Studies of the Mechanisms of Action of the Cancer Chemopreventive Agent Genistein

Submitted for the degree of
Doctor of Philosophy at the University of Leicester

by

Karen Dampier B.Sc. (Hons)
MRC Toxicology Unit
University of Leicester

October 2000

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Studies of the Mechanisms of Action of the Cancer Chemopreventive Agent Genistein.

Karen Dampier.

The discrepancy in incidence of breast cancer between the Western world and South East Asia has been associated with the high content in Asian diets of chemopreventive foodstuffs such as soy. Genistein, a major constituent of soy, can both inhibit and stimulate cell growth, depending on concentration. Whilst some biochemical properties of genistein are well known, such as inhibition of tyrosine kinases and DNA topoisomerase II, the mechanisms by which it regulates cell growth are unclear.

In order to explore generic features of the modulation of human breast cell growth by genistein, its effects on cell lines MCF-7, ZR-75.1, T47-D, MDA-MB 468, MDA-MB 231 and HBL 100 were compared. Genistein at 1 μM stimulated growth only in MCF-7 cells, whilst at 10 μM it arrested the growth of all six cell types. However, in T47-D and HBL 100 cells, genistein only inhibited growth in medium with reduced (2 %) foetal calf serum. Genistein induced apoptosis only in MDA-MB 468 cells and arrested cells in the G2 phase transition of the cell cycle in all cell lines, except ZR-75.1 cells.

Cells differed in their susceptibility towards inhibition by genistein of phorbol ester-induced proto-oncogene c-Fos levels, transcription factor activator protein-1 (AP-1) activity, AP-1 DNA-binding activity and extracellular signal regulated kinase (ERK) activation. Genistein augmented anisomycin-induced protein levels of the proto-oncogene c-Jun in ZR 75.1 and MCF-7 cells, but inhibited c-Jun phosphorylation.

The results suggest that induction of apoptosis, G2 cell cycle arrest, inhibition of c-Fos expression, AP-1 transactivation and binding, and ERK phosphorylation may contribute to the growth-inhibitory effect of genistein in some breast cell types, but none of these effects of genistein constitutes a generic mode of growth-arresting action.

For my family

Thanks for giving me the opportunity.

ACKNOWLEDGEMENTS

I would firstly like to thank my supervisors, Professors Andy Gescher (Centre for Mechanisms of Human Toxicity) and Rosemary Walker (Breast Cancer Research Unit, Glenfield Hospital) for their time, patience and direction throughout the three years of this study. Their ideas, supervision and optimism made this work really interesting and enjoyable and their ability to listen and motivate when things were not going to plan were greatly appreciated!

I would like to thank all the members of the Toxicity of Anti-Cancer Drugs section in which I was based for the three years, who made it an enjoyable and interesting section to be a part of, and in particular I would like to thank Drs. Maggie Manson, Ann Hudson, Richard Verschoyle for all their help and assistance, and Lynne Howells, for being a great friend and colleague, without who my early mornings would have been very dull indeed! I would also like to say thank-you to Dr. Linda Gordon (Breast Cancer Research Unit, Glenfield Hospital) for all her help, both here and at Glenfield and to Dr. Marion MacFarlane for providing help and assistance with the apoptosis studies.

I would like to extend my thanks to the Medical Research Council for supporting this study and to the British Association for Cancer Research for providing financial assistance to enable me to attend conferences.

Finally, I would like to say a huge thank-you to the members of my family for all their support and last, but not least, to Matt, for putting up with me and my moaning throughout the three years, even when he didn't have a clue what I was talking about. His constant encouragement kept me going and I couldn't have done it without him.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AJ buffer	Alec Jeffreys' buffer
AMPS	Ammonium persulphate
AMV	Avian myeloblastosis virus
ANOVA	Analysis of variance
AP-1	Activating protein 1
ATF2	Activating transcription factor 2
ATP	Adenosine 5' triphosphate
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CHAPS	(3-3-cholaminopropyl) dimethylammonium-1-propane sulphonate
CPP32	Cysteine protease protein 32kDa
dNTP	Deoxynucleotide 5'triphosphate
DCC	Dextran coated charcoal
DMBA	7,12-dimethylbenz(a)anthracene
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
DTT	Dithiothreitol
ECL	Enhanced chemiluminescense
ECM	Extracellular matrix
EDTA	Ethylene diaminetetraacetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ER	Oestrogen receptor
ERE	Oestrogen response element
ERK	Extracellular signal related kinase
FCS	Foetal calf serum
HEPES	N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonic acid]
HMEC	Human mammary epithelial cells
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
ICE	Interleukin-1-β converting enzyme
IL-β	Interleukin 1-beta
JNK	c-jun N terminal kinase
KAB	Kinase assay buffer
LBD	Ligand binding domain
LSD	Least significant difference
mRNA	Messenger ribonucleic acid
MAPK	Mitogen-activated protein kinase
MDM2	Minute double minute 2
MEK	Mitogen-activated protein kinase kinase
NFκB	Nuclear factor-κB
pRb	Retinoblastoma gene product
PAGE	Polyacrylamide gel electrophoresis

PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIPES	Piperazine-N,N'-bis[2-ethanesulphonic acid],1,4-piperazinediethanesulphonic acid)
PKC	Protein kinase C
PMSF	Phenylmethylsulphonylfluoride
PNACL	Protein and nucleic acid chemical laboratory
Poly DIDC	Poly deoxyinosinic-deoxycytidylic acid
PS	Phosphatidylserine
PTK	Protein tyrosine kinase
RNase	Ribonuclease
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SRE	Serum response element
TAE	Tris acetate EDTA buffer
TBE	Tris borate EDTA buffer
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween 20
TCF	Ternary complex factor
TE	Tris EDTA buffer
T/E	Trypsin/EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
TLB	Triton lysis buffer
TNF	Tumour necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRE	TPA response element
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase
Z-DEVD.afc	benzyloxycarbonyl Asp-Glu-Val-Asp-7-amino-4-trifluromethyl coumarin

CHAPTER 1: INTRODUCTION.

1.1 Carcinogenesis and the relationship to cancer chemoprevention

With one quarter of deaths in the Western world attributed to malignant diseases, the field of cancer research is challenging and exciting. Most chronic diseases such as cardiovascular disease and diabetes are not curable, yet great advances have been made in their control. The progress in cancer research does not only relate to cure, but also to the improvement in the quality of life of individuals.

One problem existing for cancer prevention is that we do not know the cause for over 50% of cancers (Wattenberg, 1995). Major factors that affect the incidence of cancer are age, gender and family history, but occupational, social and geographic factors are also extremely important. Chemical carcinogens such as asbestos and aflatoxins are known to increase the incidence of lung and liver cancers, respectively, but there are probable environmental carcinogens that are unidentified.

Many epidemiological studies support the view that the Western diet is one of the main factors in the incidence of so-called Western diseases (Adlercreutz et al., 1995), which includes hormone dependent cancers and coronary heart disease. One striking example is the incidence of breast cancer in South East Asian women. Asian women who consume a traditional low fat, high soy diet have a four to six-fold lower risk of developing breast cancer than women living in the industrialised Western world. Research into dietary components may help to identify factors that could decrease the incidence of cancer in the Western world. Analysis of the biochemical targets and mechanisms of action need to be elucidated, in addition to any undesirable effects. This study is concerned with identifying the mechanisms of action of such factors, present in the Western diet.

The term cancer refers to a tumour (swelling) which is malignant i.e. it can invade surrounding tissue and metastasise to other sites. Cancers arise from a cell (cells) that has undergone a series of genetic alterations, through the process of initiation and mutations resulting in growth advantages when compared to normal cells.

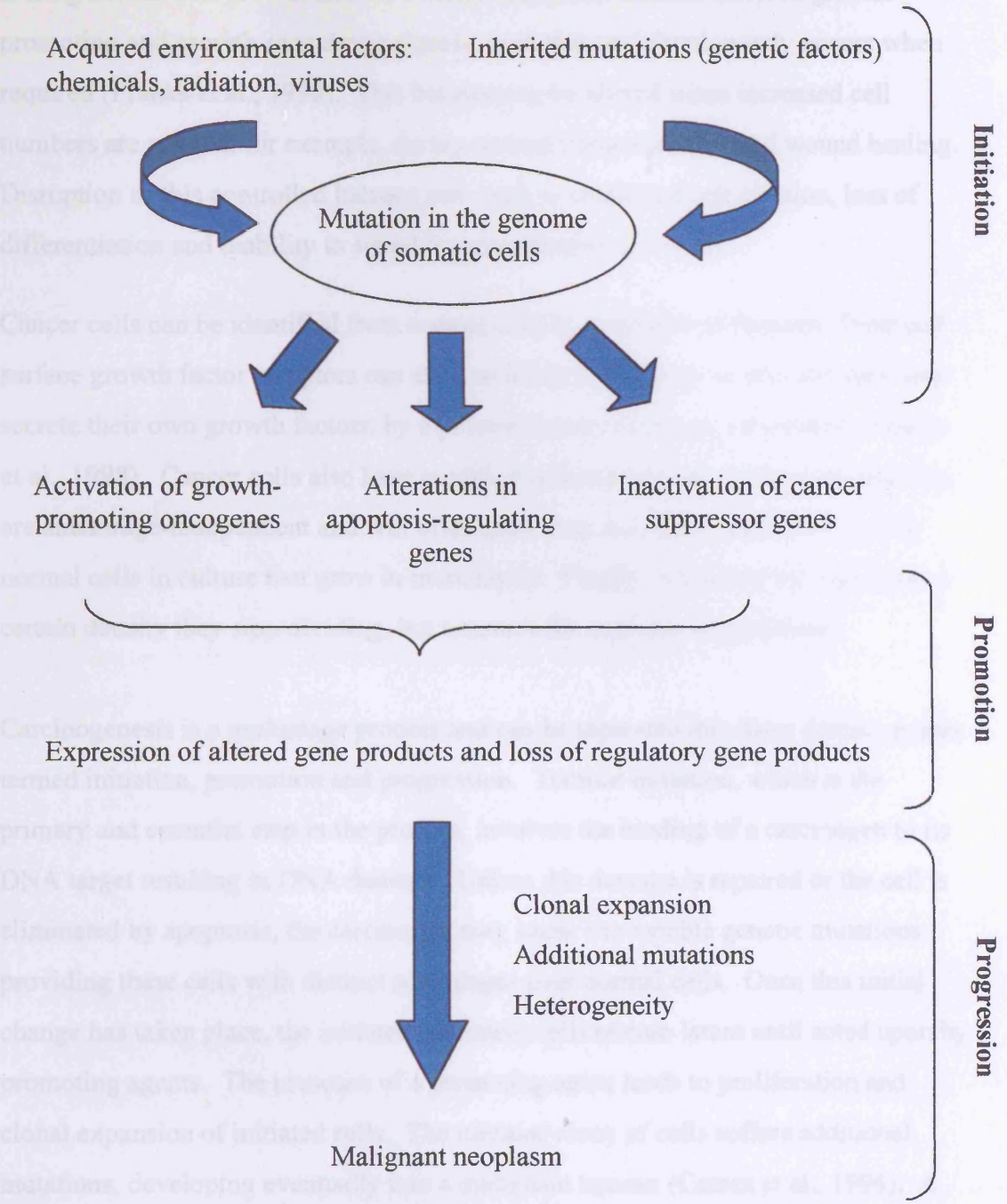


Fig. 1.1. A simplified scheme of cancer pathogenesis. Adapted from Cotran *et al*, 1994.

During normal cell growth there is a finely controlled balance between growth-promoting and growth-restraining signals, such that proliferation only occurs when required (Franks et al., 1998). This balance can be altered when increased cell numbers are needed, for example, during normal tissue turnover and wound healing. Disruption of this controlled balance can result in continued cell division, loss of differentiation and inability to signal for programmed cell death.

Cancer cells can be identified from normal cells by a number of features. Their cell surface growth factor receptors can alter, so binding no longer occurs and they may secrete their own growth factors, by a process termed autocrine stimulation (Franks et al., 1998). Cancer cells also have modified cell-cell and cell-stromal interactions, are anchorage-independent and will often grow over and under each other, unlike normal cells in culture that grow in monolayers. Finally, when normal cells reach a certain density they stop dividing, but tumour cells continue to proliferate.

Carcinogenesis is a multistage process and can be separated into three distinct events termed initiation, promotion and progression. Tumour initiation, which is the primary and essential step in the process, involves the binding of a carcinogen to its DNA target resulting in DNA damage. Unless this damage is repaired or the cell is eliminated by apoptosis, the carcinogen may cause irreversible genetic mutations providing these cells with distinct advantages over normal cells. Once this initial change has taken place, the initiated (mutated) cells remain latent until acted upon by promoting agents. The presence of a promoting agent leads to proliferation and clonal expansion of initiated cells. The initiated clone of cells suffers additional mutations, developing eventually into a malignant tumour (Cotran et al., 1994). A malignant neoplasm has several phenotypic attributes, for example, excessive growth, local invasiveness and the ability to form distant metastases (Cotran et al., 1994). These characteristics are acquired in a step-by-step fashion and this complex process is termed tumour progression. Tumour progression is associated with altered gene expression (see figure 1.1) and additional genetic damage due to progressive genomic instability (Pitot, 1989).

The optimal way of dealing with any disease is by prevention and the process of chemoprevention is one such intervention (Wattenberg, 1995). Cancer chemoprevention involves preventing, delaying or reversing cancer by intervention using either non-toxic synthetic chemicals or chemicals from natural substances prior to development of malignancy (Lee et al., 1999). Depending on which phase of carcinogenesis these chemicals affect, they can be divided into 'blocking' or 'suppressing' agents.

Blocking agents exert a barrier function by preventing carcinogenic agents from reaching or reacting with critical target sites (Wattenberg, 1995), for example, by trapping reactive carcinogenic species before they reach a critical target, whilst suppressing agents intercept promotion and progression (Gescher et al., 1998). The multifactorial nature of the carcinogenic process provides numerous pathways that may be manipulated by such agents, resulting in the inhibition or delay of tumour development (Lee et al., 1999).

1.2. Cellular mechanisms regulating growth and survival

1.2.1. The cell cycle

The eukaryotic cell cycle is comprised of a series of highly conserved events serving to ensure that firstly DNA is replicated once during S phase, and secondly, that identical chromosomal copies are equally distributed to two daughter cells (Sherr, 1996). Multiple extracellular signals control entry and exit from the cell cycle in order to both co-ordinate normal cell growth and avoid uncontrolled proliferation.

A characteristic feature of most, if not all, malignant tumours is deregulation of the cell cycle (Landberg and Roos, 1997). Cancer cells exhibit increased proliferative activity (Fernandez et al., 1998) and abnormalities in both positive and negative modulators of the cell cycle are frequent in many cancer types, including breast carcinomas.

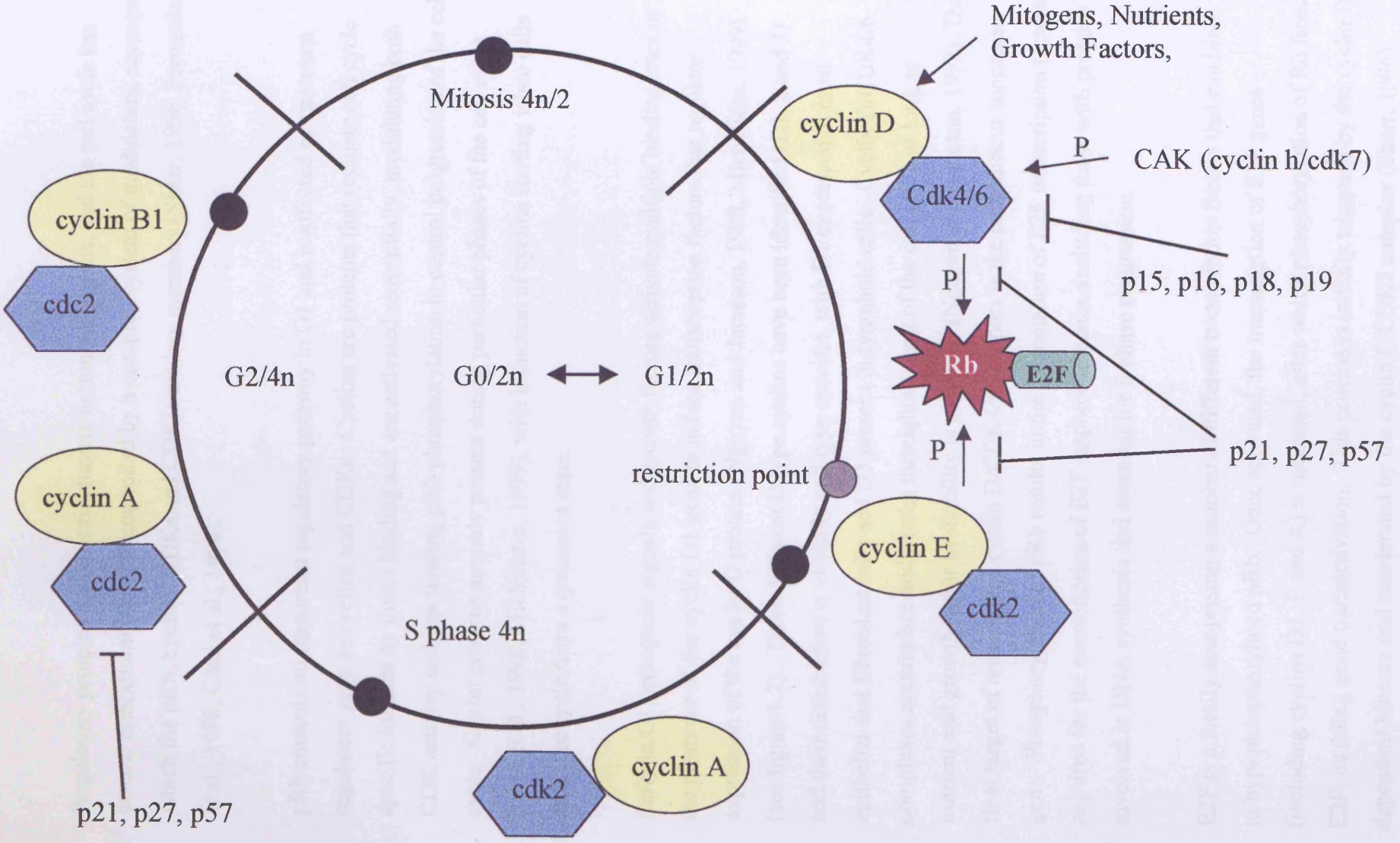


Fig. 1.2. An overview of the cell cycle. Adapted from Nguyen and Jameson, 1998. P= phosphorylation, ● = cell cycle checkpoint.

Regulatory proteins are potential targets in tumourigenesis, and the cell cycle has several checkpoints that are controlled by a complex system of modulators, amongst which the pRb, cyclins, CDKs and CDKIs are key members (Sherr, 1996, Fernandez et al., 1998, Choi et al., 1998).

pRb controls an important regulatory pathway in G1 and is affected by upstream regulators such as cyclins and CDKs. Cyclins are proteins that regulate cell cycle specific kinases by direct binding and are activated sequentially, modulating both CDK activity and inactivating pRb phosphorylation to control progression of the cell cycle. Cyclin proteins are only present during particular phases of the cell cycle (Choi et al., 1998, Michalides, 1999), with induction of cyclins starting when cells enter the cycle from a quiescent state.

Early in G1, mitogenic signals and growth factors activate multiple binding sites in the promoter of the cyclin D1 gene to induce transcription factors that regulate expression of the cyclin D protein (Nguyen and Jameson, 1998, Michalides, 1999) (see figure 1.2). Three different D type cyclins have been identified (D1, 2 and 3) and their distribution is virtually cell type specific, with D1 expressed in most epithelial and fibroblast cells and D3 present in lymphoid cells. Cyclin D/CDK4/6 complexes accumulate and persist throughout most of the cell cycle as long as nutrient and growth factor availability is sustained (Nguyen and Jameson, 1998). The final target of an activated cyclin D/CDK4/6 complex is the pRb tumour suppressor gene. Phosphorylation of pRb results in the dissociation of E2F transcription factors required for the transcription of E2F responsive genes, including those with proteins involved in DNA synthesis and essential for S phase progression.

E2F is a family comprising a number of different transcription factors that can bind to hypophosphorylated pRb. Once activated, the transcription of E2F genes (including cyclins D1, E and A) is mediated, after which phosphorylation of Rb frees E2F, enabling gene transactivation. This process is initially triggered by the cyclin D dependent kinases and accelerated by the cyclin E/CDK2 complex (Sherr, 1996).

Progression through G1 to the first restriction point requires cyclin E activity and continuation through S phase requires the activity of cyclin A, both complexed with CDK2. Activation of G1 cyclins is directly linked to the synthesis of DNA, as cyclin D or cyclin E/CDK activity releases E2F, inducing a cdc6 protein, essential for DNA replication in S phase. During G2 there are checkpoints to ensure that DNA has been properly copied before entry into mitosis. If any errors have occurred, DNA repair systems can be activated. Levels of the mitotic cyclins (A and B) increase in S phase and G2 and cyclin B complexes with unphosphorylated cdc2 (Nguyen and Jameson, 1998). Expression of B-type cyclins is maximal during G2 to M transition and they control entry into M phase.

There is great potential for increased cellular proliferation if alterations occur in these regulatory genes/proteins. Overexpression of cyclin D1, for example, allows cells to proliferate even in the absence of growth factors, perturbing tissue homeostasis and is positively associated with, among others, breast and colorectal carcinomas (Franks et al., 1998). The kinase activity of cyclin dependent kinase complexes is regulated by both phosphorylation and dephosphorylation and cyclin/CDK complexes are negatively controlled by CDKIs (Nguyen and Jameson, 1998, Fernandez et al., 1998), for example, $p21^{WAF1/CIP1}$ which acts by directly inhibiting kinase activity. The p21 and p27 proteins have been identified as inhibitors of cyclin D/CDK4, cyclin E/CDK2 and cyclin A/CDK2 and in addition, $p21^{WAF1/CIP1}$, (a cdk2 inhibitor), has been demonstrated to play a crucial role in the G1 checkpoint and appears to be required for G1 arrest following DNA damage (Fernandez et al., 1998). It is speculated that these proteins, in addition to the cyclins and their associated kinases are also potential targets for deregulation in the cancer cell cycle.

1.2.2. Signal transduction pathways

Growth factors regulate cell growth by the activation of intracellular signal transduction pathways following binding to cell surface receptors (Lange et al., 1998), resulting in changes in the transcriptional activity of genes. Among the

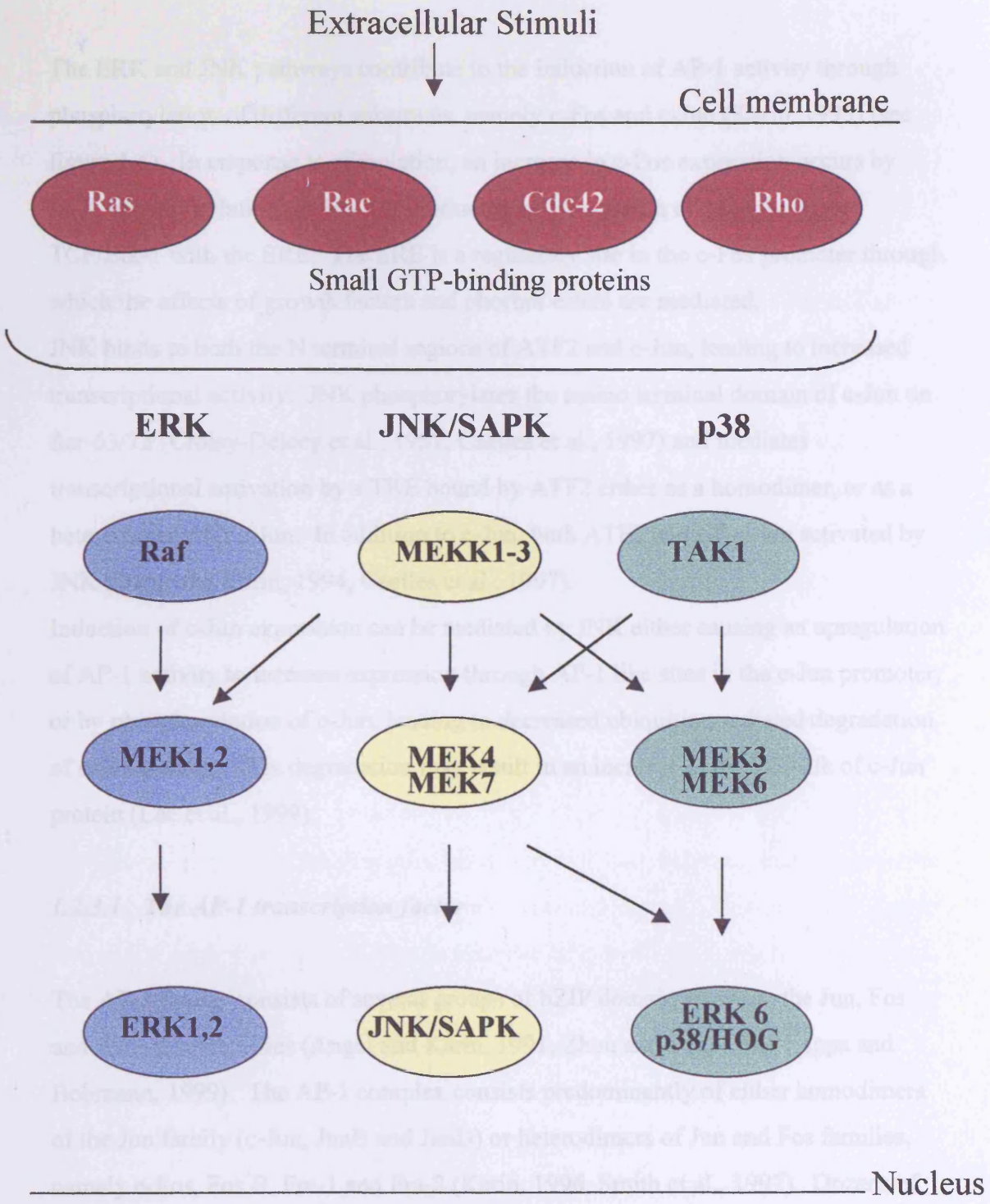
pathways often used is the highly conserved MAPK cascade (Robinson and Cobb, 1997). In their simplest form, MAPK modules consist of three distinct kinases arranged linearly, in which the upstream kinase phosphorylates and thereby activates the downstream kinase (Kieran et al., 1999). MAPK can enter the nucleus to directly modulate transcriptional regulation by phosphorylation of transcription factors, such as AP-1.

