	0.000
	PKC-α WT	164	13	0.023
	PKC-δ WT	146	13	0.632
EGF	Control	140	14	0.008
	PKC-α WT	162	22	0.049
	PKC-δ WT	169	17	0.011
HRG-β1	Control	191	20	0.000
	PKC-α WT	211	28	0.068
	PKC-δ WT	191	23	0.687
E2	Control	201	24	0.000
	PKC-α WT	238	31	0.266
	PKC-δ WT	211	35	0.523

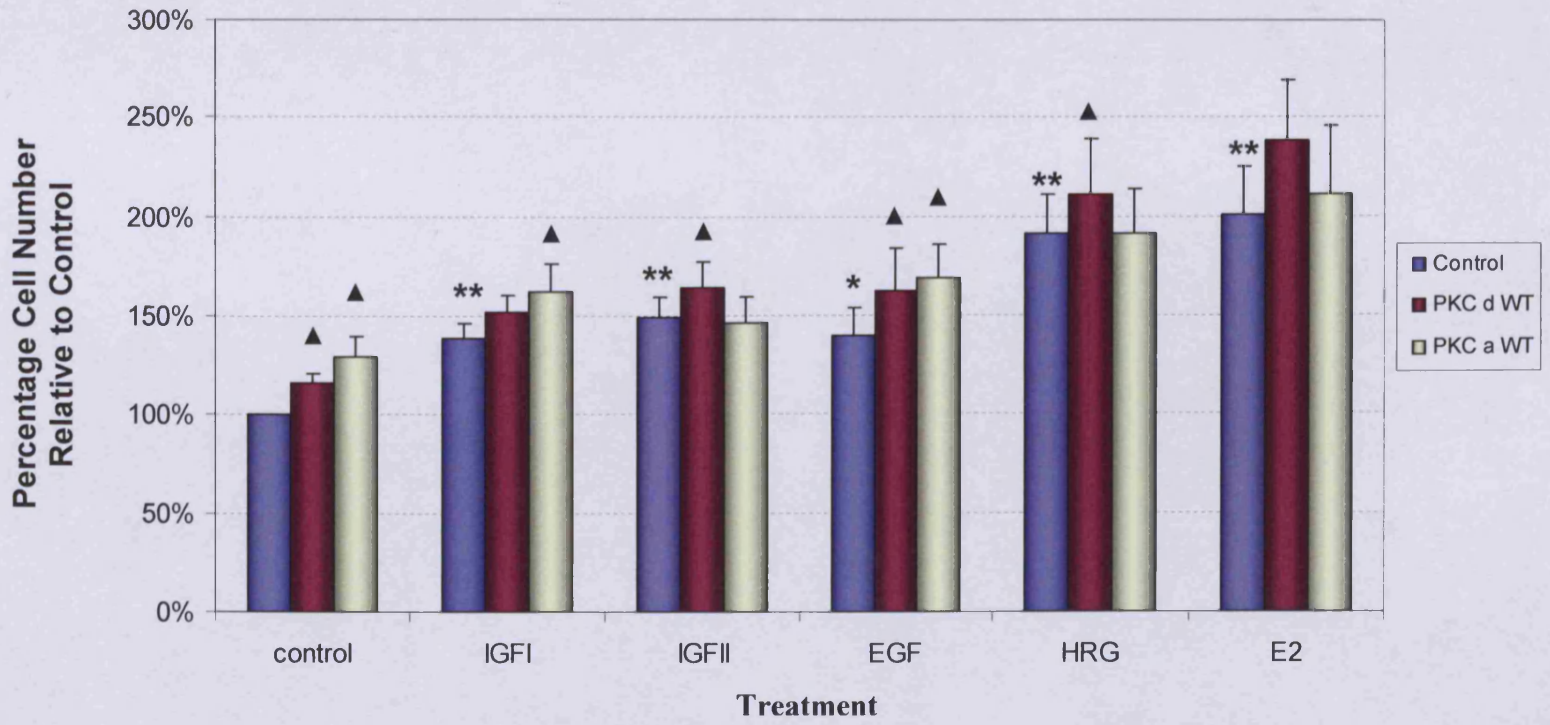


Figure 5.22. Graph showing the effect of infecting MCF-7 cells with PKC-α and δ WT overexpressing vectors on growth factor stimulated growth after four days

MCF-7 cells were adenovirally infected at MOI 100 with PKC-α or PKC-δ WT overexpressing vectors and, after 16 hours, the media changed and the cells treated with either IGF-I, IGF-II, EGF, HRG-β1 (all 10ng/ml) or oestrodiol (E_2) (10^{-9}M) for 4 days. After this time the cells were counted and expressed as the mean number of cells as a percentage relative to the uninfected, untreated control. The above graph is representative of 15 pairs of counts from 5 separate experiments ± SEM. Statistical significance was calculated from the cell numbers by paired t-test using the SPSS software package (Significance between uninfected growth factor treated cells and uninfected, untreated control: *= 0.05, **=0.005; significance between infected, growth factor treated sample and uninfected control samples treated with same growth factor: ▲= 0.05).