In mammalian cells, three MAPK cascades are important in the transmission of signals to the nucleus. The first pathway is the *ras-raf* pathway leading to the activation of the ERKs (Xing and Imagawa, 1999) and is largely responsive to mitogenic stimuli (Kieran et al., 1999). The second and third pathways result in activation of the SAPK/JNK and p38 families respectively (Karin and Hunter, 1995, Kieran et al., 1999, Ichijo, 1999,). Unlike the ERK signalling pathway, JNK and p38 pathways are not preferentially activated by mitogens, but by an array of cellular stresses including UV light, hydrogen peroxide and osmotic shock, in addition to inflammatory cytokines such as TNF and IL-1β (Ichijo, 1999). The components of each MAPK pathway are outlined in figure 1.3.

1.2.3. Transcription factors

Specific stimulatory proteins termed transcription factors interact with eukaryotic promoters to modulate transcriptional activity. The MAPK pathways culminate in the stimulation of AP-1, implicated as a critical target for many signalling pathways that regulate cell differentiation, proliferation and transformation (Angel and Karin, 1991).

The activation of ERKs is responsible for cellular proliferation and differentiation and also promotes cell survival, in contrast to JNK, which is not only involved in cell proliferation, but also contributes to cell death (Zi and Agarwal, 1999). ERK and JNK MAPK are both activated by dual phosphorylation on threonine and tyrosine residues (Croisy-Delcey et al., 1997).



Integration of signal leading to ligand- and cell type specific responses.

Fig. 1.3. Parallel MAPK cascades. Adapted from Robinson and Cobb, 1997.

The ERK and JNK pathways contribute to the induction of AP-1 activity through phosphorylation of different substrates, namely c-Fos and c-Jun (Karin, 1995) (see figure 1.4). In response to stimulation, an increase in c-Fos expression occurs by ERK phosphorylation on Ser-374 producing an association of phosphorylated TCF/Elk-1 with the SRE. The SRE is a regulatory site in the c-Fos promoter through which the effects of growth factors and phorbol esters are mediated.
JNK binds to both the N terminal regions of ATF2 and c-Jun, leading to increased transcriptional activity. JNK phosphorylates the amino terminal domain of c-Jun on Ser-63/73 (Croisy-Delcey et al., 1997, Caelles et al., 1997) and mediates transcriptional activation by a TRE bound by ATF2 either as a homodimer, or as a heterodimer with c-Jun. In addition to c-Jun, both ATF2 and Elk-1 are activated by JNK (Deng and Karin, 1994, Caelles et al., 1997).
Induction of c-Jun expression can be mediated by JNK either causing an upregulation of AP-1 activity to increase expression through AP-1 like sites in the c-Jun promoter, or by phosphorylation of c-Jun, leading to decreased ubiquitin-mediated degradation of c-Jun protein. This degradation may result in an increase in the half-life of c-Jun protein (Lee et al., 1999).

1.2.3.1. The AP-1 transcription factor

The AP-1 family consists of several groups of bZIP domain proteins: the Jun, Fos and ATF-2 subfamilies (Angel and Karin, 1991, Zhou and Lee, 1998, Leppa and Bohmann, 1999). The AP-1 complex consists predominantly of either homodimers of the Jun family (c-Jun, JunB and JunD) or heterodimers of Jun and Fos families, namely c-Fos, Fos B, Fra-1 and Fra-2 (Karin, 1996, Smith et al., 1997). Dozens of different homo- and heterodimeric combinations with different regulatory properties can therefore be formed (Leppa and Bohmann, 1999).
AP-1 proteins dimerise to form a protein complex before they can bind to their DNA target sites. These AP-1 complexes bind to a specific target DNA sites known as a TRE, found in the promoters of several cellular genes, such as human collagenase, stromelysin and plasminogen activator inhibitor, and activate transcription of these

genes (Smith et al., 1997). Activation of AP-1 has been shown to mediate growth-factor induced proliferation of breast cancer cells *in vitro* and it also upregulates a number of genes, including the p-glycoprotein multidrug resistance gene (Chen et al., 1996).

The AP-1 complex can either positively or negatively regulate transcription of target genes, depending on the composition of the heterodimers (Smith et al., 1997), and different stimuli (such as UV light or growth factor stimulation) can rapidly and transiently induce the activity and expression of Jun and Fos proteins. Total AP-1 activity may be increased by changes in the relative abundance of Jun/Fos proteins or by specific phosphorylation of c-Jun. Recent evidence also suggests that growth-promoting AP-1 activity may be co-regulated with ligand-bound ER, (see section 4) with oestrogen treatment of ER-positive cells resulting in enhanced AP-1 transcriptional activity and gene expression, mediated by DNA bound Jun/Jun or Fos/Jun dimeric complexes. ER can interact with the AP-1 complex through protein-protein interactions independent of oestrogen receptor DNA binding.

Interference in both cell cycle regulation and MAPK cell signalling pathways may be important when observing deregulation of cancer cell growth. Treatment with agents such as the tumour promoter TPA can promote cells to exhibit uncontrollable growth (Angel and Karin, 1991, Chen et al., 1996). Repeated treatment with TPA may result in the conversion of already continuously growing cells into the tumourigenic phenotype characteristic of cancer cells, which may prove useful as a model for tumour promotion.

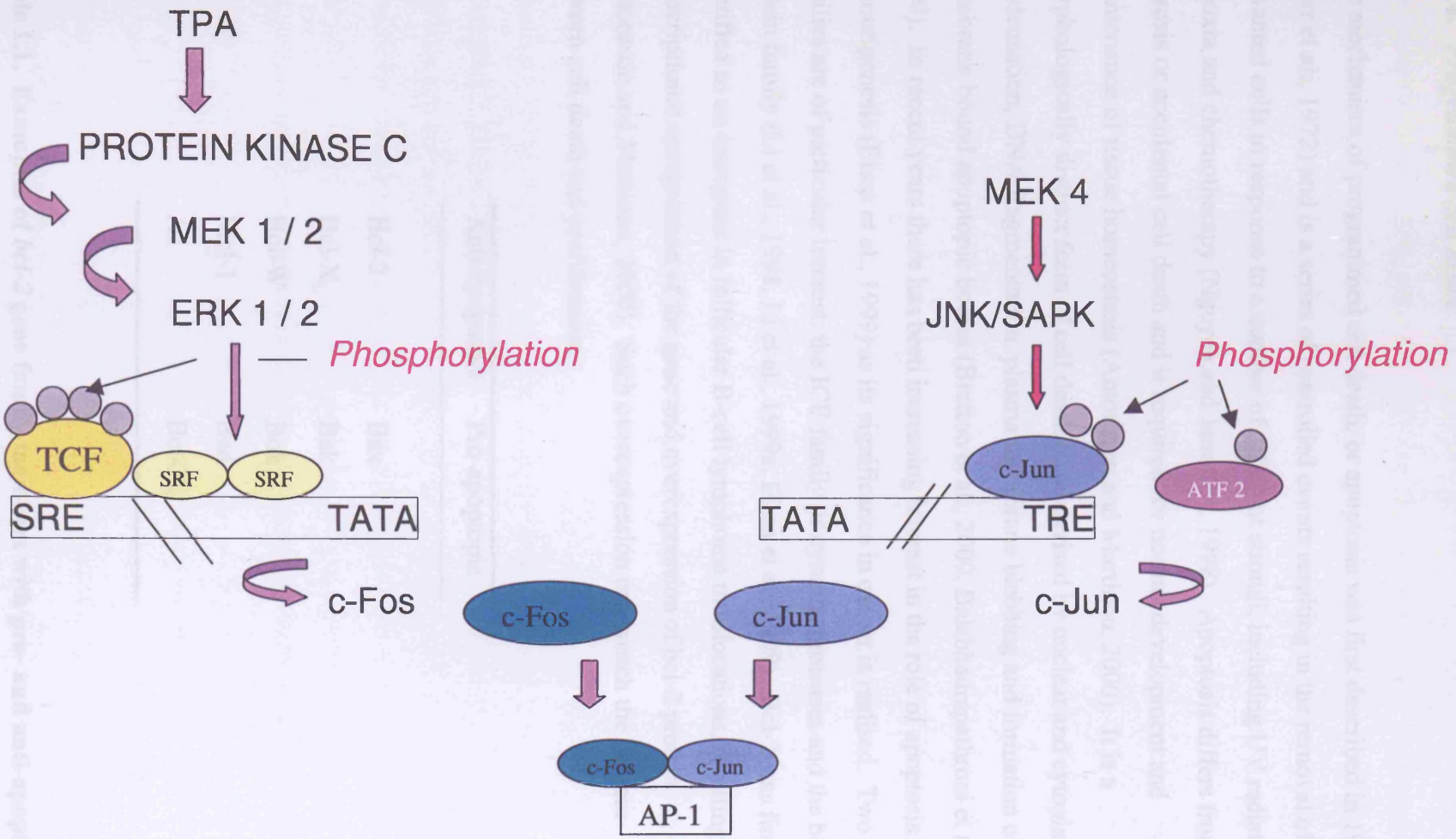


Fig. 1.4. Stimulation of AP-1 activity by MAP kinases. Adapted from Karin and Hunter, 1995.

1.2.4. Programmed cell death

The mechanism of programmed cell death, or apoptosis was first described in 1972 (Kerr et al., 1972) and is a series of controlled events resulting in the removal of unwanted cells in response to a number of different stimuli, including UV radiation, hypoxia and chemotherapy (Nguyen and Jameson, 1998). Apoptosis differs from necrosis or accidental cell death and is required for normal development and maintenance of tissue homeostasis (Antonsson and Martinou, 2000). It is a morphologically distinct form of cell death characterised by nuclear and cytoplasmic condensation, DNA fragmentation, plasma membrane blebbing and formation of membrane bound apoptopic bodies (Bratton et al., 2000, Balabhadrapathruni et al., 2000). In recent years there has been increasing interest in the role of apoptosis in tumourigenesis (Eissa et al., 1999) as its significance in cancer is realised. Two gene families are of particular interest: the ICE family of cysteine proteases and the bcl-2 protein family (Li et al., 1998, Li et al., 1999a, Eissa et al., 1999). Bcl-2 was first identified as an oncogene in follicular B-cell lymphoma translocations, resulting in transcriptional upregulation of the gene and overexpression of bcl-2 protein (Antonsson and Martinou, 2000). Such overexpression can breach the balance between cell death and proliferation.

Anti-apoptopic	Pro-apoptopic
Bcl-2	Bax
Bcl-X_L	Bak
Bcl-W	Bok
Mcl-1	Bad
A1	Bcl-X_S

Table 1.1. Examples of *bcl-2* gene family members with pro- and anti-apoptopic activity. Adapted from Antonsson and Martinou, 2000.

There are both positive and negative regulators of *bcl-2* and a number of bcl-2 related genes have been identified, some of which are outlined in table 1.1. Inhibition of cell death by alterations in crucial apoptopic genes, for example, suppression of genes that induce cell death or activation of those genes that cause cell survival, can lead to increased and uncontrolled levels of cell proliferation characteristic of cancer.

1.3. Breast cancer

Breast cancer is one of the most common cancers in women in the developed countries of the world. In 1998 it was estimated that 180,000 new cases were diagnosed in the USA, while deaths were estimated at 44,000 (Baquet, 1999). Most cases of breast cancer are diagnosed in women with no family history of the disease and there is a higher incidence post-menopausally (Welsch et al., 1998). The disease appears to be more common among Caucasians living in colder climates and highly industrialised countries of the Western world. A number of risk factors have been associated with development of the disease, such as parity status, age at first child and menopause. Mutations in BRCA 1 and BRCA 2 are associated with a family history in about 5% of cases (Walker et al., 1997).

Amplification and overexpression of the oncogene erbB2 is found in 60% of ductal carcinomas *in situ* and 20-30% of invasive carcinomas (Walker et al., 1997). Alterations in the tumour suppressor gene p53 have been identified in *in situ* and invasive carcinomas and comprise allelic imbalance and /or mutation. Recent molecular studies of breast cancer such as those using comparative genomic hybridisation have identified alterations in many chromosomal regions, illustrating the complexity of the disease.

The incidence of and deaths from cancers arising in hormone-dependent tissues such as breast and prostate are significantly lower in Asia than in Western populations (Messina et al., 1994, Barnes and Peterson, 1995, Lamartiniere et al., 1998). Yet, these rates are seen to increase when Asians emigrate to Western Countries

(Lamartiniere et al., 1998), or when the lifestyle of countries, such as Japan, become more westernised (Nagata and Shimizu, 1996).

The notion that diet derived substances can prevent the onset of cancer has received considerable interest in recent years (Messina et al., 1994, Zava and Duwe, 1997, Gescher et al., 1998). As a result from population and case control studies it is now readily accepted that many common diseases, including breast cancer, are attributed, at least in part, to differences in dietary intake (Messina et al., 1994) and may be influenced by certain dietary modifications. The human diet contains an array of phytochemicals that, if incorporated into the diet as either an integral part of the food or as a supplement, may provide individuals with significant health benefits (Setchell, 1998).

1.4. The oestrogen receptor

Oestrogen is an essential regulator of female reproductive functions (Giguere et al., 1998), for example, it regulates the growth and development of mammary glands during embryogenesis and ovarian follicles during the reproductive cycle. However, in addition to its normal roles in reproductive physiology, it is also important in bone maintenance and within the cardiovascular system (Enmark and Gustafsson, 1998). Oestrogens are produced primarily in the ovaries and adrenal glands, and the three main forms of oestrogen are 17β-oestradiol (the most biologically potent), oestriol and oestrone.

The physiological effects of these steroids are mediated by ligand-inducible nuclear binding proteins termed oestrogen receptors. Upon binding of the hormone (oestradiol) to the oestrogen receptor, there is displacement of heat shock binding protein 90 and dimerisation of two bound oestrogen receptors. The dimerised complex translocates to the nucleus and interacts with oestrogen response elements (ERE) located in promoter regions of target genes to stimulate gene transcription.

Steroid receptor proteins share a common structure and oestrogen receptors possess a similar functional organisation that can be subdivided into several structural and functional domains (Katzenellenbogen, 1996, Enmark and Gustafsson, 1998). The N terminal A/B domain contains a transactivation function (AF-1) and is highly variable in sequence and length (Enmark and Gustafsson, 1998). The most conserved region of the alpha and beta forms of the receptor is the DNA binding domain (Saunders, 1998) that contains two zinc fingers involved in specific DNA binding and receptor dimerisation (Enmark and Gustafsson, 1998, Saunders, 1998). The hinge region (D) gives flexibility to the DNA via the LBD, which is relatively large and is important for ligand binding, receptor dimerisation and interaction with transcriptional co-activators and co-repressors (Enmark and Gustafsson, 1998, Saunders, 1998). It also contains a second transactivation function (AF2) (Giguere et al., 1998). The functions of the C-terminal domain are largely unknown, but it may contribute to the transactivation capacity of the receptor (Enmark and Gustafsson, 1998).

Until recently, oestrogens were thought to mediate their actions principally through a single receptor (Leygue et al., 1998), but the first oestrogen receptor cloned in 1986 from the uterus has now been re-named ER-α, after the identification of a second oestrogen-specific receptor (Kuiper et al., 1996). This second receptor was cloned from a rat prostate cDNA library and has been termed ER-β.

In addition to its identification in the rat, ER-β has also been cloned from the mouse and humans (Mosselman et al., 1996). There is a high degree of conservation (96%) between the human β-type receptor and the ER-α receptor in the DNA binding domain and relatively high homology (58%) within the LBD (Mosselman et al., 1996). The high levels of homology between the receptors suggested ER-β was capable of binding oestradiol with high affinity (Vladusic et al., 1998) and could stimulate transcription of an ER target gene in an oestradiol dependent manner, properties duly identified by various ligand binding experiments (Kuiper et al., 1996, Mosselman et al., 1996).

The tissue distribution of ER-β mRNA has been studied (Enmark et al., 1997, Kuiper et al., 1997), and further studies have been performed to identify receptor subtypes and variants in both breast tumours and breast cancer cell lines (Dotzlaw et al., 1996, Vladusic et al., 1998, Fuqua et al., 1999, Leygue et al., 1999). One example of an ER-β mRNA variant is ER-β2, which encodes a receptor with an 18 amino acid insertion in the LBD. This variant exhibited altered ligand binding and transcriptional activation properties (Peterson et al., 1998). The kidney, lung, spleen and pituitary gland were identified to exhibit elevated levels of ER-β expression while in the breast; detectable levels were expressed in the tubular epithelium (Enmark et al., 1997). ER-α and ER-β are not generally co-expressed in breast cell lines and a four-fold increase in expression of ER-β has been observed when comparing breast cells to normal mammary tissue. Expression of the receptors may also vary between tumours, with differences between cells and tumours due to down regulation of ER-β.

The mitogenic and regulatory effects of oestrogen are known to act via its nuclear receptor(s) (Pennie et al., 1998). While the two receptors are very similar to one another and share many functional properties, they also possess individual characteristics suggesting each isoform may perform different biological functions. Differential activation of ER-α and ER-β has been observed by the anti-oestrogen 4-hydroxytamoxifen (Watanabe et al., 1997) and the receptors have also been shown to differentially activate AP-1 regulated reporter genes (Paech et al., 1997). Interestingly, ER-β has also shown a relatively high affinity for several plant-derived substances with oestrogenic activity, an affinity that is considerably higher than that of ER-α (Kuiper et al., 1997, Kuiper et al., 1998, Casanova et al., 1999). Lastly, it has been speculated that ER-α variants may play a role in breast cancer tumour progression and it is possible that the variants of the beta-receptor may also contribute to this change from a hormone-dependent to a hormone-independent phenotype (Vladusic et al., 1998). The existence of two oestrogen receptor subtypes presents another potential source of specific oestrogen regulation.

1.5. Phyto-oestrogens

1.5.1. Definition and structure

Phyto-oestrogens are plant-derived compounds of natural origin (Barnes, 1998, Setchell, 1998). The number of these plant-derived compounds identified as having biological activity is expanding rapidly, as epidemiological studies continue to indicate possible associations between diet and disease. All phyto-oestrogens are polyphenols and can be divided into two major chemical classes: isoflavones and lignans, with isoflavones found predominantly in soyabeans (Anderson et al., 1999).

One characteristic feature of the structure of phyto-oestrogens is the presence of a phenolic ring that is required for interaction with the oestrogen receptor. Phyto-oestrogens can act as either oestrogen agonists or antagonists (Barnes and Peterson, 1995) and their actions at the cellular level can be influenced by many factors including receptor status and the presence or absence of endogenous oestrogens. Phyto-oestrogens, or dietary oestrogens, are weakly oestrogenic (10^2 -10^3-fold) when compared with oestradiol or oestrone (Martin et al., 1978). Phyto-oestrogens also exhibit preferential binding to the recently cloned oestrogen receptor beta (ER-β) (Kuiper et al., 1998), suggesting they may exert their actions via distinct and separate pathways from those of classical steroidal oestrogens. The 4 hydroxyl position on the B ring and its spatial orientation with the 7 position hydroxyl on the A ring are primarily responsible for the oestrogenicity of flavanoids.

1.5.2. Phyto-oestrogens in the diet and their relationship to breast cancer

The largest concentrations of phyto-oestrogens in the human diet are isoflavanoids present in soy food and lignans found in flaxseed and linseed (Reinli and Block, 1996). The highest concentration of the major isoflavones (genistein and daidzein) are found in soyabeans and soyabean products, with the isoflavone concentration

lower in the derivative or second generation soyabean foods (Anderson et al., 1999). This decrease is due to dilution with other constituents and losses during processing, however isoflavones do appear to be resistant to degradation by common food preparation methods such as frying and baking.

Western diets do not customarily include soy products; the British intake has been estimated to be less than 1mg per day, whereas adding soy to the typical Western diet increases urinary isoflavone levels as much as 1000-fold (Setchell et al., 1984). This can be compared to the mean daily intake of soy in South East Asia that has been estimated to range between 10 and 50g (Peterson, 1995).

Epidemiological studies have supported the view that soy foods can prevent hormone dependent cancers, and this has been recognised when the incidence of breast cancer in Asian women and women of the Western world have been compared (Wei et al., 1995). Asian women who consume a traditional diet of high soy and low fat have a four-fold decreased risk of developing breast cancer compared to women in Western Europe, and it has been hypothesised that it is the consumption of soy which is responsible for the lower incidence of breast cancer due to its chemopreventive effects.

1.5.3. Soy isoflavones

The first recorded use of soy was in 2838 BC, contained in Materia Medica of the Chinese Emperor Shen Nung (Adolph and Kiang, 1920). South East Asians have used soybeans as a staple in their diet for several centuries (Dotzlaw et al., 1996) and nowadays soybeans are converted to a variety of food products (see figure 1.5).

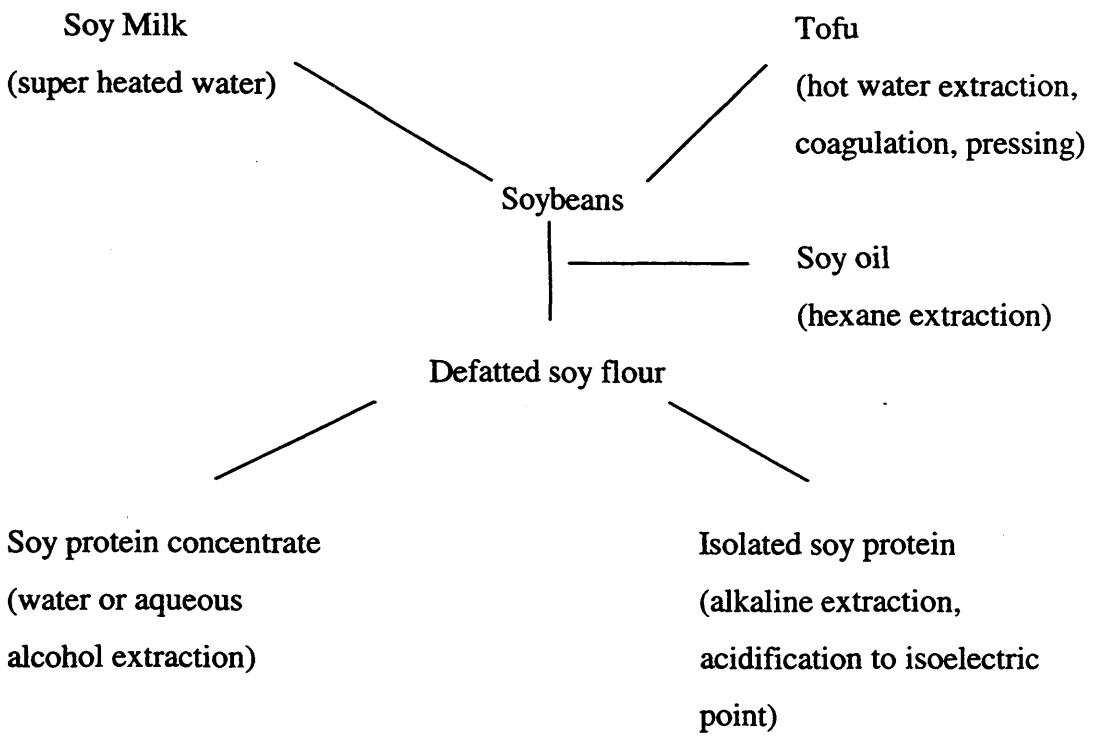
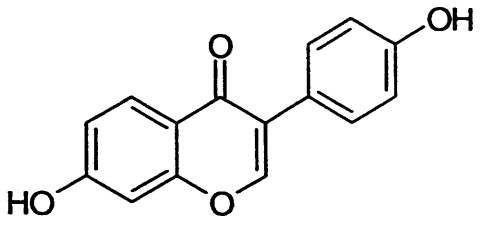


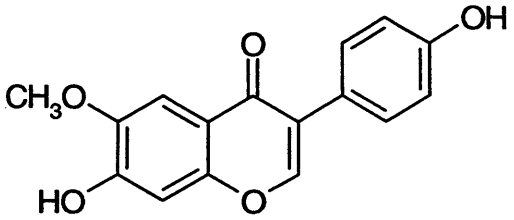
Fig. 1.5. Soy food materials prepared for the US market, adapted from Barnes, 1995a.

The major isoflavones in soybeans are the glycosides genistin, daidzin and glycitin and their aglycones genistein, daidzein and glycitein (Wahala et al., 1998) (see figure 1.6), with genistein accounting for two-thirds or more of the total soybean isoflavone content (Coward et al., 1993).

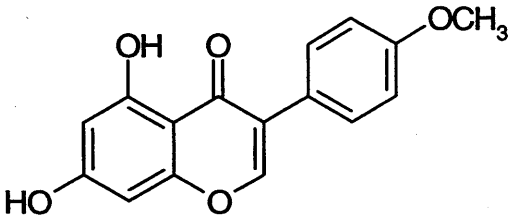
Little is known about the biological activity of the individual glycosidic conjugates of isoflavones, as they are readily hydrolysed by intestinal bacteria, making it difficult to assess their activity. Intestinal bacteria can differ between individuals and studies have shown considerable variation in isoflavone phyto-oestrogen metabolism (Wiseman, 1998), which may influence their protective activity towards breast cancer risk. Soy isoflavones undergo extensive enterohepatic



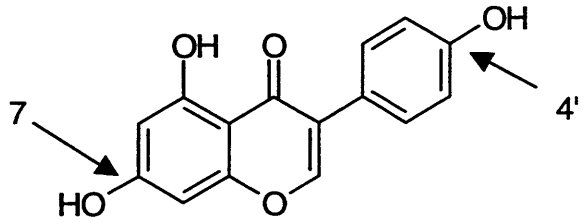
Daidzein



Glycitein



Biochanin A



Genistein

Fig. 1.6. Structure of phyto-oestrogens.

circulation, are rapidly conjugated by the liver to glucuronic acid and to a lesser extent, with sulphate (Messina et al., 1994) and are found primarily as conjugates in both blood and urine.

Genistein (4'5,7 trihydroxyisoflavone), the most abundant isoflavone in soy, is a diphenolic planar molecule with an aromatic A ring. It is found in the form of a β-glucoside called Genistin (Lamartiniere et al., 1998), with 6'-O-malonylgenistin being the principle glucoside (Barnes, 1995a). The natural intestinal microflora hydrolyse this β-glucoside to free genistein and other related flavones which are present in circulating blood, accumulate in tissues and are excreted in the urine (Fioravanti et al., 1998). Like other phyto-oestrogens, genistein is structurally similar to oestradiol and binds relatively weakly to the oestrogen receptor (Ingram et al., 1997).

1.5.4. Metabolism of genistein

The potential importance of flavanoids as chemopreventive agents requires an understanding of the absorption, distribution, metabolism and excretion after ingestion (King and Bursill, 1998). Biotransformation plays an important role in regulating the biological activity of isoflavanoids (Coldham and Sauer, 2000), with phase II biotransformations such as glucuronidation and sulphation providing mechanisms for isoflavanoid inactivation and excretion. Eight different genistein metabolites have been identified in rat urine and bile (Yasuda, 1996) and in a more recent study, seven metabolites of [^{14}C]-genistein were also observed in the rat (Coldham et al., 1999). Human pharmacokinetic studies have also been performed after ingestion of soy meals, with differences apparent between urinary excretion of genistein and daidzein (King and Bursill, 1998) and the identification of urinary metabolites (Heinonen et al., 1999).

Although the liver is regarded as the major site of biotransformation, metabolism can occur in other body compartments (Coldham et al., 1999). The metabolism of isoflavones largely occurs in organs such as the liver, small intestine and the kidney;

however, metabolism of isoflavones in cells of the breast has also been reported (Peterson et al., 1996). Peterson *et al.*, observed metabolites of isoflavones in breast cell culture media with analysis by HPLC (Peterson et al., 1996, Peterson et al., 1998). Genistein was converted in various amounts to its 7-monosulphate form in MCF-7, ZR-75.1, T47-D and BT-20 breast cell lines, in addition to a hydroxylated and methylated metabolite found in both MCF-7 and T47-D cells (Peterson et al., 1996, Peterson et al., 1998). Another isoflavone closely related to genistein, biochanin A, was demethylated to genistein, which in turn was converted to genistein 7-monosulphate (Peterson et al., 1998). Interestingly, no significant metabolism of genistein occurred in normal HMECs (Peterson et al., 1996).

1.5.5. Chemopreventive activity of genistein

Animal models of cancer have been widely used to investigate the chemopreventive effects of dietary agents (Barnes, 1995b). Different groups have performed a number of animal chemoprevention studies using soy protein preparations containing isoflavanoid conjugates.

In rodent models, breast cancer can be induced by a carcinogen such as orally administered DMBA and the animals observed for periods from 3 months to 2 years, after which tumour incidence is measured. Initial studies by Lamartiniere *et al.* showed that neonatal or prepubertal genistein (500μg/g body weight) could protect against DMBA-induced mammary cancer (Lamartiniere et al., 1995).

Further studies concluded that dietary genistein, provided early in life, reduced the number of undifferentiated terminal end buds, reduced cell proliferation and protected against mammary cancer in the adult (Fritz et al., 1998). Such data suggests a programming effect during early postnatal life on the target tissue can result in lifetime protection against mammary cancer (Lamartiniere and Wang, 1999).

1.5.6. Mechanisms of action of genistein

1.5.6.1. Biochemical targets of genistein

Cancerous cells can lose the ability to respond appropriately to extracellular signals via signal transduction pathways of which PTK are key components. Two classes of PTK are recognised: receptor tyrosine kinases and cytosolic tyrosine kinases. The former receive signals through the extracellular domain whilst the latter are downstream signal transducing elements (Gescher et al., 1998). PTK catalyse the transfer of the γ-phosphate group of ATP to the hydroxyl group of tyrosine on a number of proteins.

Genistein has received much attention in recent years, following an investigation by Akiyama *et al*, (1987). They identified genistein as a specific *in vitro* inhibitor of tyrosine kinases, and in particular EGF-R tyrosine kinase. Since this discovery, genistein has been used extensively to explore tyrosine phosphorylation events in cell signalling cascades. Genistein has also been observed to suppress protein tyrosine phosphorylation in response to cell adhesion to the ECM (Yan and Han, 1998), and subsequently to inhibit invasion through the ECM by B16-BL6 melanoma cells. Genistein has also been shown to inhibit p56/p53^{lyn}, a kinase involved in the G2/M transition of the cell cycle (Kaufmann, 1998).

Experiments performed with supercoiled plasmid DNA and DNA topoisomerase II revealed an inhibition of topoisomerase II activity by genistein. *In vitro* studies indicate that genistein, which does not intercalate with DNA, inhibits this enzyme by stabilising the DNA-topoisomerase II cleaved complex (Yamishita et al., 1990).

The production of ROS has been postulated to play a role in carcinogenesis, particularly in tumour promotion (Peterson, 1995). Genistein is a potent antioxidant capable of inhibiting TPA-induced hydrogen peroxide production in HL-60 cells, neutrophils and mouse skin (Wei et al., 1993, Wang et al., 1998), in addition to inhibition of superoxide anion production by xanthine/xanthine oxidase.

It has been shown that ER-β has a higher affinity for plant-derived chemicals with oestrogenic activity compared to the classical alpha type receptor (Kuiper et al., 1997, Enmark and Gustafsson, 1998) and genistein has been observed to have a greater affinity for ER-β in saturation ligand binding assays (Kuiper et al., 1997, Kuiper et al., 1998). In other experiments it was observed that genistein could effectively compete with oestradiol binding to rat ER-β1, but was >150-fold weaker at competing to ER-β2 (Peterson et al., 1998). Similar *in vitro* studies (Casanova et al., 1999) showed that both genistein and daidzein were complete agonists to rat ER-α and ER-β. Genistein showed a stronger interaction with rat ER-β compared to oestradiol. As genistein shows a much greater affinity for ER-β rather than ER-α, it is possible it may exert some of its actions via a different oestrogen receptor mediated pathway (Ingram et al., 1997).

1.5.6.2. Effects of genistein on cell growth and cell cycle regulation

Many studies have shown that over a range of physiological concentrations, genistein has differing effects on breast cell growth (Zava and Duwe, 1997, Fioravanti et al., 1998, Hsieh et al., 1998, LeBail et al., 1998). At concentrations between 10^{-5} and 10^{-4} M, genistein has been shown *in vitro* to inhibit cell growth in a wide range of breast cell lines (Barnes, 1995b, LeBail et al., 1998, Fioravanti et al., 1998). Paradoxically, *in vitro* at 10^{-6} M, it is seen to stimulate, rather than attenuate the growth of MCF-7 breast cancer cells (Martin et al., 1978, Fioravanti et al., 1998, Hsieh et al., 1998, LeBail et al., 1998).

The effects of genistein on the cell cycle have been very well documented in recent years. Studies from different groups have observed cycle arrest at both G2/M and G1/S phase transitions (Matsukawa and Aoike, 1993, Kuzumaki et al., 1998, Shao et al., 1998, Lian et al., 1998, Constantinou et al., 1998, Davies, et al., 2000) within a range of human and murine cancer cell lines. Genistein has been reported to arrest cell cycle progression at G2/M within MCF-7, T47-D, MDA-MB 468 and MDA-MB

231 human breast carcinoma cell lines (Choi et al., 1998, Shao et al., 1998, Constantinou et al., 1998).

One mechanism by which genistein interferes with breast cell cycle progression is by inhibiting expression of cyclin B1 protein (Choi et al., 1998, Balabhadrapathruni et al., 2000) which has been correlated with a decrease in cyclin B1 mRNA (Choi et al., 1998). Genistein has been shown to induce levels of $p21^{WAF1/CIP1}$ expression, accompanied by increased levels of apoptosis (Shao et al., 1998). In addition to G2/M arrest, genistein has also been observed to inhibit both cdc2 and cdk2 kinase activity, both associated with an increase of $p21^{WAF1/CIP1}$ expression.
As previously mentioned, genistein has also been shown to inhibit $p56/p53^{lyn}$ kinase, which is involved in the cell cycle (Kaufmann, 1998). Progression from G2 into M phase of the cell cycle is controlled by a cyclinB/CDK1 complex, which in turn is regulated by tyrosine phosphorylation. The inhibition of $p56/p53^{lyn}$ kinase by genistein inhibits phosphorylation on a tyrosine residue (Tyr15) on CDK1, thereby interfering with cell cycle progression.

1.5.6.3. Induction of apoptosis by genistein

The mechanism of programmed cell death can be measured by a variety of different methods. Fragmentation of cellular DNA at internucleosomal linker regions illustrated by DNA laddering is a widely used biochemical marker, in addition to cleavage of PARP and the activation of caspase-3, which are also early indicators of apoptosis (Li et al., 1999a). Apoptosis can also be confirmed by Hoechst 33342 staining or phosphatidylserine externalisation by Annexin V staining. Genistein-induced apoptosis has been observed in a number of different cell lines, including MCF-7 and MDA-MB 231 cells (Pagliacci et al., 1994, Choi et al., 1998,).

Genistein has been reported to cleave PARP and activate caspase-3 in MDA-MB 231 cells (Li et al., 1999a). In a number of different studies, genistein has been reported to induce apoptosis by altering the expression of apoptosis related genes. It has been

shown to upregulate levels of the apoptopic inducers Bax and p21, while downregulating both bcl-2 and p53 protein expression in MDA-MB 231 and MDA-MB 435 breast cancer cells treated with 30μM genistein (Li et al., 1999b, Li et al., 1999a). At high concentrations (150μM), genistein has also been observed to inhibit bcl-2 protein, increase levels of bcl-2 phosphorylation and cause upregulation of p53 in MCF-7 cells (Constantinou et al., 1998).

1.6. Project Aims.

This project is directed at elucidating the mechanisms involved in genistein-induced effects on cell growth. The hypothesis to be tested is that genistein affects the growth of human-derived breast cancer cell lines in a similar manner.
To do this, six breast cell lines have been used, covering a range of phenotypes. The tumour cell lines used were MCF-7, ZR-75.1, T47-D, MDA-MB 468 and MDA-MB 231, which are thought to possess different levels of hormone dependence, in addition to an immortalised, but transformed cell line, HBL 100. The study was designed to determine the generic nature or otherwise, of some of the features pertinent to the modulation of breast cell growth by genistein, and to highlight differences in the response of these cell lines to genistein.

Genistein in the 10^{-5} to 10^{-4} M concentration range has been shown to inhibit the growth and survival of breast cancer cells (see section 1.5.6.2). Here, the growth inhibitory effect was investigated using two different cell culture conditions. Studies were performed using media differing in content of serum and serum constituents, to allow observation of the role of serum in genistein-induced growth inhibition. Furthermore, it was hypothesised that genistein also elicits growth-stimulatory effects also in oestrogen receptor-positive cell lines other than MCF-7 cells. Differences between cell lines towards the effect of genistein on growth may be attributed to the presence of receptors germane to proliferation. Therefore, the presence of the EGF-R and ER (see section 1.5.6.1) were examined, as they may be targets for genistein.

The susceptibility of cell lines towards genistein-induced growth effects may be related to changes in cellular biochemistry. The mechanism of genistein-induced growth inhibition was investigated with respect to its ability to modulate the activity of cell signalling pathways and transcription factors. The effect on transcription factor AP-1, important in controlling cell growth and proliferation, and its effect on MAPK cell signalling pathways within the cell lines were examined. The hypotheses that genistein inhibits cell growth by interfering with phosphorylation of ERKs and by inhibiting levels of c-Jun and c-Fos protein were tested.

Investigation into the effect of genistein on cell cycle regulation and apoptosis was also performed, as inhibition of cell growth by genistein can occur via deregulation of such processes (see sections 1.5.6.2-3). Here, the hypothesis tested was that genistein modulates cell cycle regulation similarly in all cell lines.

The ability of genistein to inhibit tumour cell growth is important for chemoprevention and perhaps for therapy, and the mechanisms by which it inhibits growth are not necessarily identical in different cell lines. Overall, the study was designed to provide an insight into the effect of genistein on a number of cell lines and to find potential mechanisms that are common or specific to a particular cell line. The overall results may help to optimise the design of the clinical evaluation of genistein.

CHAPTER 2: MATERIALS AND METHODS.

2.1. *MATERIALS.*

2.1.1. *Source of chemicals and kits*

Acrylamide (30% acrylamide: bis acrylamide)	Anachem
AMV buffer	Promega
Benzamidine	Sigma
Beta galactosidase reporter gene assay kit	Promega
BSA	Sigma
Cholera toxin	Sigma
dNTPs	Pharmacia Biotech
DMEM	Gibco BRL
DMEM /F12 Nut Mix	Gibco BRL
DMEM /F12 (Ham 1:1)	Gibco BRL
Dextran coated charcoal	Sigma
DMSO	Fisons Analytical Reagents
Dynabead kit	Dynal
ECL	Amersham
Endofree MaxiPrep Kit	Qiagen
EGF	Sigma
Fugene transfection reagent	Boeringher Mannheim
Genistein	Sigma
Glutamax	Gibco BRL
β-Glycerophosphate	Sigma
Hybond Nitrocellulose	Amersham
Hydrocortisone	Sigma
Insulin	Sigma
Igepal CA 630	Sigma
Luciferase reporter assay kit	Promega
Miniprep DNA kit	Promega
Pierce Protein Reagent Kit	Pierce Protein Reagents
Poly DIDC	Sigma
Polyoxyethlene sorbitan monolaurate (Tween 20)	Sigma

Protein A agarose beads	Sigma
RPMI 1640	Gibco BRL
Sodium Pyrophosphate	BDH
TPA	Sigma
TEMED	Sigma
Triton-X-100	Sigma

2.1.2. Protein and phosphatase inhibitors.

Aprotonin	Sigma
Leupeptin	Sigma
Pepstatin A	Sigma
PMSF	Sigma
Sodium fluoride	Sigma
Sodium vanadate	Sigma

2.1.3. Enzymes.

AMV reverse transcriptase	Promega
RNAse	Sigma
Hind III restriction enzyme (+buffer B)	Boehringer Mannheim
T4 polynucleotide kinase (+ T4 PNK buffer)	Promega
Taq polymerase	Promega

2.1.4. Antibodies.

c-Fos - specific for c-Fos, clone H-125, corresponds to amino acids 210-235 of carboxy terminus of human c-Fos.	Santa Cruz
c-Jun – specific for c-Jun, corresponds to residues 1-79 of human c-Jun	New England BioLabs
cyclin B1 – clone 7A9, specific for complete cyclin B1 molecule	Vector Laboratories

cyclin D1 – clone P2D11F11, corresponds to human cyclin D1 molecule	Santa Cruz
EGF-R – clone Z-12, specific for full length human EGF-R	Santa Cruz
JNK1 – clone C-17, corresponds to human JNK1 molecule	Santa Cruz
PARP - clone C2-10, recognises epitope in the C terminal binding domain of human, mouse and rat, raised against bovine PARP	gift from M MacFarlane (from Dr G Poirier, Laval Univ, Quebec)
phosphorylated ERK – corresponds to short amino acid sequence containing phosphorylated Tyr-204 of human ERK1	Santa Cruz
anti-mouse HRP linked secondary antibody	Sigma
anti-rabbit HRP linked secondary antibody	Sigma

2.1.5. Oligonucleotide primers, dyes and molecular weight standards.

100bp ladder	New England BioLabs
1 Kbp ladder	New England BioLabs
AP-1 consensus oligonucleotide*	Promega
Bromophenol blue	USB
Oestrogen receptor beta primers for PCR*	Genset
GL2 counter clockwise primer *	Promega
NFκB consensus oligonucleotide*	Promega
Pre-stained broad range molecular weight marker	BioRad
Xylene cyanol	BDH

2.1.6. Radioisotopes

[γ-^{32}P] Adenosine 5'-triphosphate (ATP)	Amersham

* see appendix for nucleotide sequences.

2.2. Buffers, solutions and other agents

All buffers and solutions were prepared using autoclaved water distilled to 18.5 Ohms (ultapure) or phosphate buffered saline (PBS) unless otherwise stated.

Agarose gel electrophoresis

To prepare a 2% agarose gel, 1g ultrapure electrophoresis grade agarose was dissolved in 50ml 1 x TAE buffer by heating in a microwave. The solution was cooled to 70°C, poured in the casting tray and left to set at room temperature.

AJ buffer	*final concentration*
167µl 2M Tris-HCL pH 8.0	45mM
83µl 1M ammonium sulphate	11mM
33.5µl 1M magnesium chloride	4.5mM
75µl 100mM dATP	1mM
75µl 100mM dATP	1mM
75µl 100mM dCTP	1mM
75µl 100mM dTTP	1mM
85µl 10mg/ml BSA	113µg/ml
3.6µl 100% β-mercaptoethanol	6.7mM
3.4µl 0.01M EDTA pH 8.0	4.4mM

The components were mixed and pelleted at 3000g for 1 minute at 4°C, the supernatant was removed and stored at -20°C in 100µl aliquots.

Agar plates (with ampicillin)	*final concentration*
0.5ml 50mg/ml ampicillin	0.05mg/ml
50mg X-gal in 2ml DMF	0.05mg/ml

Agar was heated in a microwave until fully dissolved and left to cool until below 50°C. The above components were added and 20ml of agar mixture poured into sterile petri dishes and left to set for 30 minutes. The plates were then incubated for 1 hour upside down at 37°C, after which they were stored at 4°C until use.

Annexin buffer	*final concentration*
10ml 0.5 M HEPES	10mM
15ml 5M NACl	150mM
1.25ml 2M KCl	5mM
0.5ml 1M $MgCl_2$	1mM
0.9ml 1M $CaCl_2$	1.8mM

The components were mixed, the solution calibrated to pH 7.4 with sodium hydroxide and the volume adjusted to 500ml. The buffer was stored at room temperature.

Binding buffer	*final concentration*
4g Ficoll	8% (w/v)
1.6ml 0.5M HEPES (pH 7.5)	40mM

The components were added together and the volume adjusted to 50ml. The buffer was stored at 4°C.

Caspase-3 activity assay buffer	*final concentration*
11.9g HEPES	100mM
50g sucrose	10% (w/v)
0.5g CHAPS	0.1% (w/v)
The following was added prior to use:	
2M DTT	10mM

The components were mixed, the solution calibrated to pH 7 with sodium hydroxide and the volume adjusted to 500ml. The solution was stored at -20°C in 50ml aliquots.

Dulbecco's Minimal Essential Medium (DMEM)

This was purchased from Gibco Life Technologies. 500ml of media was supplemented with 2mM glutamax and 10% FCS prior to use.

Dulbecco's Modified Essential Medium (DMEM) / F12 Ham (1:1)

This was purchased from Gibco Life Technologies. 500ml of media was supplemented with 2mM glutamax and either 2% charcoal stripped FCS or 10% FCS

prior to use.

Dulbecco's Minimal Essential Medium (DMEM) /F12 Nut Mix

This was purchased from Gibco Life Technologies. Media (500ml) was supplemented with 5% horse serum, 2mM glutamax, 20ng/ml EGF, 10μg/ml insulin, 0.5μg/ml hydrocortisone and 100ng/ml cholera toxin prior to use.

Electrophoresis running buffer	*final concentration*
30.2g Tris base	25mM
188g glycine	250m
100ml 10% SDS	1% (v/v)

The first two components were mixed in 850ml water, after which 100ml 10% SDS was added. The volume was adjusted to 1000ml to give a x10 stock buffer. This buffer was diluted 1:10 with water prior to use and stored at room temperature.

Freezing medium	*final concentration*
5ml heat inactivated FCS	25% (v/v)
1.5ml DMSO	7.5% (v/v)
0.5ml Glutamax	2.5% (v/v)
13ml relevant medium	65% (v/v)

The components were mixed and used to re-suspend cell pellets prior to freezing.

Genistein

Genistein was purchased from Sigma Chemical Corp. Stock solutions of 500mM and 50mM were prepared in DMSO and aliquots were stored at -20°C. Cells were treated with genistein by addition to cell culture media at desired concentrations.

Kinase assay buffer	*final concentration*
0.6g HEPES	25mM
0.31g β glycerophosphate	25mM
2.5ml 1M $MgCl_2$	25mM
100μl 0.5M EDTA	0.5mM

The components were mixed, volume adjusted to 100ml and stored at 4°C. The

following were added immediately prior to use:

100mM Na_3VO_4	0.5mM
500mM DTT	0.5mM

Nuclear preparation buffer A	*final concentration*
2ml 0.5M HEPES pH 7.8	10mM
0.5ml 2M KCl	10mM
200µl 1M $MgCl_2$	2mM
50µl 2M DTT	1mM
20µl 0.5M EDTA	0.1mM
0.4M PMSF	0.4mM
20µl 0.5M NaF	0.2mM
200µl 100mM Na_3VO_4	0.2mM

The components were mixed, the volume adjusted to 100ml and stored at 4°C.

The following was added immediately prior to use:

0.3 mg Leupeptin

Nuclear preparation buffer B	*final concentration*
10ml Igepal CA630	10% (v/v)

This was added to 90ml water and stored at 4°C.

Nuclear preparation buffer C	*final concentration*
10ml 0.5M HEPES pH 7.8	50mM
2.5ml 2M KCl	50mM
6ml 5M NaCl	300mM
200µl 0.5M EDTA	0.1mM
200µl 2M DTT	1mM
10 ml Sterile glycerol	10% (v/v)
0.4M PMSF	0.4mM
400µl 1M NaF	0.2mM
200µl 0.1M Na_3VO_4	0.2mM

The components were mixed, volume adjusted to 100ml and stored at 4°C.

Oestrogen stripping buffer	*final concentration*
500ml 0.5M sucrose	250mM
3ml $MgCl_2$	1.5mM
20ml HEPES pH 7.4	10mM

The components were mixed, the volume adjusted to 1000ml and stored at room temperature.

PMSF	*final concentration*
0.35g PMSF	100mM

PMSF is hazardous, therefore weighing out of the powder was performed in a fume hood. A 100mM stock solution was prepared in 20ml 100% methanol, protected from light and stored at 4°C.

PIPES cell lysis buffer	*final concentration*
12.5ml 2M PIPES/KOH pH 6.5	50mM
2ml 0.5M EDTA	2mM
CHAPS	0.1% (w/v)

These components were combined, the volume adjusted to 500mls and stored at 4°C.

The following were added to 5ml immediately prior to use:

1 M DTT	5mM
Aprotonin	10µg/ml
10µl 10mg/ml Leupeptin	10µg/ml
10µl 10mg/ml Pepstatin A	10µg/ml
100µl 100mM PMSF	2mM

Polyacrylamide gel for gel shift assay

8 ml 30% acrylamide:bis acrylamide (37.5:1)

49.75ml water

3ml 5X TBE

400µl 10% AMPS

100µl TEMED

The components were combined, with TEMED added last, mixed thoroughly and

poured into a large gel casting system (Hoeffer Scientific Instruments, San Francisco, CA) and left to set.

Polyacrylamide separating gel (10%)

3.3ml 30% acrylamide:bis acrylamide (37.5:1)

4ml distilled water

2.5ml 1.5M Ttris pH 8.8

100µl 10% SDS

100µl 10% AMPS

4µl TEMED

The components were combined with TEMED added last, mixed thoroughly and poured into a Hoeffer mini gel casting system (Hoeffer Scientific Instruments). The gel was overlaid with water and left to set.

Polyacrylamide separating gel (8%)

4ml 30% acrylamide

3.4ml distilled water

2.5ml 1.5M Tris pH 8.8

100µl 10% SDS

100µl 10% AMPS

6µl TEMED

The components were combined with TEMED added last, mixed thoroughly and poured into a Hoeffer mini gel casting system (Hoeffer Sceintific Instruments). The gel was overlaid with water and left to set.

Polyacrylamide stacking gel

0.83ml 30% acrylamide

3.4ml distilled water

0.63ml 1 M Tris

50µl 10% SDS

50µl 10% AMPS

5 µl TEMED

The components were combined with TEMED added last, mixed thoroughly and

poured on top of the stacking gel, a comb inserted and left to set.

RPMI 1640 medium

This was purchased from Gibco Life Technologies. 500ml of media was supplemented with 2mM glutamax and 10% FCS prior to use.

TPA

TPA was purchased from Sigma Chemical Corp. A 5mM stock solution was prepared in DMSO and aliquots stored at -20°C. Cells were treated with TPA by addition to cell culture media at desired concentrations.

TAE buffer	*final concentration*
242g Tris	2M
57.1ml glacial acetic acid	57.1mM
100ml 0.5M EDTA pH 8.0	5mM

The components were dissolved in a volume of 1000ml, making a x 50 stock solution. The solution was autoclaved, stored at room temperature and diluted with distilled water to give a 1x working solution prior to use.