Infecting the MCF-7 cells with the PKC-α and δ overexpressors caused small but significant increases in growth. Treatment with each of the growth factors induced increases in cell growth, with the greatest increase induced by oestrogen which resulted in a cell number around 250% that of control cells at day 4. The treated cells infected with the PKC-δ overexpressor saw small increases in growth compared to uninfected controls. The cells treated with IGF-I or EGF also had increased growth rate when infected with the PKC-α overexpressor, though the cells treated with IGF-II, HRG-β1 or oestrogen displayed little if any increase in growth compared with the treated, but uninfected, controls. Though some of the increased growth observed in the treated cells infected with the overexpressors is statistically significant when compared to their uninfected but growth factor treated controls, the increases are not greatly different than those seen when the untreated cells are infected with the overexpressors alone. Therefore the increased growth of the treated cells when infected with PKC-α and δ WT may be due to effects other than those signalling caused by each of the growth factor ligands.

5.2.13. The effect of upregulating PKC-α and δ on tamoxifen sensitivity in the MCF-7 cell line

We have determined that the TAM-R cell line displays higher levels of PKC-α and PKC-δ expression and phosphorylation, and that when they are inhibited it causes a reduction in serum induced proliferation. Therefore if the TAM-R cells are utilising pathways involving PKC-α and δ we wanted to see if they were causative or a consequence of tamoxifen resistance and whether upregulation of these isoforms would confer a reduction in tamoxifen sensitivity to the MCF-7 cell line. We infected the MCF-7 cells with the PKC-α and δ overexpressing viruses and, 16 hours after infection, changed the media and treated them with 10^{-7}M tamoxifen for four days. After this time the cells were counted by Coulter counter (section 2.2.6). The results of 6 counts from an experiment carried out in triplicate are shown graphically in figure 5.23. It can be seen that after 4 days growth there was a slight reduction in growth of the tamoxifen treated cells. By contrast the cells overexpressing the WT PKC-α or WT PKC-δ displayed an increase in cell growth compared to their

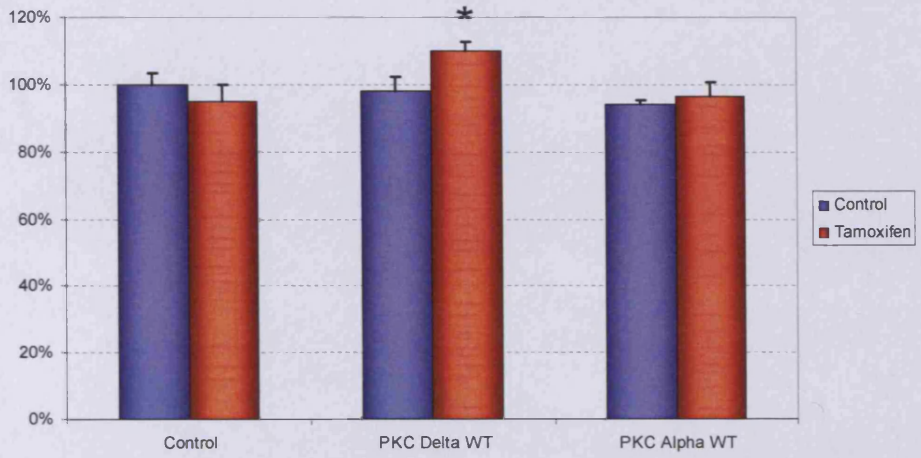


Figure 5.23. The effect of PKC-δ and PKC-α wild type (WT) overexpression on MCF-7 cells response to Tamoxifen

MCF-7 cells were infected with adenovirus containing PKC-α or δ WT overexpressing vectors at a MOI of 100 (section 2.7.4) with control cells left uninfected. The cells were then either treated with tamoxifen (10^{-7}M) or left untreated, and counted 4 days later by Coulter counting (2.2.6). The graph represents the cell number relative to the untreated, uninfected control cells ± SD from 6 counts.

matched control cells. However there was only a modest inhibition caused by tamoxifen at this time point. To establish for certain whether PKC-α or δ overexpression can confer tamoxifen resistance, the experiment would have to be repeated, possibly with longer duration of tamoxifen treatment and with the adenoviral infection repeated periodically throughout that time period so that the transient nature of the infection is not grown out with time.