TBS	*final concentration*
50ml 1M Tris	5mM
8.78g sodium chloride	15mM

The components were dissolved in a volume of 1000ml and stored at room temperature.

TBS-T	*final concentration*
50ml 1M Tris pH 7.5	5mM
8.78g sodium chloride	15mM
1ml polyoxyethylene sorbitan monolaurate (Tween 20)	0.1% (v/v)

The components were dissolved in a volume of 1000ml and stored at room temperature.

Triton lysis buffer	*final concentration*
10ml 1M Tris pH 7.4	20mM

4g NaCl	137mM
1.575g β glycerophosphate	25mM
0.45g NaPPi	2mM
2ml 0.5M EDTA	2mM
50ml sterile glycerol	10% (v/v)
0.156g benzamidine	2mM

The components were combined to make a 2x solution, the volume adjusted to 250ml and stored at 4°C. This was diluted with water to give a 1x working solution before use.

The following were added prior to use:

100mM Na_3VO_4	1mM
100mM PMSF	1mM
10mg/ml pepstatin A	2μg/ml
10mg/ml leupeptin	2μg/ml
2mg/ml aprotonin	2μg/ml
Triton X-100	1% (v/v)
500mM DTT	0.5mM

Urea sample buffer	*final concentration*
0.5ml 1M Tris pH 7.4	50mM
3ml 20% SDS	6%
3.6ml glycerol	36% (v/v)
5ml16M urea	8M

The components were dissolved in a volume of 15ml and the following protease and phosphatase inhibitors were added prior to use:

10mg/ml pepstatin	2μg/ml
10mg/ml leupeptin	2μg/ml
2mg/ml aprotonin	2μg/ml

Western blotting sample buffer	*final concentration*
1.51g Tris base	50mM
75ml 20% SDS	6%
90ml glycerol	36% (v/v)

The components were dissolved in a volume of 250ml and the following protease and phosphatase inhibitors were added to 5ml buffer prior to use:

10mg/ml pepstatin	2μg/ml
10mg/ml leupeptin	2μg/ml
2mg/ml aprotonin	2μg/ml
50μl 100mM PMSF	1mM
50μl 100mM Na3VO4	1mM
500μl 1M NaF	10mM

Western blotting transfer buffer	*final concentration*
5.8g Tris	48mM
2.9g glycine	39mM
200ml methanol	20% (v/v)
1.85ml 20% SDS	0.037%

The components were dissolved in a volume of 1000ml and stored at room temperature.

Western blotting stripping buffer	*final concentration*
50ml 20% SDS	2% (v/v)
3.78g Tris base	62.5mM

The components were combined, the volume adjusted to 500ml and 400μl β-mercaptoethanol added per 50ml prior to use.

Western blotting BSA blocking buffer

A 5% stock was made by dissolving 5g electrophoresis grade BSA in 100ml TBS-T and stored at 4°C.

Western blotting non-fat milk blocking buffer

A 5% solution of non -fat milk (Marvel) was prepared by mixing 5g Marvel and 100ml TBS-T, immediately prior to use.

2.3 METHODS.

2.3.1. Cell lines

MCF-7, ZR-75.1, T47-D, MDA-MB 468 and MDA-MB 231 breast carcinoma cell lines in addition to HBL 100 and MCF-10A immortalised breast cell lines, were a gift from the Breast Cancer Research Unit, Glenfield Hospital, Leicester, UK courtesy of Prof. R. A. Walker and Dr L. Gordon, and were originally obtained from American Type Culture Collection (Manassas,VA). For details of cell lines see table 2.1.

2.3.2 Routine cell maintenance

MDA-MB 468 and MCF-7 cells were grown and routinely passaged in RPMI-1640 medium supplemented with 10% FCS and 2mM glutamax. T47-D, HBL 100 and MDA-MB 231 cells were passaged in DMEM medium supplemented with 1g/litre glucose, 2mM glutamax, 110 mg/litre sodium pyruvate and 10% FCS. ZR-75.1 cells were grown in DMEM/F12 media F12 (Ham) 1:1, containing 15mM HEPES and supplemented with 10% FCS and 2mM glutamax. MCF-10A cells were routinely passaged in specially defined DMEM/F12 Nut Mix media. This was supplemented with 20ng/ml EGF, 0.5μg/ml hydrocortisone, 10μg/ml insulin, 100ng/ml cholera toxin, 2mM glutamax and 5% horse serum. All cell lines tested negative for mycoplasma infection and were cultured without antibiotics. Cell culture was performed using a class II laminar flow cabinet, where cells at 70-80% confluency were washed with PBS, trypsinised with 1x T/E and pelleted at 200g for 3 minutes. Cells were re-seeded at required densities in T125 flasks and maintained in a Sanyo incubator at 37°C in 5% CO_2.

2.3.3. Cell storage and resurrection

Cells in log phase of growth (70-80% confluent), were washed with PBS, trypsinised with 1x T/E and pelleted at 200g for 3 min. The cell pellet was resuspended in

Cell Line	Derived from	Culture and Morphology	Other comments	Reference:
HBL 100	Milk of nursing mother, 27 yrs, Caucasian	Continuous culture as monolayer, epithelial-like, will dome when heavy	Cell line of normal origin, contains SV40 virus genome	Gaffney *et al.*, 1979
MCF-7	Adenocarcinoma, from pleural effusion, 69 yrs, Caucasian	Continuous culture as monolayer, epithelial-like, will dome when heavy	Retained several characteristics of differentiated mammary epithelium, including the ability to process oestradiol	Soule *et al.*, 1973
ZR-75.1	Ductal carcinoma, 63 yrs, malignant effusion, Caucasian	Continuous culture as monolayer, epithelial-like	Oestrogen receptor alpha positive	Engel *et al.*, 1978
T47-D	Infiltrating ductal carcinoma, 54 yrs, substrain of T47 cell line	Continuous culture as monolayer, epithelial-like, tendancy to multilayer	Contains cytoplamic junctions and oestrogen receptor alpha	Lamp *et al.*, 1981
MDA-MB 468	Pleural effusion of metastatic adenocarcinoma, 51 yrs, Negro	Continuous culture as monolayer, epithelial-like	Oestrogen receptor alpha negative	Brinkey *et al.*, 1980
MDA-MB 231	Pleural effusion of adenocarcinoma, 51 yrs, Caucasian	Continuous culture as monolayer, epithelial-like	Tumourigenic in nude mice	Cailleau *et al.*, 1974

Table 2.1. Cell lines used in this study.

10mls freezing mixture and aliquoted into 1ml cryovials. The vials were stored overnight at -80°C, after which they were transferred to liquid nitrogen for long-term storage.

To revive cell lines, a vial of frozen cells was defrosted at 37°C and added to 9ml media. The cells were pelleted at 200g for 3 min, resuspended in 1ml media, added to a T25 flask containing 9ml fresh media and incubated at 37°C in 5% CO_2.

2.4. Cell growth assays

Cells in log phase of growth (60-70% confluent), were washed with PBS and trypsinised with 1x T/E. The cells were pelleted at 200g for 5 min and seeded at densities of 1.5 x 10^4 for HBL 100, MCF-10A, MCF-7, ZR-75.1, MDA-MB 468 and MDA-MB 231 cells and 2 x 10^4 for T47-D cells in 1.75cm wells. Cells were incubated for 48 hrs after which the medium was removed and cells were washed twice with PBS. Fresh medium (DMEM/F12 with either 10% FCS or 2% DCC-S FCS or DMEM Nut Mix with 5% horse serum for MCF-10A cells) containing genistein (10 and 1μM) was added to the wells and cells incubated for 96 hrs. Control cells were incubated with DMSO, the volume of which did not exceed 0.01%, and which on its own did not affect cell growth. After 96 hrs, the media was removed, cells washed twice with PBS and trypsinised. Media (1ml) was added to cells and thoroughly mixed to form a single cell suspension. An aliquot of cells (200μl) were diluted in 9.8ml isoton and counted using a Coulter Counter (Beckman Coulter, Luton, UK) (model ZM, settings: current 100, full scale 10, polarity auto, upper threshold 99.9, preset gain 1, volume 500μl).

Further studies using HBL 100 cells cultured in medium containing either 2% FCS or 10% FCS were performed as above. Experiments were also performed culturing HBL 100 cells in medium containing 2% FCS for 48 hrs, after which FCS was added to adjust the final serum content to 10% for a further 48 hrs. Cells were harvested as described above.

2.4.1. Growth assays in HBL 100 cells cultured in media supplemented with various serum components

HBL 100 cells were seeded as before, but in DMEM media supplemented with 2% DCC-S FCS either with or without four components of serum. Oestradiol (10^{-5} M), hydrocortisone (0.5μg/ml), insulin (10μg/ml), and EGF (2μg/ml) were added to media (100ml) and incubated together with genistein (10μM) for 96 hrs. Control cells were grown in 2% DCC-S FCS media for the same period of time, after which all cells were harvested and counted as described above.

2.4.2. Stripping foetal calf serum using DCC

To prepare serum free of endogenous oestrogens, DCC was used to 'strip' FCS. DCC was added (0.25% w/v) to oestrogen stripping buffer and incubated overnight at 4°C. A volume of DCC equivalent to the amount of serum to be stripped was taken and spun at 500g for 20 min to pellet the charcoal. The supernatant was removed and replaced with the same volume of FCS and mixed thoroughly. This mixture was incubated for 2 x 45 min at 56°C to produce heat inactivated DCC stripped serum and spun for 20 min at 500g to pellet the DCC. The serum supernatant was removed, filtered into 10ml aliquots and stored at -20°C.

2.5. Analysis of EGF-R expression by western blotting

HBL 100, T47-D, MDA-MB 468 and MDA 231 cells were seeded at $5x10^5$ cells per 3.5cm well, whilst MCF-7 and ZR-75 cells were seeded at $4x10^5$ cells per 3.5cm well in media supplemented with 10% FCS the day prior to use. Cells were allowed to adhere, after which the media was removed and replaced with that containing 0% FCS and cells incubated overnight at 37°C. Culture medium was removed and replenished with fresh 1hr prior to commencing treatment.

2.5.1. *Harvesting cells for western blotting*

Sample buffer was boiled and an aliquot (50µl) added to each well. The resulting cell lysates were sonicated to shear DNA, after which 10µl was retained for protein determination using the Pierce protein assay (see section below for details). DTT (2M) and bromophenol blue were added, samples spun for 10 sec, vortexed and boiled for 5 min prior to loading.

2.5.2. *Pierce protein assay*

The cell lysate (10µl) was diluted 1:5 with distilled water, to which 1ml Pierce protein reagent (1:50 copper sulphate and BCA reagent) was added. Once added, the samples were vortexed and incubated at 37°C for 30 min. Sample absorbance was read at 562nm using a spectrophotometer (Perkin Elmer UV/V15 Lamda 2S, Beaconsfield, UK) and protein yields were calculated from a standard curve.

2.5.3. *Gel electrophoresis and protein transfer*

A 10% acrylamide separating gel was poured using a mini gel cast (Hoeffer Scientific Instruments, San Francisco, CA), overlaid with water and left to set (based on a method by Sambrook *et al.*, 1989). Once set, a stacking gel was poured. An equal amount of protein was loaded into each well, alongside a molecular weight marker. The samples were run through the stacking gel at 80V for 15 min, then at 150V through the separating gel until the dye front ran off the bottom of the plate. Gels were removed from the gel tank, floated in transfer buffer to equilibrate for 15 min, after which protein was transferred to a nitro-cellulose membrane (Hybond, Amersham Life Sciences Ltd, UK) using a wet blotting system (BioRad Laboratories, Hemel Hempstead, UK) running at 100V for 90 min. The membrane was blocked overnight at 4°C in 5% BSA in TBS-T.

2.5.4. Detection of EGF-R expression

An antibody specific for the EGF-R was used at a dilution of 1:2000 in 0.5% BSA in TBS-T, for 90 min. Membranes were washed with TBS-T (4 washes x 10 min) and incubated with an anti-mouse HRP-linked secondary antibody (1:1000 dilution) in TBS-T for 1 hr. The signal was detected using the ECL detection system. Membranes were exposed to ECL hyperfilm (Amersham) for approximately 30 sec and developed using the X-ograph Compact X2 developer. Scanning and densitometry were performed using the Molecular Dynamics Densitometer Instrument and quantified by Image Quant Analysis software.

2.6. Detection of ER-β mRNA levels

2.6.1 mRNA extraction from cell lines

The extraction of mRNA was performed using the Dynabead Kit purchased from Dynal. Cells were trypsinised, counted, 1 x 10^6 cells were resuspended in 1ml lysis binding buffer (LBB) and divided into four samples. Resuspended dynabeads (40µl) were added to each tube, a magnet used to 'pellet' the beads and the pellet re-suspended in LBB (40µl). The mixture was re-pelleted via the magnet and the cell lysate added after mixing to reduce viscosity and to shear the DNA. The lysate/bead mix was incubated at room temperature for 10 min, pelleted via the magnet and the liquid removed. Wash/SDS buffer (200µl) was added and the mixture re-pelleted via the magnet. This wash step was repeated twice, but the second time the mixture was resuspended in wash buffer alone. This step was repeated three times with the final re-suspension performed with 30µl sterile water.

2.6.2. RT-PCR of extracted mRNA

This process produces complementary DNA from mRNA. A mastermix containing 5µl AMV buffer, 4µl dNTP mix and 1µl RNasin was made, 10µl was aliquoted into each tube, after which, 14.2µl of resuspended beads were added. Finally, 0.8µl

reverse transcriptase was added to each tube and incubated at 42°C for 1 hr. The mixture was pelleted via a magnet and washed with 1 x TE, the TE removed and the pellet resuspended in 25µl sterile water.

2.6.3. Detection of ER-β by RT-PCR

A mastermix was made by combining 5µl 10 x AJ buffer, 2µl forward ER-β primer (10pmol/µl), 2µl reverse ER-β primer (10pmol/µl) and 42µl PCR water per sample (for primer sequences, see appendix). An aliquot (48µl) of this mix and 1µl of relevant resuspended bead mix (RT-PCR product) were added to each sample tube (using 1µl water for the negative control). A drop of sterile paraffin oil was added to the top of each reaction to ensure successive rounds of heating and cooling did not result in sample evaporation. A 'hot start' to the reaction was performed by placing the samples in the PCR machine after the program reached 95°C, thus avoiding amplification of non-specific PCR products that may be produced as the temperature rises from room temperature at the start of the program. Once at 95°C, 0.2µl Taq polymerase was added to each sample to start the reaction. The conditions used were: 95°C for 5 min (amplification), 95°C for 1 min (denaturing), 60°C for 1 min (annealing) and 72°C for 1 min (extension of the DNA). The last three steps were repeated for 30 cycles with a final extension step at 72°C for 7 min.
A 1:50 dilution of first round PCR products were used and an identical reagent mix made, with the exception of different primers (see appendix). Conditions for the second round PCR were slightly different, with a hot start performed at 95°C for 5 min, followed by 95°C for 40 sec, 60°C for 40 sec, 72°C for 1 min, with the last three steps repeated for 30 cycles. A final extension step at 72°C for 7 min followed and fragments of approximately 429 base pairs were produced at an exponential rate. The PCR products were stored at 4°C prior to visualisation on a 2% agarose gel.

2.7. *Preparation of DNA for transfections*

The PBL-Luciferase/TRE plasmid (5.3kB) was kindly donated by Dr. P. Parker, ICRF Laboratories, London, UK. The PBL-Luciferase/TRE construct was generated from a Sca I and Bgl II digest ligating a large fragment of a pGL2 basic vector with a small fragment of PBLCAT2 plasmid. The TRE plasmid comprises a PGL Basic backbone, a TK minimal promoter and three tandem TRE sites upstream of a luciferase coding region.

2.7.1. *Preparation of the TRE pGL2 plasmid*

The plasmid glycerol stock for the TRE pGL2 construct (1µl) was added to 10ml L- Broth medium (containing ampicillin 50mg/ml) and incubated overnight in an orbital shaker at 37°C. An aliquot of the starter culture (50µl) was added to 400ml L Broth medium (containing ampicillin) in a large conical flask and the overnight incubation repeated. The following day the plasmid DNA was extracted using the endo free maxiprep kit. The bacterial culture (250ml) was spun at 5000g for 10 min at 4°C, the supernatant removed and the pellet resuspended in 10ml Buffer P1. Buffer P2 (10ml) was added and the suspension incubated at room temperature, after which 10ml Buffer P3 was added, mixed, poured into a QIAfilter maxi cartridge and incubated for 10 min, again at room temperature. During this time a precipitate containing proteins, genomic DNA and detergent floats to the top of the solution to ensure convenient filtration. The plunger was inserted into the cartridge and the cell lysate filtered into a 50ml tube.

Buffer ER (2.5ml) was added to the filtered lysate, mixed and incubated on ice for 30 min. A qiagen tip-500 was equilibrated by applying 10ml Buffer QBT and allowing the column to empty by gravity flow. The cleared lysate was added to the qiagen tip-500 and washed twice with 30ml Buffer QC. The DNA was eluted using 15ml Buffer QN into endotoxin free polypropylene centrifuge tubes, precipitated with 10.5ml isopropanol and thoroughly mixed before being spun at 15000g for 30 min at 4°C. The supernatant was carefully removed, the DNA washed with 2.5ml endotoxin-free 70% ethanol and re-pelleted at 15000g for 10 min at 4°C. The

supernatant was removed, the DNA pellet air dried for approximately 1 hr and resuspended in 300µl Buffer TE.

2.7.2. Restriction digest of TRE plasmid DNA

To check the DNA prepared in section 2.7.1 was the correct size, a restriction digest was carried out. This was performed by combining DNA (2µl), buffer B (1µl), water (6µl) and finally Hind III restriction enzyme (1µl). This mixture was digested for 2 hrs at 37°C, after which the products were visualised on a 2% agarose gel against a 1Kbp molecular marker.

2.7.3. Determination of plasmid concentration

Plasmid concentration was determined by measuring the optical density of a solution containing a 1:100 dilution (plasmid: water). The absorbance was read at 260, 280 and 320nm using a spectrophotometer (Perkin Elmer UV/V15 Lamda 2S). The following calculation was used to provide the DNA concentration:

absorbance (260nm) x 100 (dilution) x 50 (for dsDNA) = [plasmid]/ µg/ml.

The amount to be used in subsequent transfection experiments was determined and DNA was aliquoted accordingly to reduce freeze/thaw cycles.

2.7.4. Sequencing of TRE pGL2 plasmid DNA

The TRE plasmid was sequenced using the facilities of PNACL, housed in Centre for Mechanisms of Human Toxicity. The plasmid was cultured in L-Broth medium (10ml) containing ampicillin for 18 hrs to an OD_{600nm} of between 1.5 and 4 units, pelleted and the supernatant removed. The DNA was purified by PNACL, after which the sequencing was performed using an ABI prism 377 sequencer. The GL2 primer was used, which sequences counter clockwise across the multiple cloning sites, upstream of a luciferase coding region.

2.7.5. Transformation of pGL2 basic vector into JM109 competent cells

Competent transformed cells (100µl) were added to 1µg cloned DNA (pGL2 basic vector), thoroughly mixed and incubated in ice for 10 min. The cells were heat shocked for 45 sec in a water bath heated to 42°C, after which they were placed on ice for 2 min. L-Broth medium (900µl) was added and the cells were incubated in an orbital shaker for 1 hr at 37°C.

Cells were dispensed onto 5 agar plates (200, 100, 50, 10 or 0µl cells per plate) and incubated overnight at 37°C. A single white colony was selected (containing pGL2 basic DNA) and incubated overnight in a 10ml culture of L-Broth medium. A DNA miniprep was performed, the resulting DNA was digested using a Hind III restriction enzyme (see section 2.7.2) and visualised by agarose gel electrophoresis. The DNA from the miniprep was used as the starter culture in a maxi-prep (see section 2.7.1 for details) to obtain enough DNA to use in transfections as a control for TPA-induced AP-1 activity.

2.8. Transient tranfections to detect AP-1 activity

2.8.1. Transfection by electroporation

Cells were trypsinised, pelleted at 200g for 5 min and resuspended in serum free media. An aliquot of cells was counted using a haemocytometer and the concentration of cells adjusted to $8x10^7$ cells/ml. DNA was added to cuvettes (TRE pGL2 2.5pmol, pGL2 basic 2.5pmol, pCMVβ 0.075pmol), incubated on ice, followed by addition of 200µl of cell suspension. The cell suspension was gently mixed and cells were electroporated (BioRad Gene Pulser, Hemel Hempstead, UK) at a voltage of 0.25kV and a capacitance of 960µF. Immediate addition of 800µl of media (containing 10% FCS) was required to help the cells recover after the shock of the electrical pulse. The cuvettes were left to stand for 5 min, after which the transfected cells were seeded onto 5 cm plates. Media (4ml) containing 10% FCS was added to each plate and incubated for 5 hr at 37°C, to continue cell recovery.

After this incubation period, the media was removed and replaced with 4ml of serum free media, after which the plates were incubated for a further 48 hr.

2.8.2. Transfection by fugene

Cells were trypsinised, pelleted at 200g for 5 min and resuspended in 10% FCS media. An aliquot of cells was counted using a haemocytometer and the concentration of cells adjusted to $2x10^5$ cells per 3.5cm well. Once cells had adhered to the wells, media was removed and replaced with that containing 0% FCS for 24 hr. A mastermix of 0% media and fugene reagent (97:3µl per transfection) was prepared and incubated for 5 min at room temperature. A DNA mastermix containing desired plasmids (TRE pGL2 0.29pmol, pCMVβ 0.23pmol) was prepared, to which the media/fugene mixture was added and incubated for 15 min. An aliquot (100µl) was added to the cell cultures and incubated for 24 hr.

2.8.3. Treatment and lysis of cells

Cells were pre-treated with genistein (1, 10 or 50µM) for 1 hr followed by addition of TPA (0.2µM) for 4 hr at 37°C. Cells were washed twice with PBS before being lysed with 300µl reporter lysis reagent (β-Galactosidase Enzyme Assay Kit) for 15 min at room temperature and assayed using both systems described below.

2.8.4. β - Galactosidase enzyme assay system

pCMVβ is a mammalian reporter vector that is designed to express β galactosidase from the human cytomegalovirus immediate early gene promoter. This vector produces very high levels of β-galactosidase expression and can therefore be used as a reference or control plasmid when monitoring transfection efficiency. This expression was measured using a β-Galactosidase Enzyme Assay Kit. An extract (25µl) was mixed with 25µl buffer containing the substrate (o-nitrophenol β-d galactopyranosidase) and incubated at 37°C for 30 min, in which time β-

galactosidase hydrolyses the substrate to produce o-nitrophenol that is yellow in colour. The reaction was terminated by the addition of 75µl sodium carbonate (1M) and absorbance read by plate reader (iEMS reader MF, Lab Systems UK Ltd) at a wavelength of 414nm.

2.8.5. Luciferase assay system

The luciferase assay yields luminescence through ATP-dependent oxidation of beetle luciferin in the presence of magnesium and oxygen, resulting in light emission. The total amount of light measured over a given time interval is proportional to the amount of luciferase reporter activity in the chosen sample. This activity was measured using the luciferase assay kit. Luciferase assay reagent (50µl) was added to a cell extract (10µl) and light emission measured using a liquid scintillation counter (Wallac 1450 Microbeta Plus, Milton Keynes, UK). These results were normalised with values obtained for β-galactosidase and represented against untreated values (100%).

2.9. Detection of AP-1 DNA binding by gel shift assay

2.9.1. Seeding and treatment of cells

HBL 100, ZR-75.1, T47-D and MDA-MB 468 cells were seeded at 4 x 10^6 cells on 9cm plates in media supplemented with 10% FCS, the day prior to treatment. Cells were allowed to adhere, after which the media was removed and replaced with that containing 0% FCS and cells incubated overnight at 37°C. Culture media was replenished with fresh 1 hr prior to commencing treatment. Cells were pre-treated with genistein (50µM) for 1 hr, followed by treatment with TPA (0.2µM) for 4 hr, after which cells were harvested as described below.

2.9.2. Preparation of nuclear extracts

Culture media was removed from plates and cells were washed twice with ice-cold PBS, scraped off and transferred to an eppendorf tube. Cells were pelleted at 100g for 10 min at 4°C. The supernatant was removed, the pellet washed with 1ml PBS and re-spun at 15000g for 15 sec at 4°C. The supernatant was again removed, the pellet washed in 0.4ml Buffer A and incubated on ice for 15 min. Buffer B (25µl) was added to the sample, vortexed and spun at 15000g for 30 sec at 4°C. Pelleted nuclei were resuspended thoroughly in 50µl Buffer C, mixed for 20 min at 4°C and sonicated. Samples were spun at 15000g for 5 min at 4°C, the supernatant collected and protein concentration determined by Bradford Assay (see below).

2.9.3. Bradford protein assay

This procedure was based on a method by Bradford, 1976. An aliquot (5µl) of the nuclear protein supernatant was added to 1ml distilled water, vortexed, 200µl removed and replaced with 200µl protein reagent (Biorad). Sample absorbance was read at 595nm using a spectrophotometer (Perkin Elmer UV/V15 Lamda 2S) and protein yields were calculated from a standard curve.