5.3. DISCUSSION

To determine the importance of the PKC-α and PKC-δ isoforms in the acquisition of tamoxifen resistance we aimed to devise a strategy where their activity or expression could be ablated and the outcomes arising from that ablation studied in our TAM-R cell model. We attempted this endeavour with chemical inhibitors but met with problems relating to specificity and efficacy in our cell line models. This lead us to examine the technique of RNAi through the use of chemically synthesised siRNAs to induce gene specific silencing of PKC-α and PKC-δ. However we were hindered by the cells lack of permissiveness to transfection with the lipid / polymer based reagents we had at our disposal and the transient nature of the transfections. This lead us to the use of adenoviral technology which provided a far higher rate of gene transfer than the lipid / polymer based reagents, as illustrated by the MCF-7 and TAM-R cells adenovirally infected with the β-Gal expressing plasmid achieving nearly 70% infection rates compared to a transfection efficiency of only around 35% when the β-Gal plasmid was introduced using Lipofectamine 2000. Additionally the use of the adenovirus at a MOI of 100 displayed no cytotoxicity and had no effect on growth rates after four days infection.

The superior level of gene transfer associated with the adenovirus allows the introduction of gene vectors into a far greater proportion of cells within a population. This not only means that their overall effect on the cell population will be greater but also that the effect will take longer to be diluted through cell growth. We have established that we can utilise the adenoviral technology to introduce plasmids that express PKC-α or PKC-δ DNs into our model cell lines and that they can inhibit the activity of the endogenously expressed isoforms. Additionally we have shown that we

can use adenoviruses to introduce plasmids that express WT PKC-α and PKC-δ and thereby induce overexpression of these isoforms in our model cell lines. Therefore the adenoviral technology at our disposal not only confers the advantage of superior degrees of gene transfer but also the ability to study the functions of PKC-α and δ in terms of their overexpression as well as its inhibition.

We have previously shown that inhibition of PKC-α and δ with the inhibitor bisindolylmaleimide IX (bis) retards growth in the TAM-R cells to a much greater extent than the MCF-7 cells insinuating that PKC-α and δ play a more crucial role in the growth of the TAM-R cells than the MCF-7 cells. However as bis is a non-specific inhibitor that has been shown to impinge on several kinases the importance of these isoforms in isolation could not be assessed. When we infected the MCF-7 cells with the PKC-α or PKC-δ DN expressing adenoviruses we showed that they had very little effect on growth after four days even when used in combination. However the PKC-α and PKC-δ DN adenoviruses significantly inhibited TAM-R cell growth after 4 days with a slight additive effect when used in combination (Figure 5.14). This therefore confirms their importance as implied in the bis growth studies, but is far superior as it unpicks the isoforms from each other and from the multitude of other kinases affected by bis.

5.3.1. Growth Factor Signalling

The importance of growth factor signalling in the acquisition of tamoxifen resistance has been well established, whether mediated through the increase in growth factor ligand, the upregulation of the target receptor or the increased recruitment of their downstream signalling elements. One of the most markedly over-expressed growth factor receptors in our TAM-R model is the epithelial growth factor receptor (EGF-R) which displays 40 fold higher levels of membrane EGF-R staining than the parental MCF-7 cell line (Nicholson *et al.*, 2004). The importance of this large increase in receptor level is dramatically illustrated by the 95% inhibition of growth incurred by the TAM-R cell line when treated with the highly selective EGF-R inhibitor Gefitinib. This is in marked contrast to the <10% growth inhibition observed in the parental MCF-7 cell line (Nicholson *et al.*, 2001). This increased dependence on EGF-R

ligands in cells deprived of ER signalling is confirmed in MCF-7 derivatives resistant to the pure anti-oestrogen fulvestrant which also display sensitivity to Gefitinib (McClelland *et al.*, 2001).

The increase in EGF-R is mirrored by an increase in its related receptor family member, and preferred heterodimer partner, erbB-2 (Nicholson *et al.*, 2004). It has previously been shown that forced overexpression of the erbB-2 receptor in the MCF-7 cell line can promote ligand-independent down regulation of the ER (Pietras *et al.*, 1995) and confers a degree of tamoxifen resistance on the cells (Kurokawa *et al.*, 2000). The expression of the erbB-3/4 ligand heregulin (HRG), which can also transactivate erbB-2 (Keshamouni *et al.*, 2002), has been shown to be positively correlated with a more aggressive breast cancer phenotype *in vitro* and *in vivo*. Oestrogen sensitive breast cancer cells engineered to constitutively express heregulins also display oestrogen independent growth and tamoxifen resistance (Lupu *et al.*, 1996). We have shown that treatment with HRG significantly increases TAM-R cell growth (Figure 5.20)