2.9.4. Labelling of the oligonucleotide

Consensus AP-1 oligonucleotide (8.75pmol), T4 polynucleotide kinase (1µl), T4 polynucleotide kinase buffer (2µl) and water (7µl) were added to a screw top eppendorf and transferred to the radioactivity room. [γ-^{32}P] ATP (5µl) was added to the mix and incubated at 37°C for 30 min. TE buffer (80µl) was added to stop the reaction, after which 100µl of the labelled oligonucleotide was dispensed onto the top of a NAP '5' column and eluted into a fresh tube. The oligonucleotide was eluted with subsequent 100µl aliquots of TE buffer and collected in 12 fractions. The DPM of each fraction was measured and the three fractions containing the highest DPM were combined. The oligonucleotide was precipitated with 2.5x the

volume of ethanol and 1/10 volume of NaCl and incubated overnight at -20°C. The following day, the oligonucleotide was thawed, spun at 14000g for 30 min and the resulting pellet resuspended in 50µl water.

2.9.5. Preparation of samples

A polyacrylamide gel was poured and left to set for 1 hr. Protein (5µg) from the nuclear extracts was added to buffer C to give a volume of 4µl, which was adjusted to a final volume of 6µl with water. For competitive and non-competitive inhibitors, 8.75pmol of cold AP-1 and NFκB oligonucleotides were used respectively. To each sample, binding buffer (10µl), poly DIDC (1µg), 10nM $MgCl_2$, 10nM DTT and 1.6µl water was added. Once samples were transferred to the radioactivity room, the labelled oligonucleotide (1µl) was added, mixed and incubated at room temperature for 30 min.

2.9.6. Loading and running the gel

The combs were removed from the gel, the wells washed out with water and a syringe used to remove excess water from the wells. TBE buffer (800mls) was poured into the bottom of the gel tank, the cooling system switched on and the gel moved into place. Buffer was poured into the top of the tank and the gel was pre-run for 15 min at 120V, after which loading buffer (2µl) was added to each sample and loaded onto gel. The gel was run at 240V for 10 min after which the voltage was reduced to 120V for approximately 2 hrs.

2.9.7. Detection of AP-1 DNA binding

Gels were transferred onto blotting paper and dried (Hoeffer Gel Drier SR, San Francisco, CA) at 65°C for 1 hr. When completely dry, gels were transferred to a phosphorimager cassette (Molecular Dynamics) and left overnight after which the image was scanned into the phosphorimager (Molecular Dynamics). Gels were

exposed to film for between 3 and 24 hr at -80°C, depending on the signal and visualised by autoradiography.

2.10. Analysis of ERK phosphorylation

2.10.1. Seeding and treatment of cells

HBL 100, T47-D, MDA 468 and MDA 231 cells were seeded at $5x10^5$ cells per 3.5cm well, whilst MCF-7 and ZR-75 cells were seeded at $4x10^5$ cells per 3.5cm well, in medium supplemented with 10% FCS on the day prior to use. After cells had adhered to the wells, the media was removed and replaced with that containing 0% FCS and cells incubated overnight at 37°C. Serum-free media was removed, cells washed with PBS and fresh serum free media added 1 hr prior to cell treatments.

Cells were pre-treated with genistein (50μM) or DMSO for 30 min, after which 0.2μM TPA was added for a further 5 min. The media was removed and cells given a final wash with PBS. Cells were harvested as described in section 2.5.1, samples subjected to polyacrylamide gel electrophoresis and proteins transferred onto a nitrocellulose membrane (section 2.5.3).

2.10.2. Antibody detection of levels of ERK phosphorylation

A phospho-specific antibody, pERK, was used to detect levels of ERK 1 and 2 phosphorylation at a dilution of 1:2000 in 0.5% BSA in TBS-T, for 90 min. Membranes were washed with TBS-T (4 washes x 10 min) and incubated with an anti mouse HRP-linked secondary antibody (1:1000 dilution) in TBS-T for 1 hr. Detection of protein was as for EGF-R (see section 2.5.4).

2.11 Western blot analysis of c-Fos and c-Jun protein

2.11.1. Seeding and treatment of cells

Cell lines were seeded at 4 x 10^6 cells on 15cm plates the day prior to harvesting in media supplemented with 10% FCS. Cells were allowed to adhere, after which the media was removed and replaced with that containing 0% FCS and cells incubated overnight at 37°C. Culture medium was replenished with fresh 1 hr prior to commencing treatment. Cells were pre-treated with genistein (50μM) for 30 min, followed by treatment with either TPA (0.2μM) or anisomycin (100nM) for 2 hr, for c-Fos and c-Jun, respectively, after which cells were harvested as described in section 2.9.2. Samples were subjected to polyacrylamide gel electrophoresis as described in section 2.5.3 and the proteins transferred to a nitrocellulose membrane. The membranes were blocked in either 5 or 10% non- fat milk in TBS-T for c-Jun and c-Fos respectively, for 24 hr at 4°C.

2.11.2 Detection of c-Fos and c-Jun protein

Antibodies specific for c-Jun and c-Fos were used at a dilution of 1:2000 in 5 and 10% non-fat milk, respectively, in TBS-T for 2 hr. Membranes were washed in TBS-T (4 x 10 min) and incubated with an anti mouse and anti rabbit HRP-linked secondary antibody respectively, (1:1000 dilution) in TBS-T for 1 hr. Detection of proteins was as for EGF-R (see section 2.5.4).

2.12 JNK assay for detection of c-Jun phosphorylation

2.12.1. Seeding, treatment and harvesting of cells

MCF-7 and ZR-75.1 cells were seeded at a density of 3 x 10^6 cells on 9cm culture dishes, incubated at 37°C for 24 hr, after which the media was removed and replaced with that containing 0% FCS and cells incubated overnight at 37°C. Culture media was replenished with fresh 1 hr prior to commencing treatments. Cells were pre-

treated with genistein (50μM) for 30 min, followed by anisomycin (100nM) for 30 min, after which cells were harvested as described below. Culture media was removed, cells washed with PBS, 1ml TLB was added to cells and plates were incubated for 15 min on ice. Cells were scraped off plates, collected in tubes and pelleted at 1000g for 5 min at 4°C. The supernatant was retained and protein concentration determined by Bradford Assay (see section 2.9.3). Protein concentration was calculated from a standard curve and the remaining supernatant was frozen at -80°C until use.

2.12.2 Immunoprecipitation of JNK

Protein A agarose beads (0.6mg) were added to tubes representing the number of samples. PBS (1ml) was added, the mixture pelleted and supernatant removed, after which JNK1 antibody (1μg) was added to the beads in each tube, mixed and incubated for 1 hr at room temperature. Samples were defrosted and equal amounts of protein combined with TLB to adjust all sample volumes to 500μl. The beads were washed with 0.5ml PBS, pelleted, the sample added and incubated on a rotary mixer for 3 hr at 4°C. Samples were pelleted, supernatant removed and the process repeated twice with 0.5ml TLB. Samples were given a final wash with 0.5ml kinase assay buffer and the supernatant removed.

2.12.3. JNK Kinase Assay

Kinase assay buffer (30μl), GST c-Jun substrate (5μg) and cold ATP (49.98μM) were added to the pellet on ice, after which samples were transferred to the radioactivity room. An aliquot of [γ-^{32}P] ATP (13.2nM) was immediately added to each sample. Samples were incubated for 30 min at 30°C; the reaction was terminated with the addition of sample buffer (10μl). Samples were boiled for 5 min and subjected to polyacrylamide gel electrophoresis as described in section 2.5.3, after which gels were exposed to film for autoradiography after incubation for 6 hr at -80°C.

2.13. Flow-cytometric analysis

2.13.1. Seeding and treatment of cells

All cell lines were seeded at a density of 2 x 10^6 cells on 9cm culture dishes. Cells were incubated at 37°C for 48 hrs, after which genistein was added at either 1 or 10μM. Cells were also incubated with DMSO, the volume of which did not exceed 0.01% and which had no effect on the cell cycle.

2.13.2. Harvesting cells for FACS analysis

After 96 hr, cells were harvested by trypsinisation; pelleted and resuspended in PBS. Ice-cold 70% ethanol/PBS (2 ml) was added and cells were incubated for 3 hr at 4°C, after which RNAse (100μl) and propidium iodide (100μl) were added and the cells incubated for a further 24 hr at 4°C (Ormerod, 1993). Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose CA) and Cell Quest software.

2.14. Detection of cyclin B1 and D1 protein in breast cell lines

2.14.1. Seeding and treatment of cells

All cell lines were seeded at 2 x 10^6 cells on 9cm plates in media supplemented with 2% DCC-S FCS. Fresh media containing genistein (10μM) was added to the wells at 24, 48, 72 and 96 hr intervals and control cells were incubated with DMSO at the same time points. Cells were harvested for nuclear protein as described in section 2.9.2, subjected to polyacrylamide gel electrophoresis as described in section 2.5.3, after which membranes were blocked in 10% non- fat milk in TBS-T for 24 hrs at 4°C.

2.14.2. Detection of cyclin B1 and D1 protein

Antibodies specific for cyclin B1 and cyclin D1 were used at dilutions of 1:2000 in 10% non-fat milk in TBS-T for 90 minutes. Membranes were washed in TBS-T (4 x 10 min) and incubated with an anti mouse HRP-linked secondary antibody (1:1000 dilution) for 1 hr. Detection of proteins was as for EGF-R (see section 2.5.4).

2.15. Annexin staining to observe apoptosis

Annexin V is a vascular protein that binds in a calcium dependent fashion to the phospholipid PS, present on the external membrane surface of apoptotic cells.

2.15.1. Seeding and treatment of cells

PS externalisation was assessed using the Annexin V staining method adapted for adherent cells (Freathy et al., 2000). Cells were seeded at a density of 2 x 10^6 on 9cm culture dishes in media supplemented with 10% FCS and incubated at 37°C for 24 hr, after which genistein (10μM) was added for 96 hr. Cells were also incubated with DMSO to act as a control.

2.15.3. Harvesting cells for annexin staining

Attached cells were trypsined (0.1-0.5X trypsin/EDTA in PBS) and combined with the detached cells. Cells were pelleted at 300g for 5 min at 4°C, resuspended with fresh medium containing 10% FCS (5ml) and incubated for 20 min at 37°C. Cells (1x10^6) were pelleted and resuspended in 1ml annexin buffer. Annexin V was added to a final concentration of 100ng/ml after which the cells were incubated for 8 min at room temperature. Propidium iodide (50μg/ml) was added, cells incubated for 1 min and analysed by flow cytometry.

2.16. Western blot analysis of PARP in MDA-MB 468 cells

2.16.1. Seeding and treatment of cells

MDA-MB 468 cells were seeded at a density of $3x10^6$ on 9cm culture dishes in medium supplemented with 10% FCS and incubated at 37°C for 24 hr, after which genistein (10μM) was added for 96 hr. Cells were also incubated with DMSO to act as a control.

2.16.2. Harvesting cells for PARP protein analysis

Attached and detached cells were collected and pelleted at 300g for 5 min at 4°C. The resulting cell pellet was washed in 1ml ice cold PBS and spun at 300g for 5 min at 4°C. The cell pellets were snap frozen in liquid nitrogen and thawed at 37°C three times in succession. The pellet was finally re-suspended in sample buffer (200μl) and sonicated.

2.16.3. Gel electrophoresis and proteins transfer

Samples containing $0.5x10^6$ cells were loaded onto a 8% acrylamide separating gel alongside a molecular weight marker. The samples were run through the stacking gel at 80V for 15 min, then at 150V through the separating gel until the dye front ran off the bottom of the plate. Gels were removed from the gel tank and floated in transfer buffer to equilibrate for 15 min, after which protein was transferred to a nitro-cellulose membrane using a wet blotting system (BioRad) running overnight at 30V.

2.16.4. Detection of PARP protein

Membranes were blocked in 5 % non-fat milk in TBS-T for 1 hr at room temperature and incubated with an antibody specific for PARP (1:10 000 dilution) for 1 hr. Membranes were washed (4 x 10 min) and incubated with an anti mouse HRP-linked

secondary antibody (1:1000 dilution) for 1 hr. Detection of proteins was as for EGF-R (see section 2.5.4).

2.17. Detection of caspase-3 activity in MDA-MB 468 cells

2.17.1. Seeding and treatment of cells

MDA-MB 468 cells were seeded at $1x10^6$ in T125 flasks in media supplemented with 10% FCS and incubated for 24 hr at 37°C. Media was removed and replaced with that containing genistein (10μM) and incubated for 96 hr. Cells were seeded at a low density in T125 flasks so only extremely low levels of spontaneous apoptosis occurred.

2.17.2. Harvesting cells for caspase-3 activity

This protocol was based on a method described by MacFarlane *et al.*, (1997). Attached cells were collected by scraping the flasks and were combined with media containing any detached cells and pelleted at 300g for 5 min at 4°C. The resulting cell pellet was washed twice in 1ml ice cold PBS and spun at 300g for 5 min at 4°C. The cell pellets were snap frozen in liquid nitrogen and thawed at 37°C three times, and finally resuspended in 50μl PIPES lysis buffer. Protein concentration was determined by the Bradford Assay (see section 2.9.3) and 50μg used for the enzyme assay.

2.17.3. Enzyme activity assay

Extracts containing 50μg protein were combined with assay buffer (1.25ml) and Z-DEVD.afc substrate (10μl), a synthetic tetrapeptide based on the main cleavage site of PARP. The extracts were assayed for caspase-3 activity using a luminescent spectrophotometer (Perkin Elmer LS 50B, Beaconsfield, UK). The liberation of 7-amino-4-fluromethylcoumarin from Z-DEVD.afc was measured at excitation and emission wavelengths of 400 and 505nm, respectively, and given as a measure of

caspase-3 activity. The results were calculated as units of DEVDase activity, using UV WinLab software.

2.18. Statistical analysis

Statistical significance was assessed using the analysis of variance statistical test (Aiffi et al., 1977). The two-way ANOVA was used in experiments where an even number of data points were present and the two-way ANOVA General Linear Model was used when an uneven number of data points were analysed. The ANOVA was followed by Fischer's LSD posthoc test (Snecodor et al., 1980) or an one-way ANOVA followed by Tukey's posthoc test.

CHAPTER 3:

THE EFFECT OF GENISTEIN ON THE GROWTH OF BREAST CELLS.

INTRODUCTION

Development of the normal breast and progression of breast cancer are regulated by hormonal factors. The best defined of these factors are the endocrine steroids which by their interaction with nuclear receptors, regulate cellular function (Dickson et al., 1991, Dickson and Lippman, 1995).

The growth modulatory properties of genistein, a weakly oestrogenic compound, has been investigated extensively in recent years with regard to its effect on many different types of human-derived cells. Studies have been predominantly carried out in the breast (Pagliacci et al., 1994, Fioravanti et al., 1998, Hsieh et al., 1998, Shao et al., 1998, Miodini et al., 1999, Dixon-Shanies and Shaikh, 1999, Li et al., 1999a, Li et al 1999b, Santell et al., 2000, Balabhadrapathruni et al., 2000), but have also been extended to prostate (Davis et al., 1998, Davies et al., 2000), leukaemia (Peterson, 1995), non-small lung cancer cells (Lian et al., 1998) and bovine endothelial cells (Fotsis et al., 1993).

Almost all published studies on the effect of genistein on breast cell growth have focused on a small number of cell types, in particular the oestrogen-responsive MCF-7 cell line. My hypothesis was that a range of human breast cell lines would respond similarly to the effects of genistein, regardless of oestrogen receptor status.
This study was designed to highlight any differences between cell lines in their response to genistein, which may be important in the use of genistein as a therapeutic or chemopreventive agent. To that end, the effects of genistein on the growth of six human-derived breast cell types were compared, tumourigenic MCF-7, ZR-75.1, T47-D, MDA-MB 468 and MDA-MB 231 cells and the non-malignant, but immortalised, HBL 100 cells.

RESULTS

3.1 Phenotype of the breast cell lines

In order to examine whether the growth effects elicited by genistein are attributable to an effect on receptors germane to cell proliferation, the cell lines were screened for the presence of EGF-R and ER-β mRNA. The ER-α protein status of human-derived breast cell lines has been reviewed in many studies and will be discussed later.

The EGF-R is a transmembrane tyrosine kinase that is activated by tyrosine autophosphorylation after ligand-induced dimerisation (Xing and Imagawa, 1999). The ligand-bound receptor is capable of phosphorylating multiple signal transduction molecules leading to the activation of protein kinase cascades. Its function is inhibited by genistein (Akiyama et al., 1987). Elevation in EGF-R levels are known to play a role in mammary tumour progression from hormone dependence to independence, illustrated by murine mammary tumours (Keinhuis et al., 1993) and human ZR-75.1 breast cells (van Agthoven et al., 1992). The EGF-R serves to regulate the proliferation of multiple tissues in adults and thus may be a target for the growth-modulatory effects of genistein. Knowledge of the ER-β status of the cell lines may help to understand the mechanism of action of genistein if growth inhibition occurs via an ER-mediated pathway. Expression of EGF-R and ER-β was determined by western blot analysis and by RT-PCR, respectively.

3.1.1 EGF-R expression

Figure 3.1 shows EGF-R expression in the six breast cell lines, as detected by western blotting. Both MDA-MB 468 and MDA-MB 231 cell lines express elevated levels, with the remaining four cell lines expressing very low or undetectable levels of the EGF receptor.

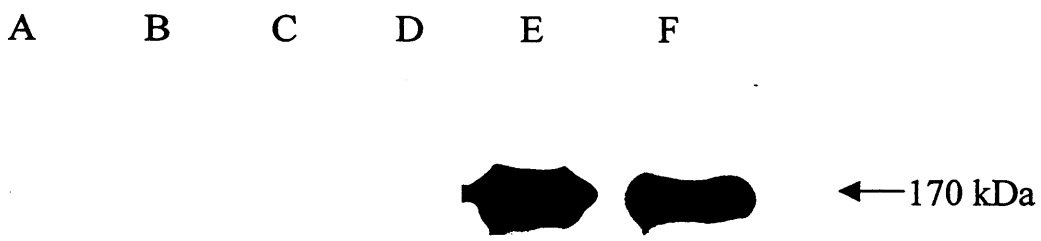


Fig. 3.1. EGF-R protein expression in breast cell lines: (A) HBL 100, (B) MCF-7, (C) ZR-75.1, (D) T47-D, (E) MDA-MB 468 and (F) MDA-MB 231 cells by western blot analysis.

For details of western blot analysis see section 2.5.3.

3.1.2. ER-β status

Figure 3.2 shows the ER-β mRNA status of the breast cell lines. Four cell lines exhibited the presence of ER-β mRNA, namely HBL 100, MCF-7, MDA-MB 468 and MDA-MB 231 cells.

3.2 The effect of genistein on breast cell growth

A number of experiments were performed to investigate the effect of genistein on cell growth in varying culture conditions. The first experiments were designed to investigate the effect of genistein on cell growth in medium supplemented with 10% FCS. The results in figures 3.3, 3.4 and 3.5 show that the growth-accelerating effect of genistein was only observed in MCF-7 cells, in which 1 μM increased cell numbers by 30 % over controls (figure. 3.3). Genistein (10 μM) inhibited growth of all cell types with the exception of HBL 100 and T47-D cells, with MCF-7 cells the most sensitive to the growth-modulatory effects of genistein. The lower concentrations of genistein (0.1 and 0.01μM) did not exert any effect on growth in any of the cell lines.

To test the hypothesis that cell growth modulation by genistein is influenced by serum constituents, growth experiments were repeated using medium supplemented with charcoal stripped serum (see chapter 2.4.2 for details of protocol), with cells grown in medium containing 2% DCC-S FCS for 96 hours as before. Serum starvation did not abrogate the growth effects of genistein observed with the full serum complement, except in the case of HBL 100 and T47-D cells. Here, cell growth in the presence of 10 μM genistein, was reduced by 77 and 72 % respectively, compared to control (figures 3.3 and 3.4). In the remaining four cell lines, the extent of growth inhibition by genistein under conditions of reduced FCS was much more pronounced.

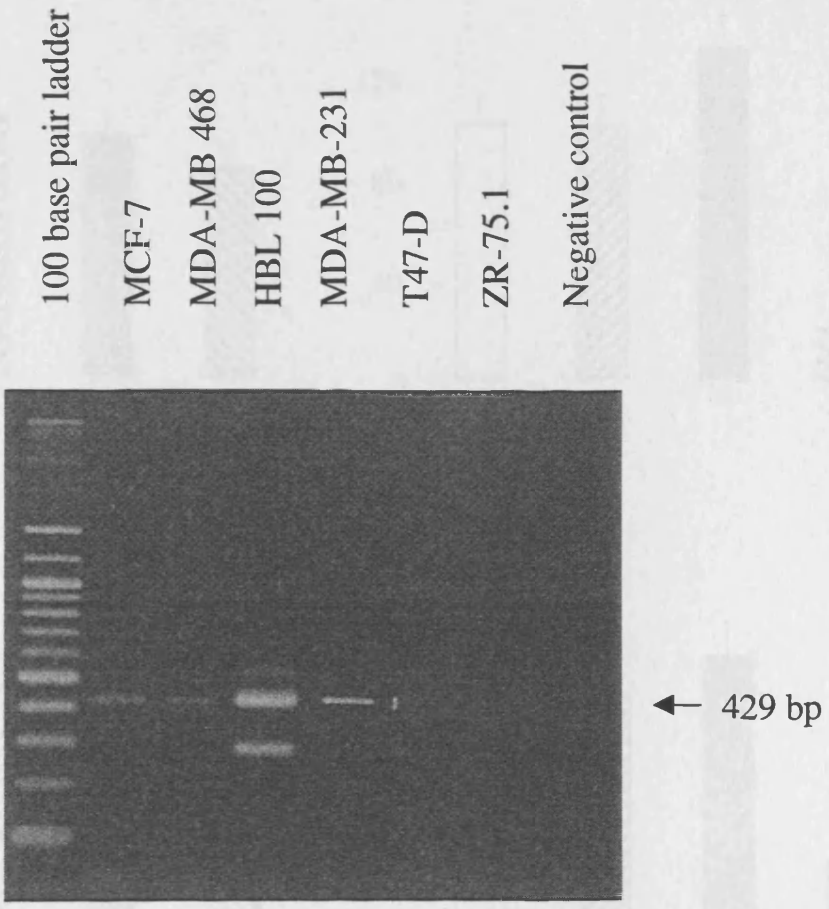


Fig. 3.2. Expression of ER-β mRNA in breast cell lines by RT-PCR.

For details see section 2.6

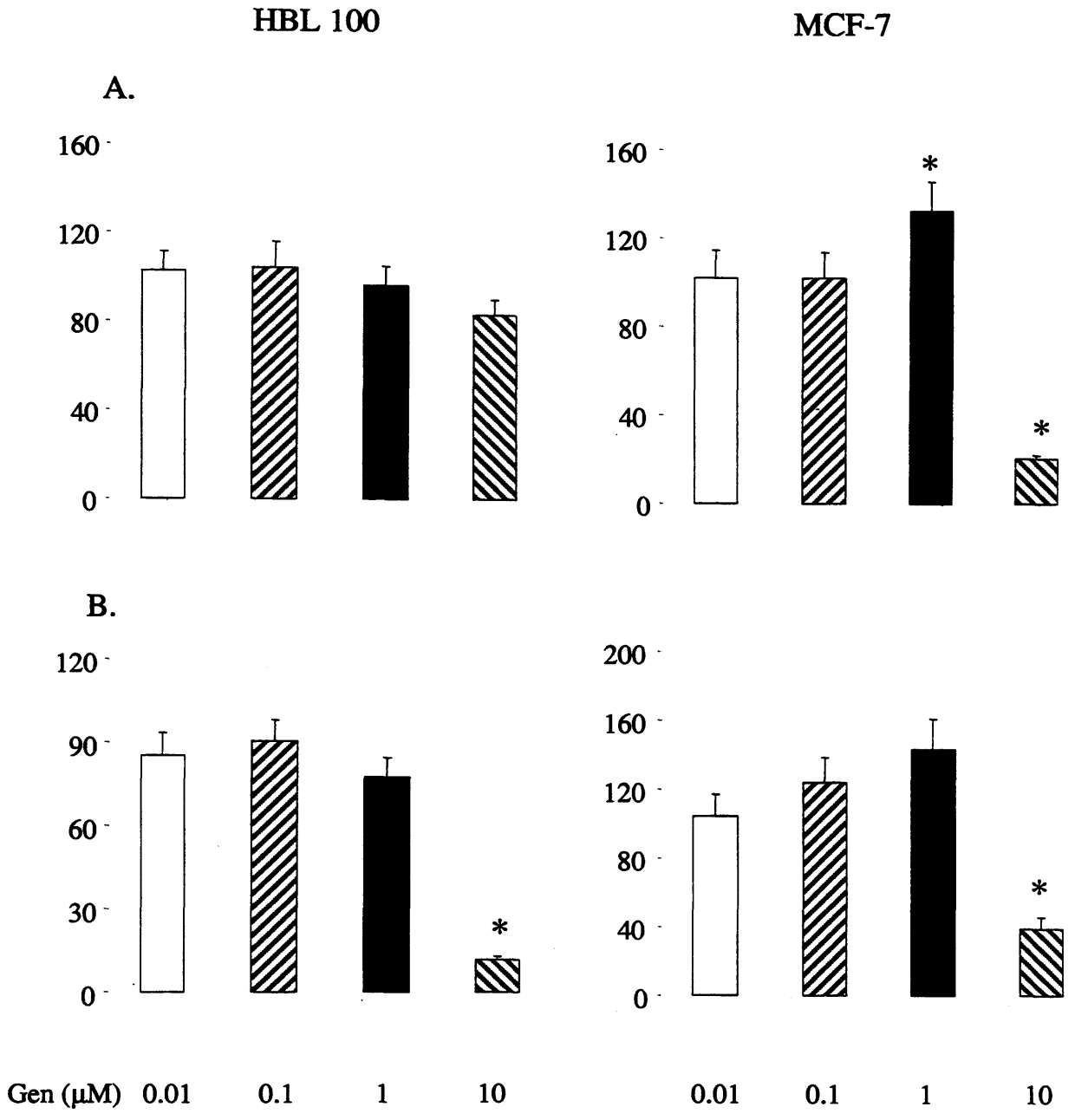


Fig. 3.3. Effect of genistein (0.01-10μM) on the growth of HBL 100 and MCF-7 breast cells in medium containing (A) 10 % FCS and (B) 2% DCC-FCS.

Results, which are presented as a percentage of cells in control cultures, are the mean±SEM of 3 independent determinations, each conducted in duplicate. Asterisks indicate that values are significantly different from controls ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of culture conditions see section 2.4.

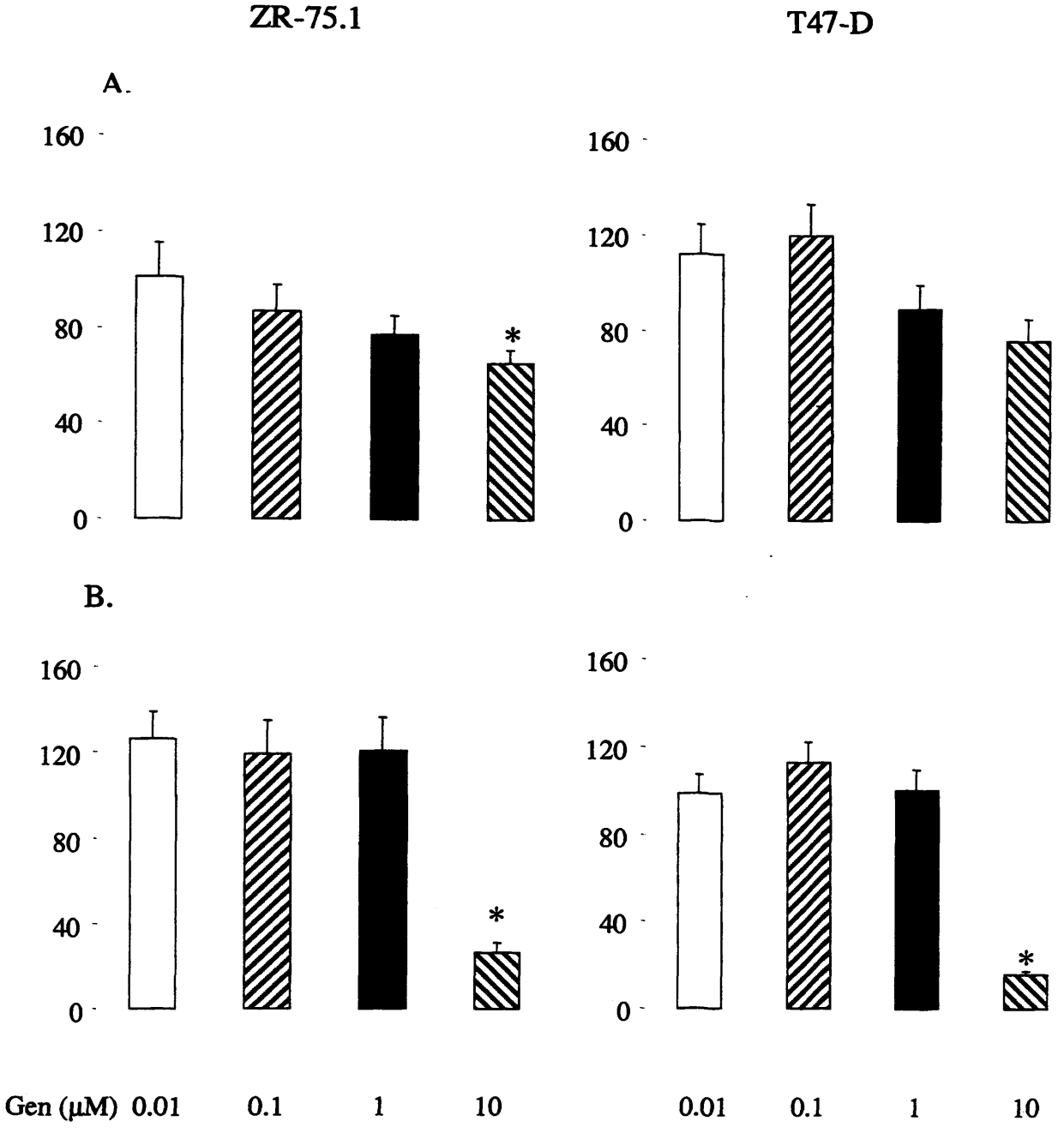


Fig. 3.4. Effect of genistein (0.01-10μM) on the growth of ZR-75.1 and T47-D breast cells in medium containing (A) 10 % FCS and (B) 2% DCC-FCS.

Results, which are presented as percentage of cells in control cultures, are the mean±SEM of 3 independent determinations, each conducted in duplicate. Asterisks indicate that values are significantly different from controls ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of culture conditions see section 2.4.

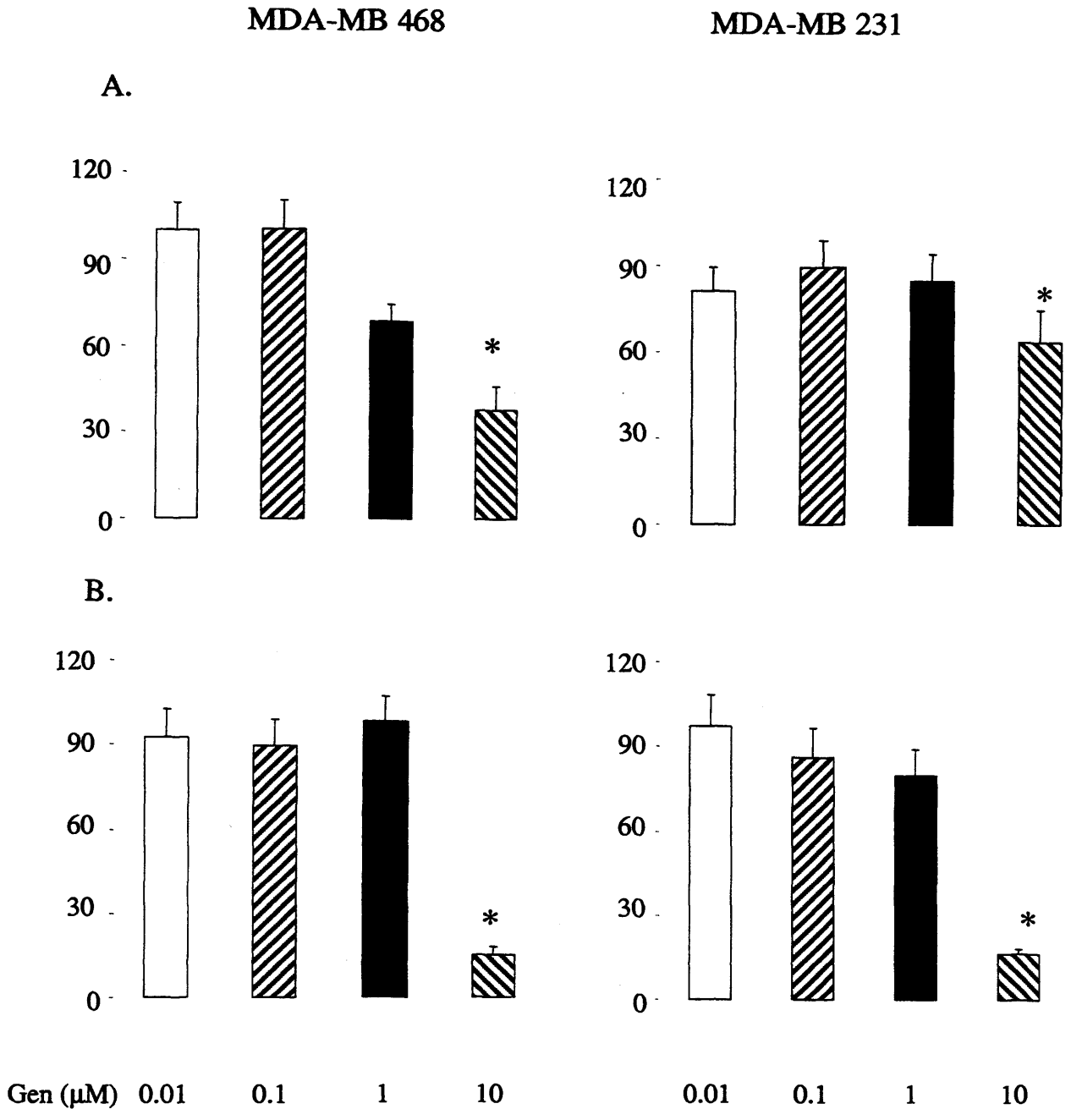


Fig. 3.5. Effect of genistein (0.01-10μM) on the growth of MDA-MB 468 and MDA-MB 231 breast cells in medium containing (A) 10 % FCS and (B) 2% DCC-FCS.

Results, which are presented as percentage of cells in control cultures, are the mean±SEM of 3 independent determinations, each conducted in duplicate. Asterisks indicate that values are significantly different from controls ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of culture conditions see section 2.4.

As differences were observed in genistein-induced HBL 100 and T47-D cell growth in medium containing 10% FCS and 2% DCC-S FCS, further experiments were performed in order to investigate the effect on cells grown in medium containing 10% DCC-S FCS and 2% FCS. The results from these studies would indicate if either charcoal stripping or the amount of serum, or both, were required for genistein to exhibit its growth-inhibitory effects.

Genistein did not elicit a growth inhibitory effect in T47-D cells grown in either type of culture condition (figure 3.6). Therefore, in this cell line it appears that in order for genistein to significantly modulate growth, the culture medium needs to contain a reduced amount of serum which is charcoal stripped, as growth inhibition was only observed when cells were grown in medium containing 2% DCC-S FCS.

In contrast, in HBL 100 cells, genistein elicited a growth-inhibitory effect in both types of culture condition (figure 3.6). These results suggest that the growth inhibitory effect on HBL 100 cells does not depend on reduced serum, nor does it rely on the removal of hormones from the serum. It would appear that the presence of (undefined) serum proteins interfere with the effect of genistein on cell growth modulation. In further experiments, the culture condition '2% DCC-S FCS' will be known as just '2% FCS', as the process of serum stripping did not have any effect on cell growth. This will hopefully cause less confusion when interpreting results.

3.2.1. Comparison of genistein-induced growth inhibition between two non-malignant cell lines

Further experiments were performed, comparing the effects of genistein on HBL 100 cells and MCF-10A cells, another non-malignant cell line. MCF-10A cells express very low levels of mRNA of ER-α (Dickson and Lippman, 1995) and are cultured in specially defined medium (see section 2.3.1 for details). They normally require 5% horse serum, as compared to 10% FCS in order to proliferate, therefore the experiments could not be repeated identically. In addition, experiments were not

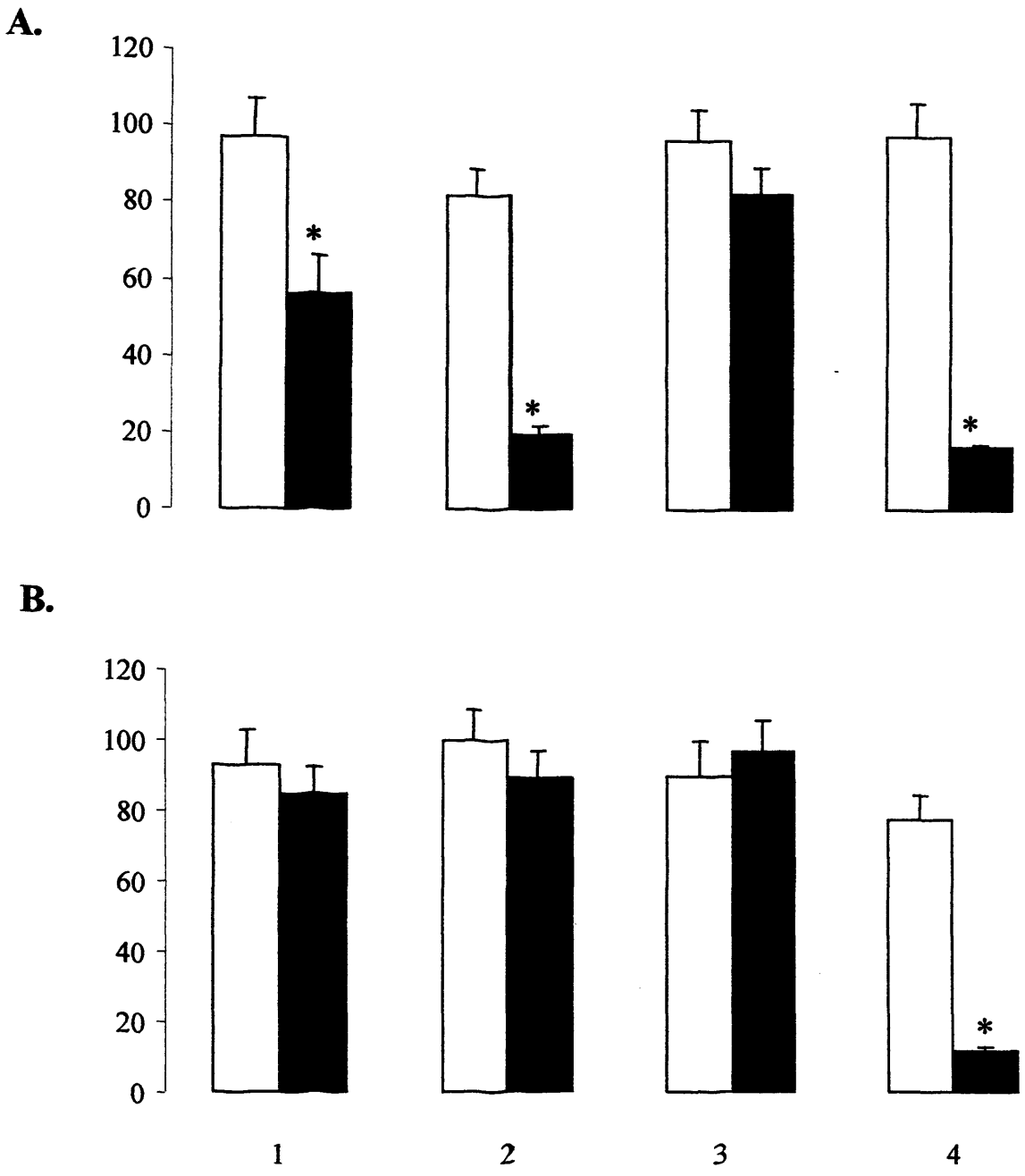


Fig. 3.6. Effect of genistein 1 μM (open bars) and 10 μM (closed bars) on the growth of HBL 100 (A) and T47-D (B) breast cells in medium containing 10 %FCS (1), 10% charcoal stripped FCS (2), 2% FCS (3) and 2% charcoal stripped FCS (4).

Results, which are presented as percentage of cells in control cultures, are the mean±SEM of 3 independent determinations, each conducted in duplicate. Asterisks indicate that values are significantly different from controls ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of culture conditions see section 2.4.

performed using medium containing charcoal stripped horse serum, as preliminary studies using this culture condition resulted in large numbers of dead cells after just 24 hours. MCF-10A cells obviously require essential nutrients present in horse serum that are removed during the process of charcoal stripping. Therefore, experiments were performed studying the effect of identical concentrations of genistein on cells grown in medium containing either the full complement (5%) or reduced (2%) horse serum. This would provide a comparison to the results gained with HBL 100 cells grown in medium with full and reduced serum concentrations.

The results in figure 3.7 show the growth of MCF-10 A cells treated with genistein (0.01-10μM). Genistein (10μM) significantly inhibited growth of cells in medium containing either 5% or 2% horse serum, with inhibition much more pronounced when cultured in the latter medium. Inhibition of cell growth by genistein was also observed at 1μM in medium supplemented with 2% serum.

3.3. Differential response of HBL 100 cell growth to the effects of genistein

The following experiments performed using HBL 100 cells were an extension of previous investigations, and were carried out in an attempt to elucidate why the behavior of genistein (10μM) differed between culture conditions.

Genistein (10μM) elicited a different inhibitory response on the growth of HBL 100 cells, depending on the type of culture conditions, so further experiments were performed to test the hypothesis that the inhibitory action of genistein could be reversed in line with changes in the culture conditions. Cells were primarily incubated in medium containing 2% FCS and after 48 hours, serum was added to the medium to adjust the serum content to 10%.

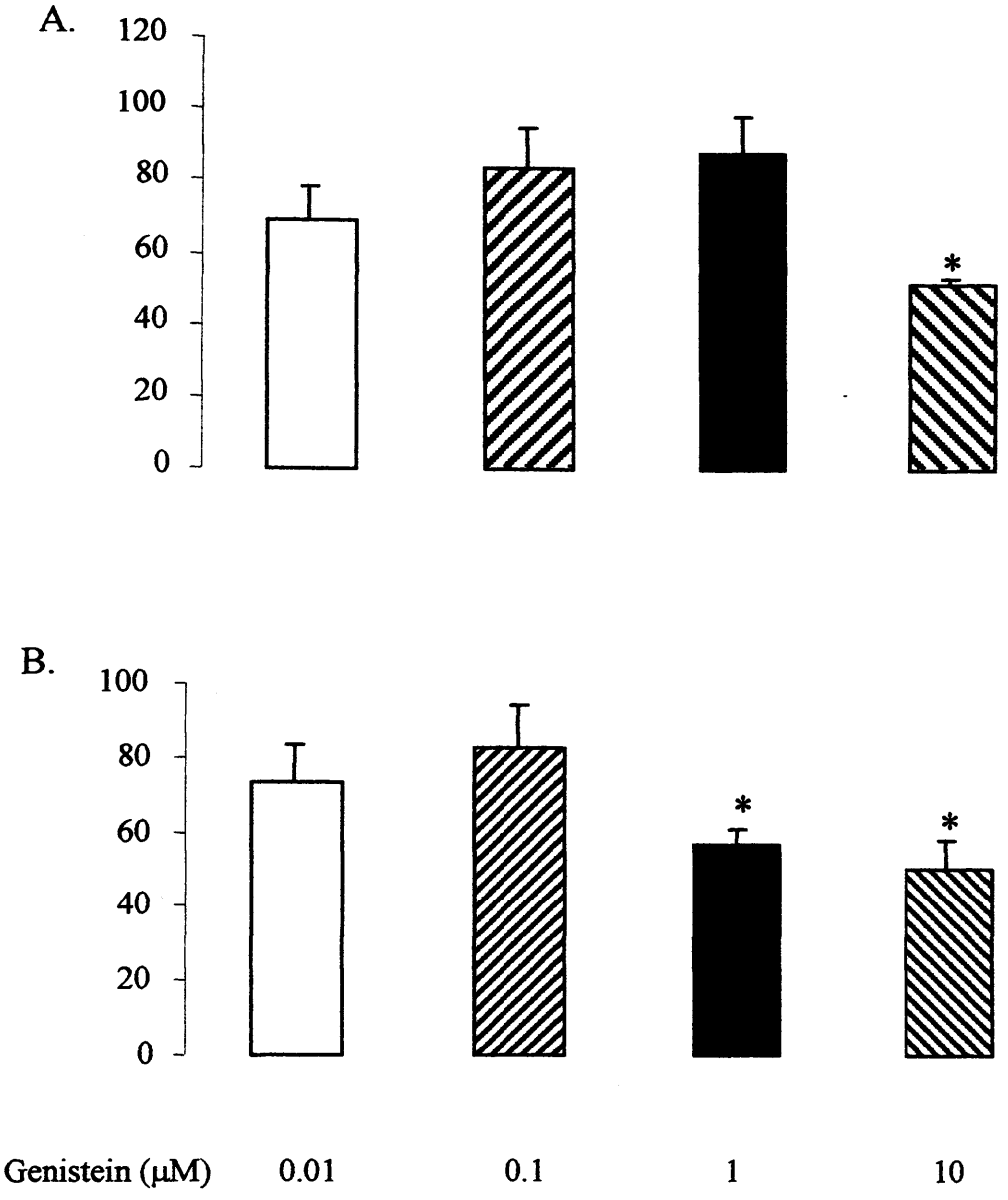


Fig. 3.7. Effect of genistein (0.01-10μM) on the growth of MCF 10A breast cells in medium supplemented with (A) 5% or (B) 2% horse serum.

Results, which are presented as percentage of cells in control cultures, are the mean±SEM of 3 independent determinations, each conducted in duplicate. Asterisks indicate that values are significantly different from controls ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of culture conditions see section 2.4.1.

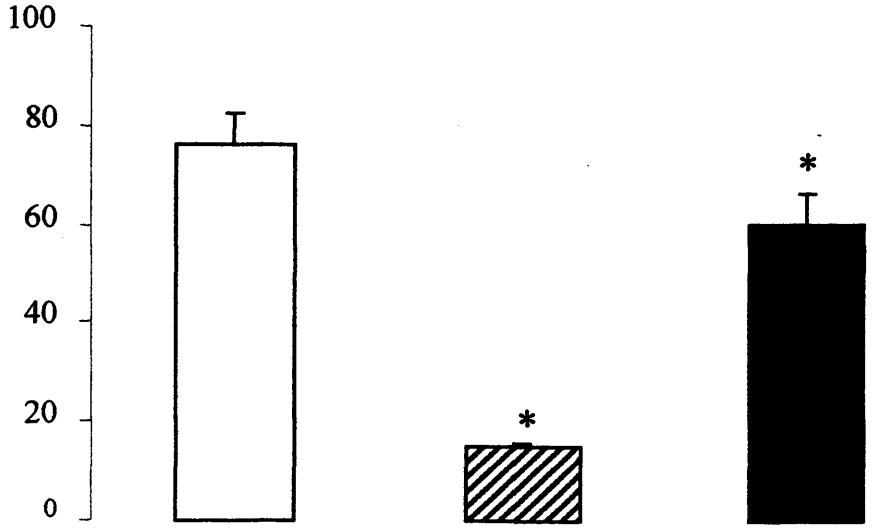
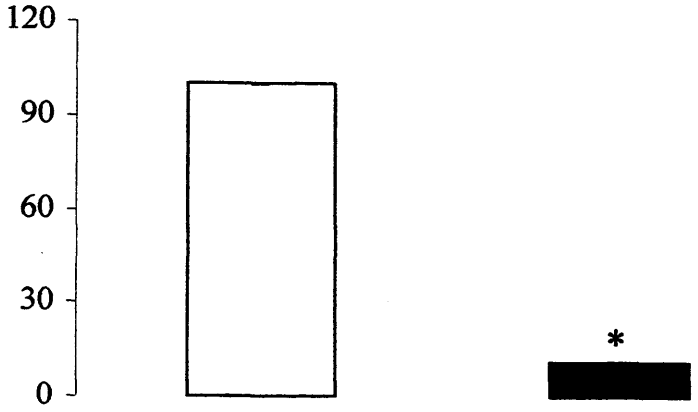


Fig. 3.8. Reversal by 10% FCS of the effect of genistein (10μM) on the growth of HBL 100 cells in 2% FCS.

Open bars represent cells grown in media containing 10% FCS, hatched bars 2% charcoal stripped and closed bars serum change from 2 to 10% FCS. Results, which are presented as percentage of cells in control cultures, are the mean±SEM of 3 independent determinations, each conducted in duplicate. Asterisks indicate that values are significantly different from controls ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of culture conditions see section 2.4.

A.



B.

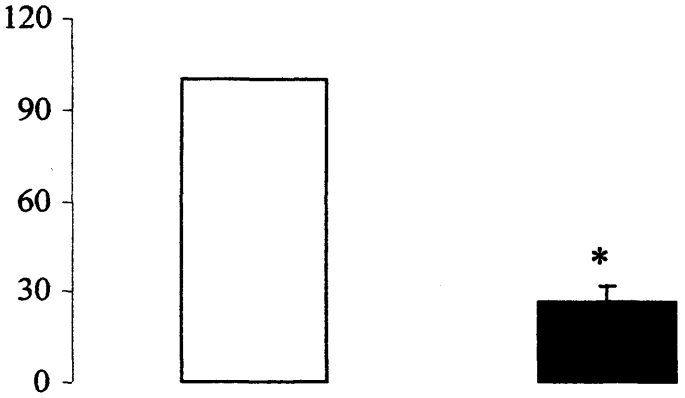


Fig. 3.9. Effect of genistein on the growth of HBL 100 cells in medium containing 2% DCC-S FCS (A) supplemented with EGF, insulin, hydrocortisone and oestradiol, and (B) without supplements.

Open bars represent cells treated with DMSO and closed bars represent cells treated with genistein (10μM). Results, which are presented as percentage of cells in control cultures, are the mean±SD of 3 independent determinations, each conducted in duplicate. Asterisks indicate that values are significantly different from controls ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of culture conditions see section 2.4.

As seen in previous results (see section 3.2), genistein (10μM) had no effect on HBL 100 cell growth in medium supplemented with 10% FCS, but when cells were grown in medium containing 2% DCC-S FCS, genistein inhibited cell growth by 85% compared to the control. Interestingly, when HBL 100 cells were incubated with medium containing serum changing from 2 to 10%, the inhibitory action of genistein was reversed (figure 3.8).

Further experiments were performed to test the hypothesis that the growth sensitivity of HBL 100 cells to genistein relates to specific serum components. It is possible that a constituent of serum may mask the growth-inhibitory effects of genistein in this cell line. Four components of serum were chosen: EGF, hydrocortisone, insulin and oestradiol. Experiments were performed assessing the effect of genistein on cells grown in medium containing 2% DCC-S FCS alone and with the supplements listed above. The results shown in figure 3.9 provide evidence that the addition of these components to culture medium did not affect the ability of genistein to inhibit cell growth, as significant growth inhibition was observed in cells grown in medium both with and without the supplements.