We have shown that both EGF and HRG can induce significant increases in TAM-R cell growth (Figure 5.20). This correlates with the evidence stated above for their importance in the tamoxifen resistant and oestrogen deprived context. It has recently been shown that MCF-7 cells engineered to overexpress erbB-2 express significantly enhanced levels of both PKC-α and PKC-δ (Nabha *et al.*, 2005) and that inhibition of PKC-δ in these cells inhibits HRG mediated ERK 1/2 activation (Keshamouni *et al.*, 2002). We have shown that the TAM-R cell line, known to overexpress erbB-2, also express significantly increased levels of PKC-α and PKC-δ (Figure 3.4; Figure 3.5). Furthermore we have demonstrated that phosphorylation of these isoforms can be induced by HRG (Figure 5.19) and confirmed that ERK 1/2 activation is indeed downstream of PKC-δ (Figure 5.15) supporting the work of Keshamouni *et al.*, (2002). In addition, we have shown, through the use of the adenovirally infected dominant negatives, that TAM-R cell growth, induced by the erbB receptor family ligands EGF and HRG, can be suppressed through the inhibition of PKC-α and PKC-δ (Figure 5.20) providing direct evidence that these isoforms play a functional role in erbB receptor signalling mediated growth.

We have also shown that IGF-I and II are able to phosphorylate PKC-α and PKC-δ and significantly stimulate growth in the TAM-R cell line (Figure 5.20). The importance of IGF-IR signalling in the tamoxifen resistant context has been previously shown in *in vitro* studies by Parisot *et al.* (1999) that show that whilst IGF-I is unable to stimulate MCF-7 cell growth it can stimulate the growth of their tamoxifen resistant model and that this growth is inhibited by an IGF-IR monoclonal antibody. It has also been shown that treating IGF-IR overexpressing MCF-7 clones with IGF-I greatly reduces their requirement for oestrogen for growth (Guvakova and Surmacz, 1997) and that increased IGF-IR can confer oestrogen independence on long term oestrogen deprived MCF-7 cells (Stephen *et al.*, 2001). However, we have shown for the first time that the growth stimulatory effects of IGF-I and IGF-II in TAM-R cells can be markedly reduced through the selective inhibition of either PKC-α and PKC-δ (Figure 5.20), providing evidence that in addition to playing a role in erbB receptor mediated cell growth, these isoforms also play a critical role in growth signalling through the IGF-IR.

Many layers of complexity are added to the mechanisms underlying growth factor induced signalling through PKCs in the TAM-R cell line, due to the phenomenon of receptor cross-talk between the IGF-IR and the erbB family of receptors. For example, it has been shown within our group that treatment with IGF-II induces the activation of both the IGF-IR and the EGFR in the TAM-R cell line but only IGF-IR in the MCF-7 cell line. It has also been shown that the IGF-IR inhibitor AG 1024 reduces EGF-R phosphorylation in non-primed TAM-R cells (Knowlden *et al.*, 2005). It has been suggested that this cross talk may even be mediated through a direct interaction between the IGF-IR and the erbB receptors (Balana *et al.*, 2001) though this has yet to be established in the TAM-R cell line. An interesting extension of our work would therefore be to test whether IGF-II stimulation of the PKC in the TAM-R cells is direct or mediated via EGF-R activation.

5.3.2. Oestrogen Receptor Signalling

Whilst growth factor signalling has been shown to play a major role in tamoxifen resistant growth it has been shown that the TAM-R cells are sensitive to the pure anti-oestrogen fulvestrant (Robertson *et al.*, 2001) and therefore must retain ER functionality. Certainly, the ER can be phosphorylated at the serine 118 and 167 residues through EGF-R dependent EGF or TGF-α stimulation (Britton *et al.*, 2002). In addition, inhibition of the IGF-IR with the selective inhibitor AG 1024 causes a reduction in ER phosphorylation (Nicholson *et al.*, 2004). Interestingly there is also evidence that the erbB-2 receptor may even associate directly with membrane ER to confer tamoxifen resistance (Chung *et al.*, 2002). Whilst this cross talk between growth receptor signalling and the ER appears to play a vital role in tamoxifen resistant growth, we have shown that, even in the presence of tamoxifen, oestrogen itself can still significantly stimulate growth of the TAM-R cell line and that this growth stimulation is suppressed by inhibition of PKC-α and PKC-δ (Figure 5.20). This oestrogen induced proliferation of tamoxifen resistant MCF-7 derivatives has also been shown by Nabha *et al.* (2005), who also showed that this effect can be suppressed by PKC-δ inhibition. Surprisingly they did not further investigate the role played by PKC-α in this pathway, even though they observed a very large increase in PKC-α expression in their tamoxifen resistance model over the MCF-7 cells from which they were derived, mirroring the PKC profile of our TAM-R cell model (Chapter 3).

Oestrogen stimulated growth has also been shown to occur in tamoxifen resistant xenographs derived from MCF-7 cells (Berstein *et al.*, 2004). However, in that study the oestrogen induced cell growth was observed over 7 weeks after the removal of tamoxifen from the micro-environment whereas the growth stimulation observed in our TAM-R cell model occurs in the presence of tamoxifen (Figure 5.20). Therefore oestrogen stimulated growth of the TAM-R cells appears to be occurring through a non-classical ER pathway. Studies from several laboratories have demonstrated rapid non-genomic effects of oestradiol through membrane receptors distinct from ERα and ERβ (Benton *et al.*, 2001; Doolan and Harvey, 2003; Sylvia *et al.*, 2000). For example Walsh *et al.* (2005) have shown that oestradiol is capable of inducing a rapid and

maintained increase in intracellular calcium ion concentration in MCF-7 cells, which express ERα and β, but also in SKBR-3 cells, which express neither receptor. This effect was also unaffected in both cell lines by pre-treatment with the pure anti oestrogen fulvestrant.

Another study by Doolan *et al.* (2000) has shown that oestradiol can also stimulate the activation of PKA in rat distal colonic epithelium and that this effect is not inhibited by tamoxifen but is inhibited by the PKC inhibitor, bis. Interestingly they also showed that PKC-α and PKC-δ, but not PKC-ε or PKC-ζ, can be stimulated by oestradiol in a cell free environment independent of ER. Therefore there is evidence of an ER independent mechanism for oestradiol induced activation of PKC-α and PKC-δ leading to PKA activation. This ER independent utilisation of PKC-α and PKC-δ provides a possible mechanism through which oestradiol is able to promote growth in the TAM-R cell line even in the presence of tamoxifen and also provides a possible explanation for the efficacy of systemic oestrogen ablation through aromatase inhibition on breast cancers that have become resistant to tamoxifen.

However, our studies also show that the ER still appears to play a role in the activation of PKC-α and PKC-δ in the TAM-R cells, even in the presence of tamoxifen, as ER downregulation mediated by prolonged treatment with fulvestrant or oestradiol leads to a marked reduction in both PKC-α and PKC-δ activation (Figure 5.21). In contrast, a smaller downregulation of ER in the MCF-7 cell line leads to a reduction of PKC-δ activation but also a concomitant increase in activation of the PKC-α isoform (Figure 5.21). This phenotype is reminiscent of the ER-ve MB-MDA-231 cells (Figure 3.3) and implies that upon blockade of ER signalling, MCF-7 cells convert to a more ER-ve phenotype. Thus there appears to be distinct differences in the response of TAM-R and MCF-7 cells to ER downregulation with regard to PKC isoform expression and activation. Furthermore, whilst we have shown that both PKC-α and PKC-δ activation can be mediated through signalling from the ER in the TAM-R cell line, thus appears to be mediated by a different pathway than that demonstrated by Doolan *et al* (2000).

One possible mechanism could be that the PKCs are activated through the oestradiol promoted expression of HRG, inducing autocrine and paracrine signalling to the erbB-2 receptor. This mechanism, described by Keshamouni *et al.* (2002) in erbB-2 overexpressing MCF-7 cells, leads to the PKC-δ dependent activation of ERK 1/2. This concurs with our observation that PKC-δ activation increases with HRG treatment (Figure 5.19), that PKC-δ is an important downstream target in HRG induced TAM-R cell growth (Figure 5.20) and that PKC-δ is an important upstream component of ERK 1/2 signalling (Figure 5.15). Another possibility is that tamoxifen itself is acting as the agonist, with the ER effectively functioning as the receptor for tamoxifen mediated signalling. This explanation is especially feasible since tamoxifen invokes agonistic or antagagonistic properties in a tissue specific manner. For example it can function as an antagonist in the breast but an agonist in the bone and uterus (Graham *et al.*, 2000). If the breast cancer cells are developing mechanisms that allow them to arrogate the agonistic properties of tamoxifen then it may further explain the sensitivity of TAM-R to fulvestrant (Robertson *et al.*, 2001). It has recently been shown that tamoxifen can indeed induce cell proliferation in a tamoxifen resistant MCF-7 derived cell line and that this effect is inhibited by transfection with PKC-δ specific siRNA (Nabha *et al.*, 2005). Therefore in our TAM-R cell model it would follow that ER down regulation in the presence of tamoxifen would lead to the downregulation of downstream effectors such as PKC-α and PKC-δ.