DISCUSSION

The results presented here show that the growth of all six breast cell lines investigated in my thesis were affected by genistein at 10 μM, which is consistent with a number of published studies including Zava et al., 1997, Fioravanti et al., 1998, LeBail et al., 1998 and Dixon-Shanies and Shaikh, 1999. Nevertheless, differences were apparent between cell lines in terms of their sensitivity towards genistein, with MCF-7 cells proving most sensitive when grown in medium containing 10% FCS (table 3.1).

The enhancement of breast cell growth elicited by genistein (1 μM) in MCF-7 cells is not a generic feature of human-derived breast cells, as growth stimulation was not detected in any of the other cell lines.

The observation that genistein in the 10^{-6} M range can promote the growth of MCF-7 cells is consistent with those reported by Martin *et al.*, (1978) and Wang *et al.*, (1996), in which growth stimulation was observed at concentrations of 200nM and 10nM-1μM, respectively. These studies indicate there are two mechanisms by which genistein can modulate cell proliferation. The first mechanism, in which genistein stimulates cell growth at low concentrations, may be mediated via the oestrogen receptor (Hsieh et al., 1998) and the second, anti-proliferative mechanism, evident at higher concentrations, may be mediated through a number of pathways, possibly independent of the ER, including deregulation of the cell cycle and/or apoptosis.

Stimulation of breast cell growth by genistein has raised concern that physiologically achievable concentrations of genistein might support - rather than counteract - the progression of breast cancer in women with pre-malignant changes. Studies by Hsieh *et al.*, (1998) showed genistein enhanced the growth of MCF-7 cell tumours implanted subcutaneously into ovariectomised athymic nude mice *in vivo*, with plasma levels of genistein reaching 2.1μM. Thus, there is the potential for dietary genistein to stimulate the growth of oestrogen dependent tumours in postmenopausal women, who have low circulating levels of endogenous oestrogens. Such an effect would severely confound any potential benefit of genistein consumption and emphasizes the importance of fully characterising phyto-oestrogens in terms of biological activity, the balance between agonistic and antagonistic properties, natural potency and long and short term effects (Whitten et al., 1995).

Growth promotion is associated with both EGF and ER. The work of Aikyama *et al.*, (1987) showed that genistein exhibited specific activity against tyrosine kinases, for example, EGF–R kinase, $pp60^{v\text{-}src}$ and $pp110^{gag\text{-}fes}$ kinases. In the same study, genistein was also shown to inhibit the tyrosine kinase activity of the EGF-R in cultured A-431 cells. It is therefore possible that genistein may elicit an inhibitory effect on cell growth by specific inhibition of tyrosine kinases. In a different study, genistein treatment (6.25-100μM) for 5 days was shown to decrease EGF-dependent mammary epithelial cell proliferation in a dose-dependent fashion (McIntyre and

Sylvester, 1998), whereas identical concentrations had little or no effect on EGF-induced EGF-R tyrosine autophosphorylation. This would indicate that the antiproliferative effect of genistein was not directly associated with a reduction in EGF-R activity.

Experiments with transgenic mice have implicated the overexpression of several growth factors and receptors, including EGF, in mammary cancer (Dickson and Lippman, 1995). Hormone unresponsive cells differ from their steroid receptor positive counterparts by possessing higher proliferative and invasive rates both *in vivo* and *in vitro*, in addition to altered expression of certain growth factor receptors, for example EGF-R (Dickson and Lippman, 1995).
Clinically, high levels of EGF-R in breast tumours strongly correlate with a poor prognosis, and high expression of EGF-R is often accompanied by a low or absent expression of ER-α (Sainsbury et al., 1985). The cell lines used in this study were characterised in terms of their EGF-R and ER status to provide an insight into their nature and to examine whether genistein could utilise either EGF-R- and /or ER-mediated pathways to inhibit cell growth.

Figure 3.1 shows the level of EGF-R protein expression in the cell lines. MDA-MB 468 and MDA-MB 231 cell lines both exhibit elevated levels of this receptor, consistent with the work of Sainsbury *et al.*, (1995) in that high EGF-R expression correlates with the absence of ER-α. The inhibitory effect of genistein on cell growth is therefore probably not mediated through inhibition of EGF-R activity, as growth inhibition was also observed in EGF-R negative cell lines, which suggests other mechanisms must be involved.

Work by Peterson *et al.* in cells of the breast (Peterson and Barnes, 1996) and prostate (Peterson and Barnes, 1993) showed neither EGF-stimulated EGF-R tyrosine autophosphorylation nor EGF-stimulated tyrosine phosphorylation of intracellular target proteins such as MAPK were affected at concentrations of genistein that inhibited proliferation of such cells. This data suggests that genistein

affects these cells by mechanisms other than inhibition of EGF-R PTK activity and this is probably also true for the breast cell lines tested in this study.

Oestrogen receptors play an important role in proliferation of breast cells. The modulation of cell growth by genistein has been associated with the interaction of genistein with oestrogen receptors (Kuiper et al., 1998) and this may be the mechanism by which low concentrations of genistein stimulate the growth of MCF-7 cells. Genistein is known to have a greater affinity for ER-β, as opposed to ER-α, therefore experiments were performed to investigate the ER-β mRNA status of the six breast cell lines by RT-PCR, to test the hypothesis that the growth stimulatory effects of genistein are somehow mediated via oestrogen receptors.

The results in figure 3.2 represent ER-β mRNA expression. It would have been preferable to detect ER protein in addition to mRNA to ensure that ER-β mRNA was translated, but protein experiments using ER-β antibodies in western blotting were unsuccessful. However, as more antibodies become available, it would be important to screen the cell lines for ER-β protein levels.

Four cell lines exhibited levels of ER-β mRNA, namely HBL 100, MCF-7, MDA-MB 468 and MDA-MB 231 cells. The ER-α protein status of the cells lines used in this study have been described extensively in the literature and are summarised together with ER-β mRNA results, from both this study and others, in table 3.3.

Cell line	ER-α protein	ER-β mRNA
HBL 100	- Nakatani et al., 1999	+ Dampier (unpublished)
MCF-7	+ Brooks et al., 1973	+ Enmark et al., 1997
ZR-75.1	+ Enmark et al., 1997	– Enmark et al., 1997
T47-D	+ Enmark et al., 1997	+ Dotzlaw et al., 1996 + Enmark et al., 1997
MDA-MB 468	– Shao et al., 1998 – Balabhadrapathruni et al., 2000	+ Dampier (unpublished)
MDA-MB 231	– Fioravanti et al., 1998	+ Dotzlaw et al., 1996 + Vladusic et al., 1998

Table 3.1. ER status of breast cell lines used in this study.

MCF-7 cells are positive for ER-α protein and also express ER-β mRNA. T47-D and ZR 75.1 cells express ER-α protein, but differ in their ER-β status. Both MDA-MB 231 and MDA-MB 468 cells harbour only ER-β mRNA. This comparison between the cell lines renders the possibility unlikely that the growth promotion caused by genistein specifically in MCF-7 cells is intrinsically linked to expression of either estrogen receptor-α or -β or both.

Some breast cell types, exemplified by HBL 100 and T-47 D cells, were only sensitive to the growth inhibition exerted by genistein under conditions of low serum concentration in the culture medium. From further investigations of these two cell lines it was concluded that certain culture conditions were required to enable genistein to inhibit cell growth. Genistein did not inhibit growth of HBL 100 and T47-D cells cultured in medium containing the full complement of serum, suggesting

that certain serum proteins may play a role in protecting these cells from the effects of genistein.

Serum binding proteins, such as the sex-hormone binding protein, are known to provide a mechanism to limit cell uptake of, and response to natural oestrogens in target tissues (Welshons et al., 1999), and several xeno-oestrogens, including genistein, are known to exhibit decreased levels of binding to serum binding proteins (Martin et al., 1978). A study by Nagel *et al.*, (1998) found several xeno-oestrogens, including genistein, showed greater access to oestrogen receptors than oestradiol in MCF-7 cells, in the presence of 100% serum. Genistein exhibited a 10-fold increase in relative binding affinity in 100% serum, compared to serum free media. This study suggests that in the presence of serum, genistein may have a greater proportion of its total concentration free to react with intracellular oestrogen receptors and the overall biological impact, relative to oestradiol, may be overestimated in serum free assays. The biological activity of natural oestrogens, such as genistein, can therefore be influenced by the degree to which they bind serum proteins and this may be a mechanism by which genistein can modulate breast cell growth.

The sensitivity of individual cell lines to the growth modulating effects of genistein may also be attributed to their ability to metabolise the isoflavone. Studies by Peterson *et al.* (1996, 1998) have investigated the metabolism of genistein in a number of breast cell lines, including MCF-7, T47-D, ZR-75.1 and HME cells using radiolabelled genistein. Extensive metabolism was observed in MCF-7 cells, with genistein 7-sulphate and a hydroxylated, methylated form of genistein sulphate detected. HME cells, which can be compared with both HBL 100 and MCF-10A cells, did not significantly metabolise genistein. If the growth inhibitory action of genistein is somehow related to its metabolites, then this may be why there is no inhibition of HBL 100 cells grown in media containing 10% FCS, although growth inhibition was seen in MCF-10A cells. It can be suggested that the 'normal' cell lines may differ from others in their ability to metabolise genistein. However, the growth of T47-D cells, which metabolise genistein to both genistein 7-sulphate and a

hydroxylated and methylated metabolite, is only affected by genistein when cultured in medium supplemented with 2% DCC-S FCS. This renders the possibility of growth-inhibition by an active metabolite unlikely and the mechanism by which genistein inhibits growth must be via a different mechanism.

Genistein may be converted to active metabolites in some cancer cell lines, but this metabolism does not necessarily affect growth inhibition. It may be a mechanism by which genistein inhibits growth in one cell line, but it is clear that a number of different pathways and mechanisms are involved for each individual cell line. However, it must be noted that metabolism of genistein *in vitro* and *in vivo* may differ and genistein may not be converted to sulphate esters in normal breast or breast carcinoma (Peterson et al., 1998).

To conclude, the growth of all breast cell lines in this study were inhibited by genistein with the degree of inhibition dependent on the culture condition in particular cell lines. The growth-stimulatory effect of genistein was not a generic feature of human-derived breast cells. The mechanism by which genistein elicited its growth inhibitory effects in this study is not mediated via the ER or through the EGF-R as the same degree of growth inhibition was observed in cell lines both with and without the receptors. It does not mean that these receptors are not important in cell growth, just that genistein may utilise other cellular pathways in order to modulate proliferation.

CHAPTER 4: INVESTIGATION INTO THE EFFECT OF GENISTEIN ON CELL SIGNALLING PATHWAYS.

INTRODUCTION

The results presented in chapter three provide evidence that genistein (10μM) inhibits the growth of breast cell lines, which is consistent with many published studies. The mechanisms by which genistein elicits this inhibitory effect, however, need defining. Intracellular signalling pathways, acting as positive mediators and negative regulators of cell growth have the potential to be modulated by disease processes such as cancer. Over the past several years it has become clear that perturbation of signalling pathways is a frequent event during the acquisition of the transformed phenotype evident in these disease processes (Franks et al, 1998).

Gene expression in a cell is constantly modulated by a variety of extracellular stimuli. These stimuli transmit signals through the cell membrane, cytoplasm and nucleus to mediate gene expression (Chen et al., 1996) via the regulation of transcription factor activity (Johnson and Vaillancourt, 1994). For example, activation of the MAPK cell signalling cascade results in activation of AP-1 activity. The regulation of gene expression by MAPK therefore implicates these kinases in disease processes such as cancer (Davies, 1999). The role of both MAPK and the AP-1 transcription factor complex are important in cell growth and differentiation, and it is conceivable that genistein may elicit its growth-inhibitory effects via modulation of such cellular components.

Experiments described in this chapter were designed to test the hypothesis that genistein interferes with transcription factors and cell signalling pathways to modulate cell growth similarly in all cell lines. The chapter concentrates on investigations into the effect of genistein on AP-1 transcription factor activity and AP-1 DNA binding, levels of c-Jun and c-Fos protein and also MAP kinase activity.

RESULTS

4.1. The TRE reporter construct

The TRE construct was grown and DNA purified using the endofree maxiprep kit (section 2.7.1) and a restriction digest using Hind III was performed to check the size of the DNA corresponded to 5.3kB. The DNA was digested and visualised by agarose gel electrophoresis (see figure 4.1). Lanes 2 and 4 represent uncut DNA of the correct size, although the bands are difficult to see as only a small amount of DNA was loaded onto the gel. The TRE plasmid can just be detected at 5.3kB, which was the expected size and could therefore be used in transfection experiments. For more details of the TRE reporter construct, see section 2.7.

4.1.1. Sequencing the TRE construct

The TRE reporter construct was sequenced in order to ascertain that the three TRE /AP-1 binding sites were present. A method using PCR to amplify the amount of DNA prior to sequencing was employed, using the *fmol*™ DNA Sequencing System. This system used the dideoxy method developed by Sanger *et al*, (1977) suitable for sequencing plasmid DNA. The extension/labelling protocol was used together with [α-^{35}S] dATP, a low energy nucleotide which provided reduced exposure to radioactive emissions, better band resolution and possibility of long-term storage without loss of isotope activity, as the half life is 87 days. This sequencing system had several advantages over conventional sequencing strategies, in that firstly, a linear amplification of template DNA was gained, reducing the amount of template DNA required to achieve a detectable sequence ladder. Secondly, the high temperatures employed in each denaturing cycle eliminated the need for ethanol precipitation of dsDNA templates and also reduced problems associated with PCR reaction products. Lastly, the high annealing temperature increased the stringency of primer hybridisation.

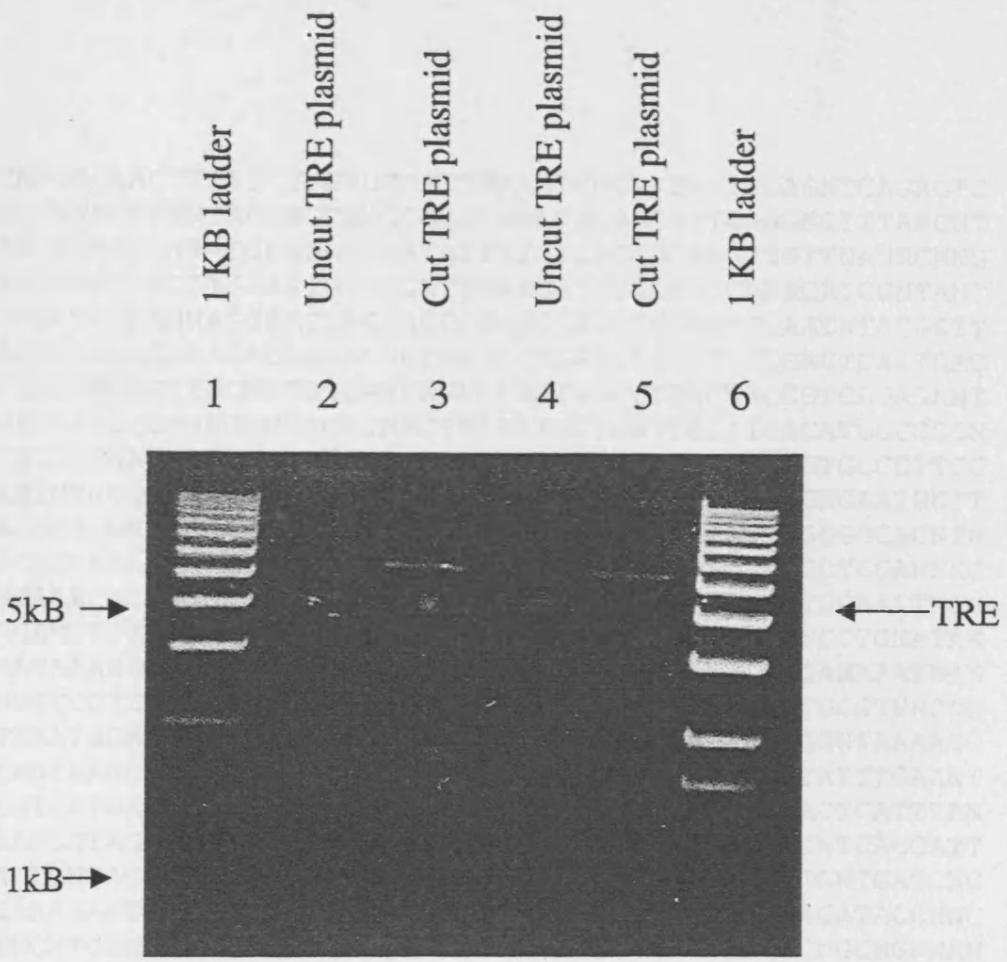


Fig. 4.1. Agarose gel electrophoresis of the restriction digest of the TRE plasmid.

The uncut TRE plasmid migrates through the gel as relaxed circular DNA, whilst the cut plasmid migrates as linear DNA. The size of the TRE plasmid is 5.3kB. For details of restriction digest method see section 2.7.2.

CGGCANCGCAACTTCNTCTNNTCTNNTTNAANTNGCNNTNGTTCAGNTCAGAGTC
GCANANNNNCNTNNTACCNGTNNGCGACCNAATGAGGCTTTGAGGNCTTTANCNT
CTTCGCAGACCGNTATGCNNNTCGATATTATCTCNCGGCANGNTGTTNANNCNNG
CNAAACGNATCNCTTGAATTGTCTGNTTNANGATCTGANGCCANACATCGNTANC
ATANATATGCNANNAGGAACTAGAACCCGCGCCNCCCCGANTGCAATNTGCGGTT
NCNAANNCGNACAAAGACANGACNCCNNGCGAGNNCCGGATCCTGACTCACTGAC
TCACTGACTCACTTNCNTTGGCANTNGNCNGATGGCTTTACNNCCGTCAGAGANT
TGGGATAAACCCTGNCGNTACCCNACTTAAATNGTCNTTGCATCACATGGCCCCN
TTTCGCCAGNATGGCGNAAATANCGNAATAGTCCCAGCACCCGATCTGCCCTTCC
AACANTNTGCGCANATCTNNNAAGNGCGAAANGGGNNCCNGATGACNGAATGNTT
CTCNCATACGNCACAATGGTCNGNTANTTGCACCACNTNCAAATTGGGGCACNTN
TCAGTNCAAAANTNTGCNNNNGATGCNCGCNAAANTAAAGGCANGCCTGGANNNC
CCGNCAAACACCTCGNNTGGACNNNCCCNTGATGGGGCTTNGANNTGCAACTNGG
NATACANTTTTNCANAANTAACNTGTNNCCCTGCCAATNCCGNNANCCTGNATAN
NTGNATAAANGGNTTTTTAANCCNTANTNNNCCTGANNTANGCGTCANAAATNAN
AGGNGGTCCTCGNANNAANCTCCANAATATTNCAANATGGGNNANTGGGTNNCNN
AANTAAATGGNTCCNTTATAAACNCNANGNNGGNGANCCATNTCGGGNTAAAAGG
CGNTNNTAANCCNNANTTNGGNNANNTCTCNTNACTTNNNTCCAATATTTGAANT
CGNCGTCATNAAANANACNNAANCNAGANATAATGGCGTCCTAATACTCATTTAN
ANGAANGNTAGANNNNTANGNGTANCTCTAAAAATATCTNCGNGACNTCACCATT
NNANTTCNTAGTTTATGGGGACTATTTNTCNCCTNNTGNTTGNNNGGNTCACCNC
NNACAAAGACTCTGTGTGTANNAANAAAANANANNNTCNCNTGTAACATACCNNC
GGATNCNTCANNTGNGGNACCACAACTTNCGACAGGAGTTCNATACNGCNGNNAN
AAGNANACACNTNCATNAANATGNGCACTCCNGNAATNNAGAACNTNGCNCACAN
GANACTNCNTNNTCTCATACATNNTGTATGTCTACATGGAGNGACNCGGANTATA
CTNNATCGNGGATACTCCNATNCACNTNTATNAACCGCCN

Figure. 4.2. The sequence upstream of the luciferase coding region of the TRE reporter construct as generated by a GL2 primer.

This sequence of approximately 1400bp is courtesy of PNACL, MRC Toxicology Unit, Leicester, UK, using the GL2 primer from Promega which sequences counter clockwise across the cloning sites, upstream of the luciferase coding region. For details of the TRE plasmid and a simple circle map, see section 2.7.3 and appendix, respectively.

Using this method, a sequence ladder could be detected, however, it did not give good sequence separation and was generally unreadable. The TRE plasmid was finally sequenced by means of a second method, using the automated sequencing facilities of PNACL, housed in the Centre for Mechanisms of Human Toxicity (see section 2.7.3 for details). The GL2 primer, which sequences counter clockwise across the cloning sites upstream of the luciferase coding region, generated a 1400bp sequence which is shown in figure 4.2. The three tandem TRE sites are highlighted in grey. The sequencing was repeated twice and although there were a number of N nucleotides, the presence of three TRE sites were confirmed.

4.2. The effect of genistein on AP-1 transcription factor activity

The effect of genistein on AP-1 transcription factor was investigated with respect to AP-1 activity by transient transfection experiments. The transfections were performed by two different methods; by electroporation and using the fugene reagent. Cells were treated with genistein at concentrations of 1, 10 and 50μM for 1 hour, prior to the addition of the tumour promoter TPA (0.2μM) for 4 hours.

4.2.1. Transfection by electroporation

HBL 100, MCF-7 and MDA-MB 468 cells were used in the initial electroporation experiments, with TPA inducing levels of AP-1 activity to varying extents in these TRE-transfected cell lines (figure 4.3).

TPA induced a four-fold increase in AP-1 activity in MDA-MB 468 cells, and a three-fold increase seen in both MCF-7 and HBL 100 cells (figure 4.3). Genistein significantly inhibited this induction in MDA-MB 468 cells by 75% at 50μM, but no significant inhibition was seen at the lower concentrations, as not enough data points were gained to calculate statistics. No inhibition of TPA-induced AP-1 activity was observed in either of the other cell lines at any concentration, although an induction in AP-1 activity was seen by genistein (1 and 10μM) and TPA.

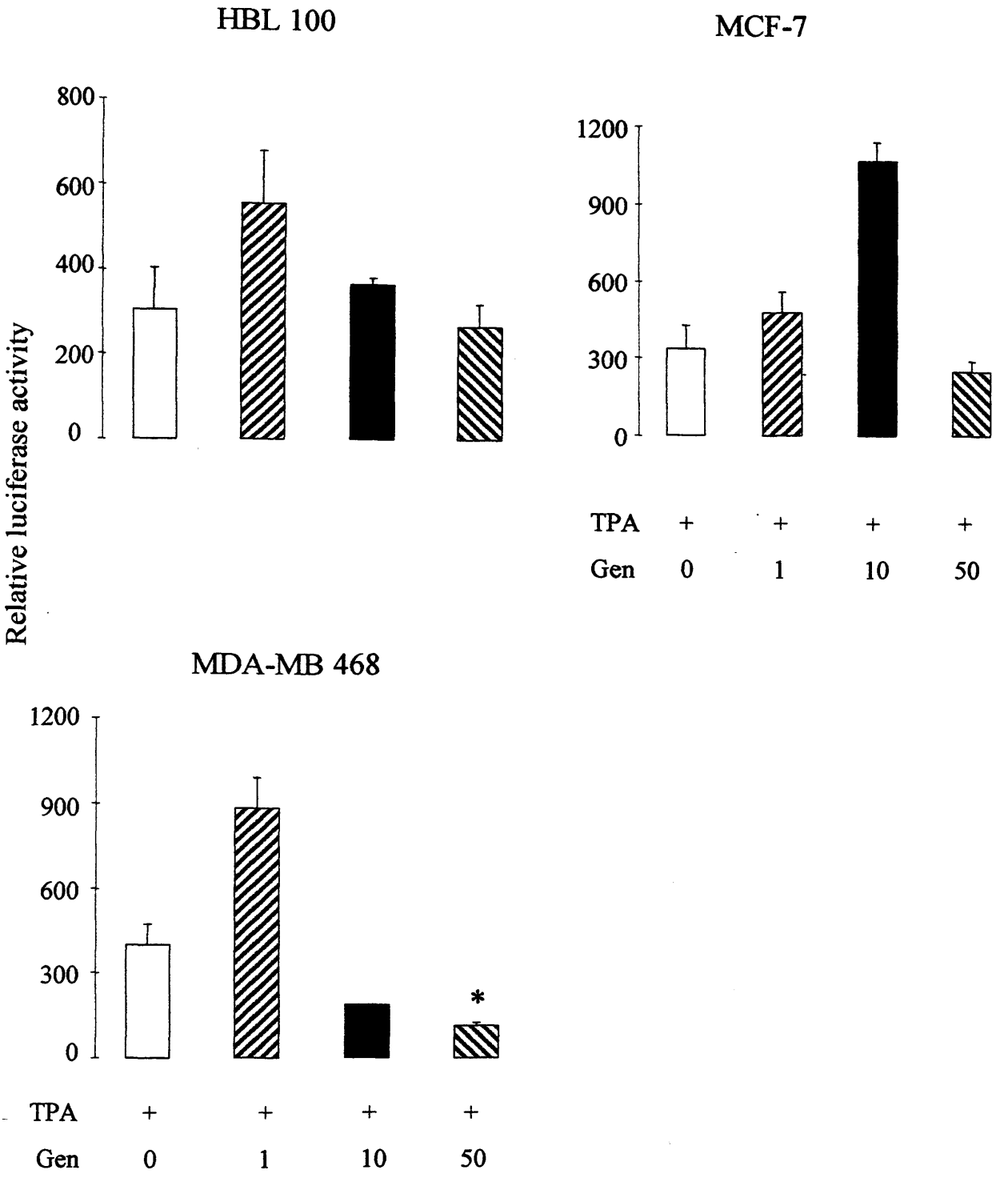


Fig. 4.3. Effect of genistein (1, 10 and 50 μM) on TPA-induced AP-1 activity using electroporation in HBL 100, MCF-7 and MDA MB 468. Open bars denote luciferase activity after treatment with TPA (0.2 μM) only. Results are the mean±SEM of between 3 and 5 independent experiments. Activity was presented as percentage of values in incubates without TPA and deduced from luciferase expression in cells into which a TRE pGL2 luciferase reporter plasmid had been transfected, together with a pCMVβ vector (which expresses β-galactosidase), present to monitor transfection efficiency. Genistein on its own did not affect luciferase activity. Asterisks indicate that values are significantly different from incubates with TPA only ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of transient transfection and culture conditions see section 2.8.1.