5.3.3. Effect of PKC-α and PKC-δ Overexpression in the MCF-7 cell line

As growth factor signalling has been shown to be mediated through PKC-α and PKC-δ in the TAM-R cell line, we investigated the effects of PKC-α and PKC-δ overexpression on growth factor signalling in the MCF-7 cell line (Figure 5.22). Overexpressing PKC-δ increased cell growth in the MCF-7 cells treated with each of the ligands whilst PKC-α overexpression only augmented growth in the cells treated with IGF-I and EGF. However this seems quite reasonable given that the MCF-7 cell line are known to express relatively little PKC-α (Chapter 3) with greater amounts of PKC-δ (Shanmugam *et al.*, 1999) and are therefore more likely to be geared towards

it's utilisation. Whilst none of the increases in growth factor induced growth are dramatic, this may be due to factors upstream and downstream of the increased PKCs. To more accurately assess the effects of overexpression of the PKC isoforms on growth factor induced growth in the MCF-7 cell line it may be a better strategy to overexpress the PKCs in cells engineered to overexpress each of the receptors. The largest increase in growth seen following PKC-δ overexpression was that induced by oestradiol treatment. This correlates with our earlier observation that the ER is upstream of PKC-δ (Figure 5.21).

Finally, having already shown that PKC-α and PKC-δ are overexpressed in the TAM-R cell line compared to the MCF-7 cell line (Chapter 3) and that the inhibition of the PKC-α and PKC-δ inhibits basal growth and growth induced by growth factors and oestrogen, we wanted to determine if the overexpression of PKC-α and PKC-δ is causative or a consequence of tamoxifen resistance. To this end we overexpressed PKC-α and PKC-δ in the tamoxifen sensitive MCF-7 cell line to see if this could confer tamoxifen resistance (Figure 5.23). After 4 days treatment, tamoxifen had inhibited MCF-7 cell growth as expected. However in the PKC-α and PKC-δ overexpressing cells, treatment with tamoxifen induced increased growth. In support of these observations, another group have shown that PKC-α and PKC-δ overexpression can confer a reduced sensitivity to tamoxifen (Nabha *et al.,* 2005). Moreover, our studies have shown that tamoxifen treatment of MCF-7 cells that overexpress PKC-α and PKC-δ can lead to tamoxifen acting as an agonist.

CHAPTER 6.

GENERAL DISCUSSION

6. GENERAL DISCUSSION

During the course of our study we have established that the expression of PKC-α and PKC-δ is significantly increased in TAM-R cells relative to the tamoxifen sensitive MCF-7 cells from which they are derived. We have also established that the increased expression is mirrored by a concomitant increase in PKC-α and PKC-δ activation inferring that these isoforms are playing a more important role in the signalling mechanisms of the TAM-R phenotype. Interestingly our parallel studies on clinical samples demonstrate that tumours overexpressing PKC-α and PKC-δ are associated with poor prognostic outcomes with respect to survival and duration of endocrine response (Assender *et al.*, 2005; in prep.). This profile differs from ER-ve cell models that possess *de novo* tamoxifen resistance. Such cells display high PKC-α levels but low PKC-δ. Therefore there appears to be distinct mechanisms that underpin acquired resistance compared to *de novo* resistant cells.

To study the importance of this increased PKC-α and PKC-δ activity in the TAM-R phenotype, relative to the MCF-7 tamoxifen sensitive phenotype, we investigated various techniques to suppress PKC functionality or ablate their expression. The most commonly used method of inhibition is through the use of low molecular weight, cell permeable chemical inhibitors. There are several inhibitors that have been reported to provide selective inhibition of the PKC family of kinases or even single isoform specificity. Two of the most commonly cited PKC inhibitors in published articles are bisindolylmaleimide IX (bis) and rottlerin. Bis has been used in over 600 published studies to demonstrate a role for PKC in many cell systems and species (McGovern and Shoichet, 2003). We showed that bis is indeed a competent inhibitor of PKC as it greatly reduced the protein expression and phosphorylation of both the α and δ isoforms in both MCF-7 and TAM-R cells. However the inherent lack of selectivity between the two isoforms is compounded by its comparable inhibition of the PKC-βI/II, γ and ε isoforms (Way *et al.*, 2000) and several non-related kinases including GSK-3 (Hers *et al.*, 1999), Rsk-1 and p70S6 kinase (Alessi, 1997). Therefore the picture is not only clouded by the pan inhibition of several PKC isoforms but also by the varying degrees of inhibition on several other kinases.

In order to try to selectively inhibit one of our target PKC isoforms, we next tried the widely reported PKC-δ selective inhibitor rottlerin. However the PKC-δ inhibition observed upon treatment with 5μM rottlerin was negligible at best. Of more concern was the observation that at low doses (500nM-1μM) rottlerin functioned as a dose dependent activator of PKC-δ. When we looked deeper into rottlerins credentials we discovered that there had already been voices of dissent over its entitlement to be called a PKC-δ specific inhibitor. Whilst first reports of its efficacy did flag up its inhibition of CAM kinase III, PKA and casein kinase II (Gschwendt *et al.*, 1994) it has since been discovered to be a potent inhibitor of PRAK and MAPKAP-K2 (Soltoff, 2001). Whilst these inhibitory characteristics can be explained by common folding motifs or conserved kinase domain structure, rottlerin has been also shown to inhibit the unrelated enzymes β-lactamase, chymotrypsin and malate dehydrogenase with at lower IC_{50} concentrations than for PKC (McGovern and Stoichet, 2003). It has also been shown that rottlerin can activate PKC-δ under certain conditions in whole cells, concurring with our observations in the MCF-7 cell line. However, this peer reviewed dissent has not been sufficient to stem the flow of publications that elaborate a role for PKC-δ from observed effects seen with rottlerin treatment with several papers published this year inferring PKC-δ involvement from rottlerin induced effects in MCF-7 cells (Zhang *et al.*, 2005; DeServi *et al.*, 2005; Nabha *et al.*, 2005). However, due to its lack of efficacy in our cell model, we decided that taking a molecular approach to selectively inhibit specific isoforms was the most appropriate option.

The lack of efficacy and selectivity of the chemical PKC inhibitors lead us to investigate the use of RNAi, through siRNA, to knock out PKC-α and PKC-δ at the mRNA level. The technique of RNAi is now one of the most widely utilised techniques in molecular biology and is currently being investigated as a possible therapeutic tool. However one of the major limitations of RNAi is the ability to introduce the siRNA into the cell model. The most commonly used method to do this is via the use of lipid / polymer based transfection reagents. However using these reagents to transfect plasmid DNA into the MCF-7 and TAM-R cell lines resulted in

less than 40% cell transfection. To determine if we could achieve better results with actual siRNAs we transfected GAPDH specific siRNA into the cells. The knockdown of around 40% total GAPDH protein suggested that the transfection efficiency was roughly on a par. Whilst we were able to incur 60% and 30% knockdowns of PKC-δ and PKC-α protein respectively with their respective specific siRNAs, we felt that the level of knockdown, married to its transient nature and requirement for high cell densities, would be a hindrance in studying longer term effects such as cell proliferation and mitogen induced signalling pathways.

We therefore optimised and validated an adenoviral system of introducing PKC-α and PKC-δ dominant negative and wild type overexpressing plasmids into our model cell lines. Using this system, we were able to infect effectively and target selectively the PKC-α and PKC-δ isoforms individually and achieve gene transfer in twice as many cells as by lipid / polymer transfection. Using this technology we have shown that PKC-α and PKC-δ inhibition has a significant effect on growth of the TAM-R cell line (Figure 5.13) whereas it has very little effect on the growth of the MCF-7 cell line (Figure 5.12). This shows that the increased expression and activation of these isoforms in the TAM-R cell line is indeed borne from an increased reliance on and functionality of these isoforms in the resistant cells.

The importance of growth factor signalling in the acquisition of breast cancer resistance has been well established. We discovered that many of the ligands implicated in these pathways are able to induce an increase in activation of PKC-α and PKC-δ in the TAM-R or MCF-7 cell lines. We therefore investigated the effect of inhibiting these isoforms on growth factor induced growth of the TAM-R cells. The growth factors IGF-I, IGF-II, EGF and HRG-β1 all induced increased growth in the TAM-R cell lines over 4 days. Moreover this growth was suppressed in each case by the inhibition of either PKC-α or PKC-δ, demonstrating that these isoforms play an important role in growth factor ligand stimulated growth in the TAM-R cell line. We also showed that oestradiol is able to promote the growth of the TAM-R cells even in the presence of tamoxifen. This seems counterintuitive as the target receptor for oestradiol would already have tamoxifen bound in its ligand binding pocket. However

it has recently been shown that oestradiol can induce rapid non-genomic effects through membrane receptors distinct from ERα and ERβ (Doolan and Harvey, 2003). This membrane receptor pathway can rapidly induce increased intracellular calcium concentrations in ER negative SKBR-3 cells, can stimulate PKA by a mechanism inhibited by bis but not tamoxifen, and can phosphorylate PKC-α and PKC-δ, but not PKC-ε or PKC-ζ, in a cell free assay; providing a possible mechanism whereby oestrogen can activate these isoforms directly and specifically (Doolan *et al.*, 2000).

Whilst these non-classical pathways merit further investigation, our studies indicate that PKC-α and PKC-δ still need nuclear ER to have a functional role in signalling. We have shown that downregulation of nuclear ER with prolonged oestradiol or fulvestrant treatment causes the concurrent downregulation of both PKC-α and PKC-δ. This is contrasted by the MCF-7 cell line after ER downregulation which appears to revert to a PKC profile akin to the ER negative *de novo* resistant cells by displaying decreased PKC-δ activation but an increase in PKC-α activation. The TAM-R cells however have more of both isoforms and appears to be utilising them in ER related signalling. A possible reason for the downregulation of PKC-α and PKC-δ on ER ablation is that tamoxifen is itself acting as an ER agonist in the TAM-R cell line. Tamoxifen is already known to function as an ER agonist in tissues such as the uterus and the bone and it would provide a further explanation for the efficacy of the pure anti-oestrogen fulvestrant on the TAM-R cells (Robertson, 2001). A recent study by Nabha *et al.* (2005) supports this hypothesis as they showed that the proliferation of their tamoxifen resistant cell model could be induced by tamoxifen and interestingly that this effect was inhibited following inhibition of PKC-δ. This study highlights a functional role for PKC-δ in tamoxifen resistance. We overexpressed PKC-δ along with PKC-α in the tamoxifen sensitive MCF-7 cell line to ascertain if this would be sufficient to confer tamoxifen resistance. Whilst the growth of the PKC-α overexpressors was inhibited to a slightly lesser degree than the non-infected control cells by tamoxifen, the PKC-δ overexpressing cells grown in tamoxifen actually demonstrate a growth stimulation compared to control. Whilst it has recently been shown that PKC-δ overexpression can confer a degree of lessened sensitivity to

tamoxifen (Nabha *et al.*, 2005) we have shown that PKC-δ overexpression may contribute to tamoxifen acting as an agonist in previously tamoxifen sensitive cells.

However the true nature of the activation and utilisation of PKC-α and PKC-δ in tamoxifen resistance is doubtlessly more complicated and involved than could be fully characterised over the time scale of this project. For example PKC-α has been shown to play a role in invasion and migration in breast cancer (Ways *et al.*,1995; Morse-Gaudio *et al.*, 1998; Tonetti *et al.*, 2000) and we know that the TAM-R cell line exhibit greater invasive properties than the MCF-7 cell line (Hiscox *et al.*, 2003) and possess greater levels of PKC-α (chapter 3). Therefore using our adenoviral system we would like to look in future at how the inhibition of PKC-α and PKC-δ would affect the invasion and migration properties of these cells. This endeavour was not possible during the course of this project due to the limited Class II level containment equipment at our disposal.

It has become clearer over recent years that growth factor receptors are much more promiscuous than previously thought with the phenomenon of receptor cross talk adding a further layer of complexity to the already formidable entanglement of signalling pathways and molecules. For example it has been shown within our group that treatment with IGF-II induces the phosphorylation of both IGF-IR and EGF-R in the TAM-R cell line (Knowlden *et al.*, 2005). Therefore it would also be interesting to test whether the activation of PKC-α and PKC-δ by IGF-II is actually mediated via activation of the EGF-R rather than signalling directly downstream of IGF-IR. This could be achieved through the use of the EGF-R specific inhibitor Gefitinib to inhibit the receptor during IGF-II stimulation.

We have also only touched on the surface of the interplay between the PKC isoforms and the oestrogen receptor. Whilst we have shown that PKC-α and PKC-δ play an important role in the growth stimulatory effect of oestradiol on the TAM-R cell line time constraints have meant that we cannot further investigate the mechanisms that underlie that involvement. For example it would be interesting to investigate whether PKC-α or PKC-δ play a role in the enhanced serine 118 phosphorylation of the

oestrogen receptor by the activation of tyrosine kinase growth factor receptors (Britton *et al.*, 2002), the possible involvement of the PKCs interaction in oestrogen receptor co-activator/ co-repressor interactions and the effect of PKC-δ on oestrogen receptor localisation and translocation in our cell models (De Servi *et al.*, 2005). Also it would be interesting to examine the role played by membrane oestrogen receptor signalling of the PKCs (Boyan et al., 2003) in the TAM-R cells, possibly through the use of E_2 conjugated to bovine serum albumin.

Much has been made of late of the obsoleteness of Tamoxifen at the hands of the emerging aromatase inhibitors. However reports of this demise have probably been somewhat exaggerated as the over 250 clinical trial in which Tamoxifen is involved attests to (www.controlled-trials.com). Tamoxifen is not only unrivalled in breast cancer therapy in terms of toxicity and therapeutic profile but also confers cardioprotective effective, reduces levels of low-density lipoprotein and total cholesterol (Love *et al.*, 1992) and has beneficial effects on bone density (Love *et al.*, 1991). In contrast there are still question marks over side effects of the aromatase inhibitors with preliminary evidence suggesting that they could have possible negative effects on cognitive function, sexual function and musculoskeletal integrity (Sing Ranger, 2005). Unfortunately there are also financial issues to consider with the cost of treatment with the aromatase inhibitors far outstripping the inexpensive tamoxifen, and sadly the availability to prescribe to the populous often appears to depend on the will of politicians not the knowledge of scientists and clinicians or, most importantly, the needs of patient.

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