When cells were transfected with the 'empty' vector (pGL2 basic), the backbone of the TRE construct, no induction by TPA was observed in any of the three cell lines (figure 4.4). This result was expected, as the pGL2 basic vector does not contain any TPA response elements.

4.2.2. Transfection by fugene

Figure 4.5 shows levels of TPA-induced AP-1 transcriptional activity in HBL 100, MDA-MB 468, T47-D and ZR-75.1 cells using fugene as the method of transfection. Similarly to electroporation, TPA stimulated AP-1 activity to different extents, ranging from a two -fold to a ten-fold increase in T47-D and ZR-75 cells, respectively. The stimulation seen by TPA using fugene was less pronounced when compared with cells transfected by electroporation, with only a 2.5-fold and 1.5-fold increase in MDA-MB 468 and HBL 100 cells, respectively.

Using this method, genistein inhibited TPA-induced transcriptional activity in MDA-MB 468 cells by 33% whilst in T47-D cells it inhibited the two-fold induction of AP-1 activity by TPA by 50%. However, the extent of these reductions was variable and not significant, and observed only at the highest genistein concentration.

In ZR 75.1 cells genistein (50 μM) augmented, rather than decreased, TPA-induced AP-1 activity, although this elevation was highly variable (figure 4.5). Genistein treatment did not result in inhibition at any concentration in HBL 100 and ZR-75.1 cells. Results obtained for three independent experiments using MCF-7 and MDA-MB 231 cell lines have not been included as they exhibited a similar pattern of activity as HBL 100 cells.

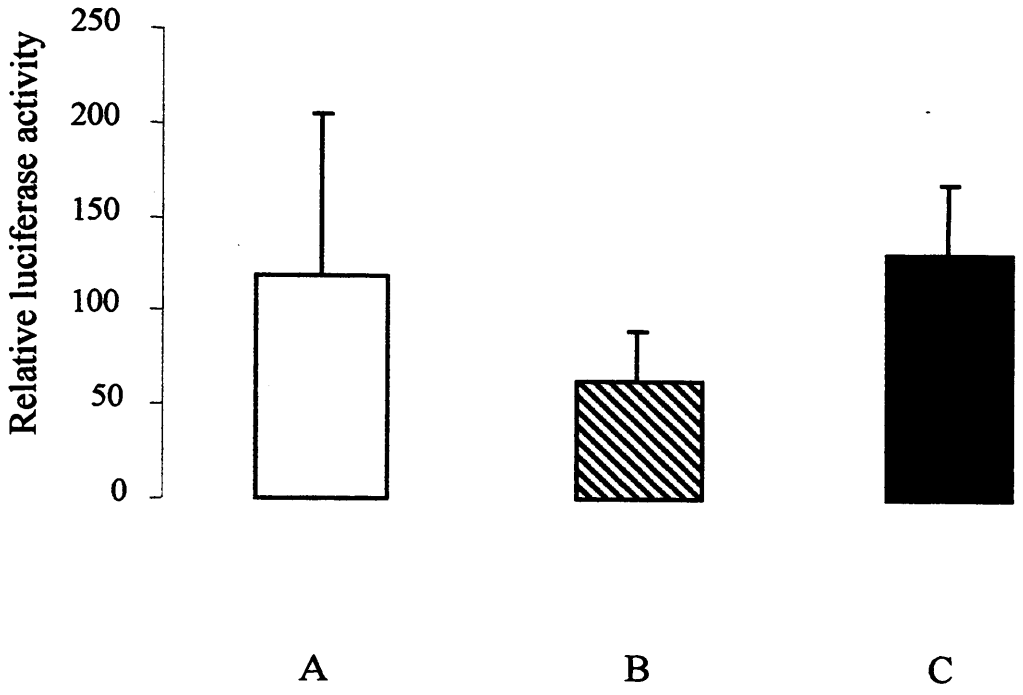


Fig. 4.4. Effect of TPA on AP-1 activity in (A) HBL 100, (B) MCF-7 and (C) MDA-MB 468 cells transfected with an pGL2 basic reporter construct.

A pGL2 luciferase reporter plasmid containing no TRE sites was transfected into breast cells together with a pCMVβ vector, used to express β-galactosidase as a control for transfection efficiency. Results are presented as luciferase activity which has been deduced from luciferase expression in cells into which a had been transfected and the results were normalised to β-galactosidase and are the mean±SEM of 3 independent experiments and are presented as percentage of values in incubates without TPA. For details of transient transfection and culture conditions see section 2.8.1.

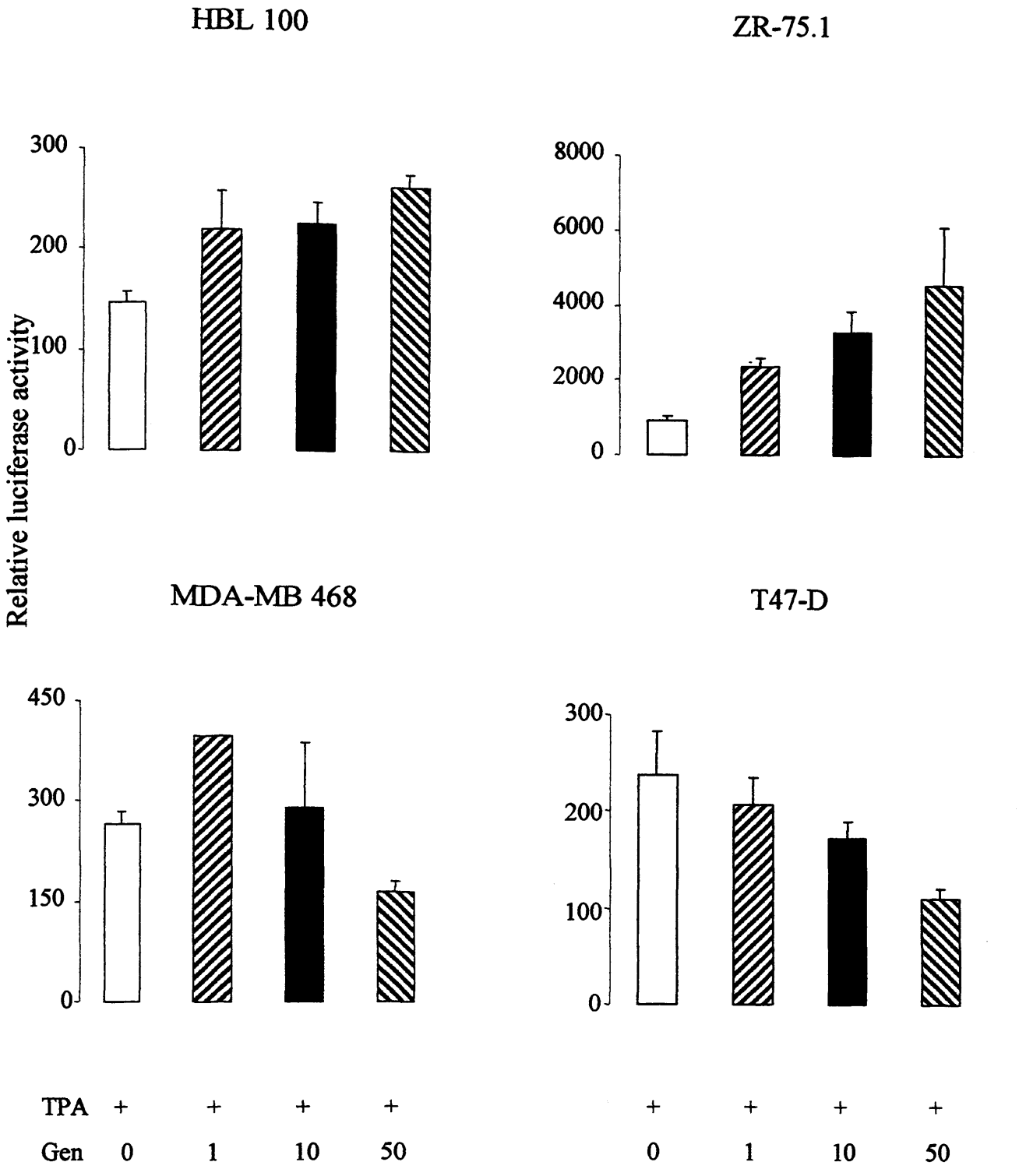


Fig. 4.5. Effect of genistein (1, 10 and 50 μM) on TPA-induced AP-1 activity using fugene in HBL 100, MDA MB 468, ZR-75.1 and T47-D cells. Open bars denote luciferase activity after treatment with TPA (0.2 μM) only. Results are the mean±SEM of between 3 and 5 independent experiments. Activity was deduced from luciferase expression in cells into which a TRE pGL2 luciferase reporter plasmid had been transfected, the results were normalised to β-galactosidase (a pCMVβ was used to express β-galactosidase as a control for transfection efficiency) and are presented as percentage of values in incubates without TPA. Genistein on its own did not affect luciferase activity. For details of transient transfection and culture conditions see section 2.8.2.

4.3. The effect of genistein on AP-1 DNA binding activity

The results from transient transfection experiments provided an insight into the effect of genistein on AP-1 transcription factor activity. In order to investigate the molecular basis for the inhibition of AP-1 activity by genistein, it was hypothesised that genistein interfered with AP-1 DNA-binding activity. The effect of genistein on AP-1 DNA-binding activity was examined by gel shift assay using an oligonucleotide containing the AP-1 consensus sequence (TGAC TCA). For details of the oligonucleotide sequence, see appendix.

Levels of AP-1 DNA-binding activity were investigated in HBL 100, ZR-75, T47-D and MDA-MB 468 cells, with the results from the AP-1 activity experiments providing the rationale for using these cell lines. HBL 100 cells were used as a negative control, as no inhibition was seen at the transactivating level. The ZR-75 cells were used as they exhibited increased levels of AP-1 activity compared to control and finally, both T47D and MDA-MB 468 cells were used as genistein was shown to elicit an inhibitory effect on their AP-1 transactivating activity. Cells were treated with genistein (50μM) for 1 hour, prior to the addition of the tumour promoter TPA (0.2μM) for 4 hours. Nuclear protein was then extracted, and subjected to gel shift analysis. Specificity for the AP-1 sequence was confirmed by competitive inhibition with an excess of unlabelled AP-1 oligonucleotide.

Figure 4.6 shows representative autoradiography of levels of AP-1 DNA-binding activity in T47-D and MDA-MB 468 cells after treatment with genistein, and figure 4.7 provides results of all experiments performed in all cell lines. TPA induced AP-1 DNA binding to varying extents, from 1.5 to 4-fold in HBL 100 and ZR-75.1 cells respectively (figure 4.7), compared to DMSO control. Genistein alone appeared to induce AP-1 DNA-binding activity when compared to control (DMSO) levels in T47-D and MDA-MB 468 cells (figures 4.6 and 4.7).

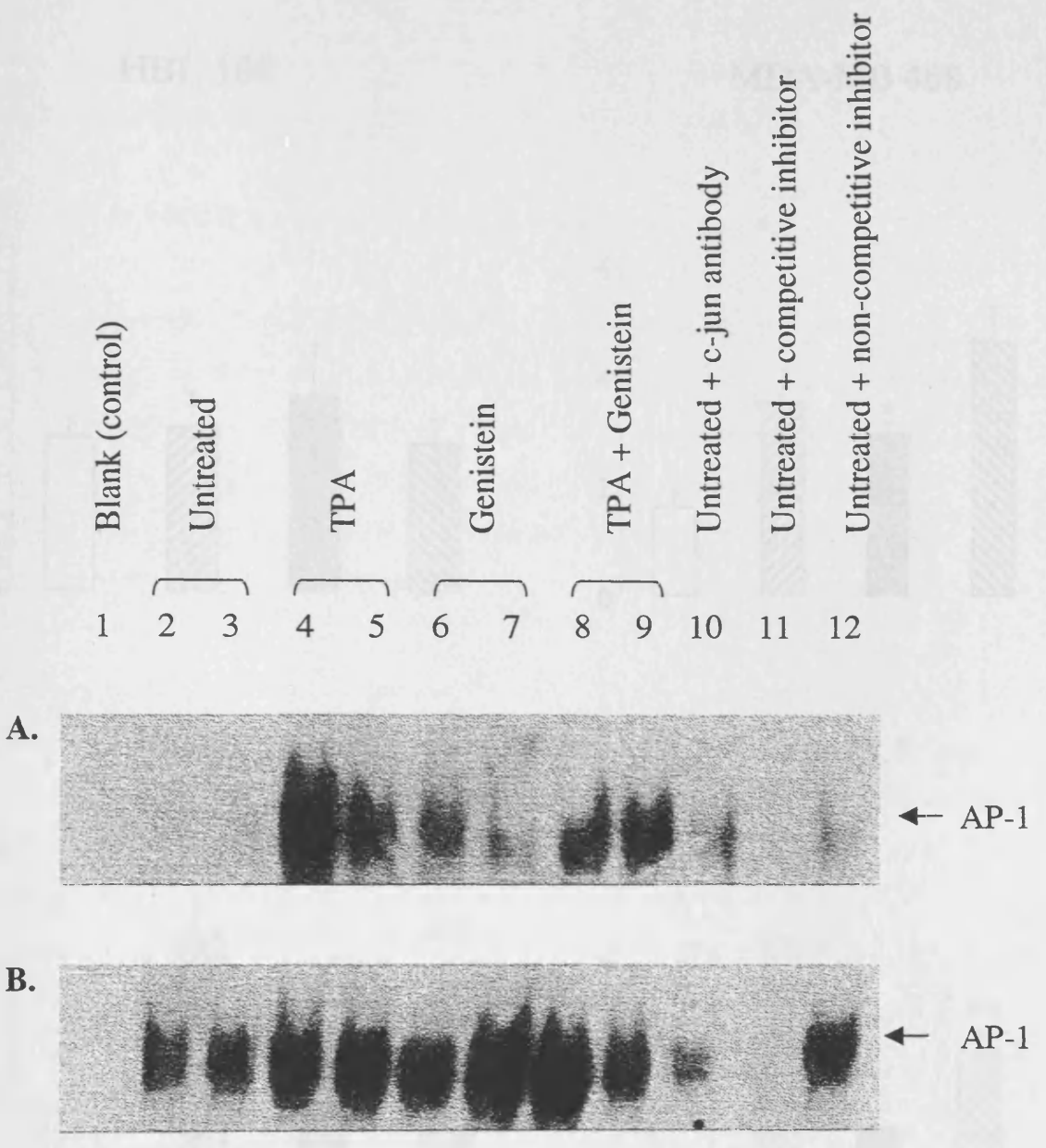


Fig. 4.6. The effect of genistein (50μM) on AP-1 DNA-binding activity in (A) T47-D and (B) MDA-MB 468 cells.

Results are presented as representative autoradiography blots of gel shift assays treated as above. Specificity for the AP-1 sequence was confirmed by competitive inhibition using an excess of the same, but unlabelled oligonucleotide (see lane 11).

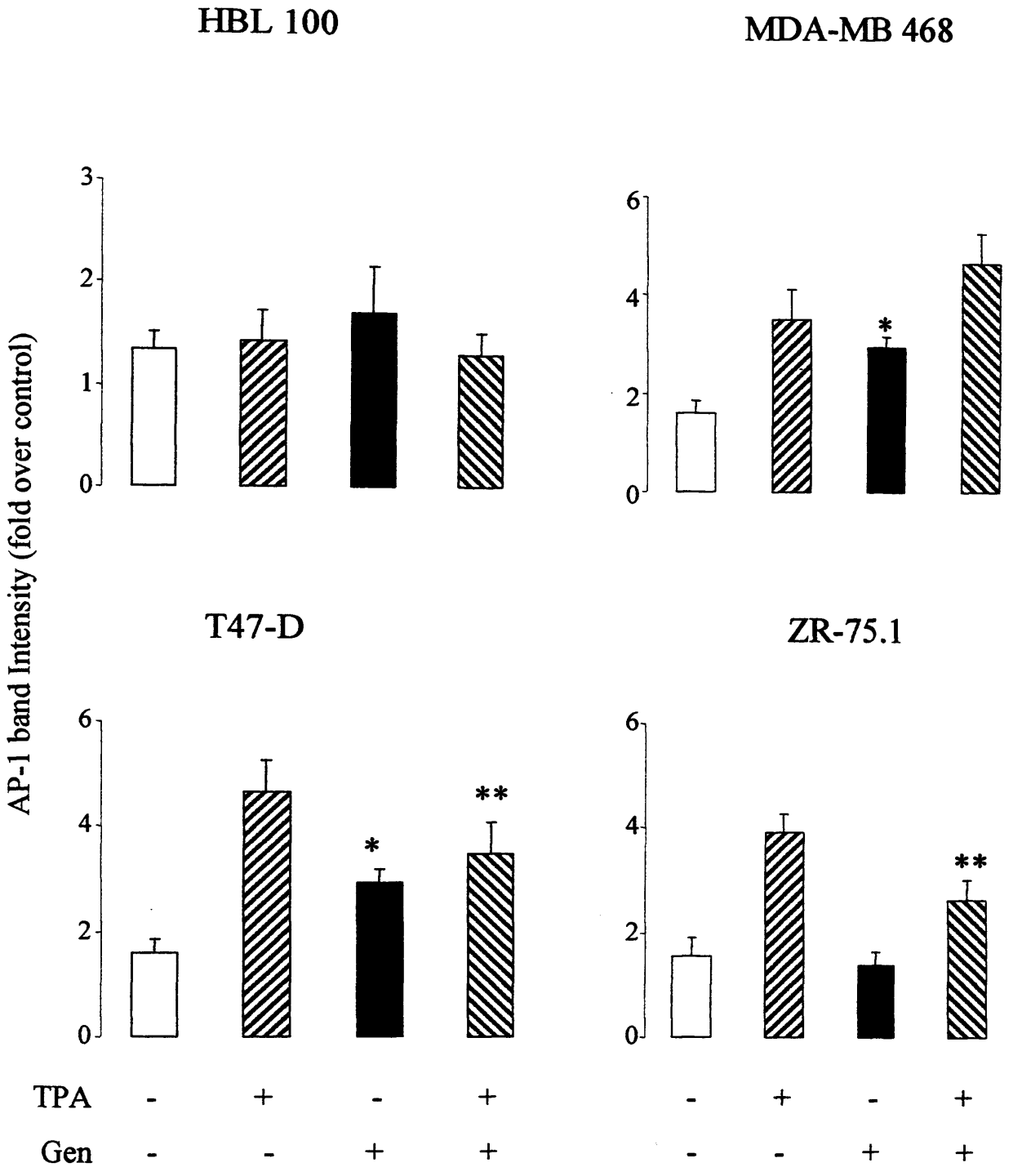


Fig. 4.7. Effect of genistein (50 μM) on TPA-induced AP-1 DNA-binding activity by gel shift assay in, HBL 100, MDA MB 468, T47-D and ZR-75.1 cells.

Results, which are presented as fold changes established by laser densitometry compared to control incubates, are the mean±SEM of 3 independent experiments. One and two asterisks indicate that values are significantly different from untreated and TPA only incubates, respectively ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of gel shift assay and culture conditions see section 2.9.

When T47-D and ZR-75.1 cells were treated with genistein in conjunction with TPA, significant inhibition was observed by 25% and 33% respectively.

4.4. The effect of genistein on levels of c-Fos and c-Jun protein

In order to examine further the mechanisms by which genistein regulates AP-1 transactivation, its effect on levels of c-Fos and c-Jun protein, major constituents of the AP-1 complex, was studied using western blot analysis. The effect of genistein on c-Fos and c-Jun were detected by pre-treating cells with genistein (50μM) for 30 minutes, followed by treatment with either TPA (0.2μM) (c-Fos) or anisomycin (100nM) (c-Jun), for a further 2 hours.

TPA stimulated c-Fos levels (compared to control) by varying extents, ranging from 8-fold to 55-fold in HBL 100 and T47-D cells, respectively (figure.4.8). Genistein inhibited TPA-induced c-Fos levels in MDA-MB 468 and T47-D cells by 77 % and 73 %, respectively, when compared to TPA alone, but was devoid of any effect on induced c-Fos levels in HBL 100, MCF-7, ZR-75.1 and MDA-MB 231 cells. In addition, genistein did not upregulate TPA-induced c-Fos protein levels in any cell line.

In contrast to the results gained for c-Fos, genistein when incubated together with anisomycin, stimulated levels of c-Jun protein by 1.5 and 2.5-fold in MCF-7 and ZR-75.1 cells, respectively, when compared to anisomycin alone (figure 4.9). Genistein did not affect levels of c-Jun in HBL 100, MDA-MB 468, T47-D and MDA-MB 231 cells. Further experiments were performed to see if genistein could upregulate levels of c-Jun protein in MCF-7 and ZR-75.1 cells when administered alone. Cells were treated with genistein at either 50 or 10μM, both alone and in conjunction with anisomycin, as described in section 4.4. Genistein (10 and 50μM), in the absence of anisomycin, had no effect on c-Jun levels in either cell lines (figure 4.10) as levels were not decreased to below control, taken as a value of 1.

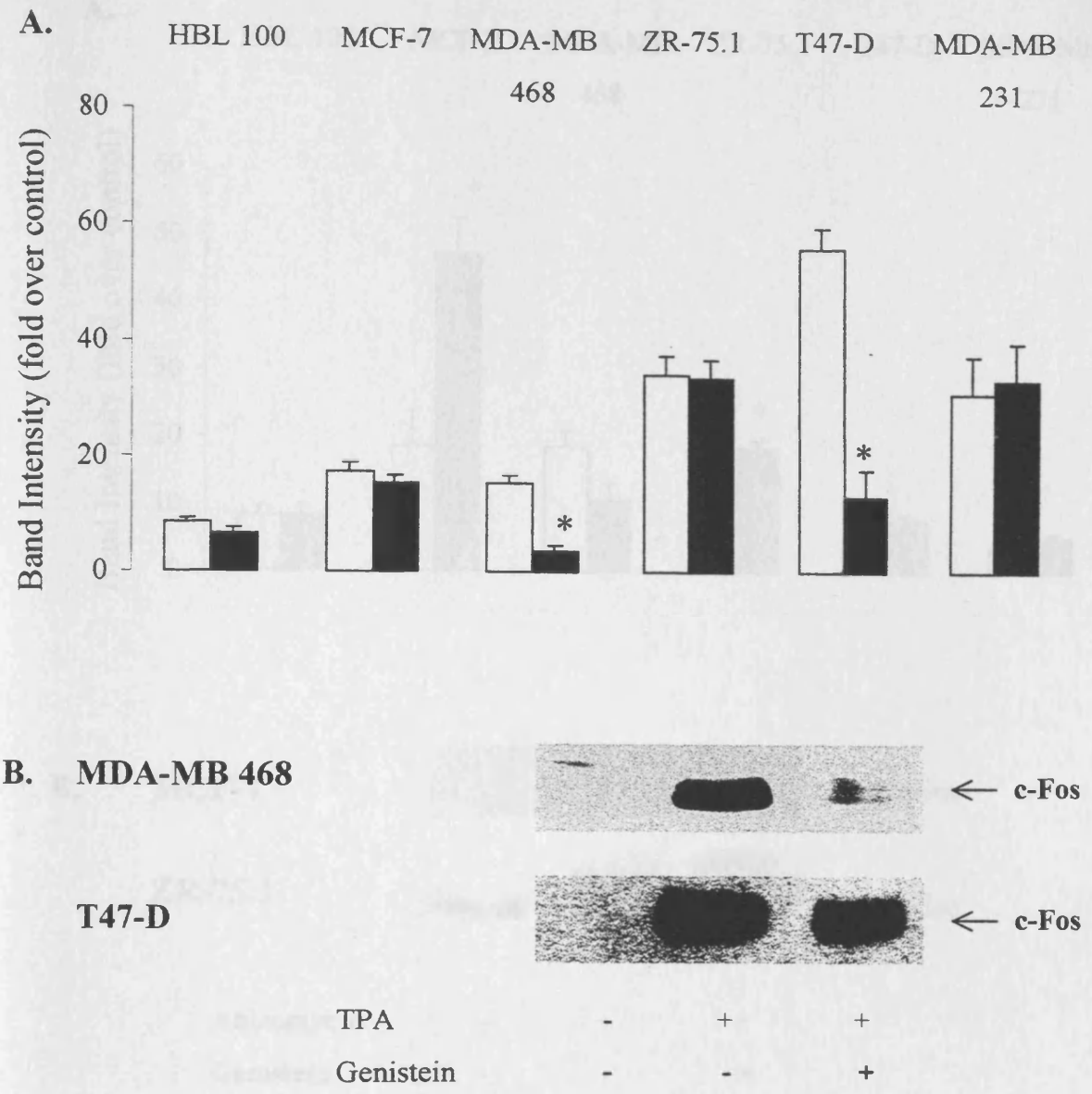


Fig. 4.8. Effect of genistein (50 μM) on TPA-induced c-Fos levels in (A): HBL 100, MCF-7, MDA-MB 468, ZR-75.1, T47-D and MDA MB 231 as reflected by densitometric analysis of western blots and (B) MDA-MB 468 and T47-D cells as shown by representative western blots. Open bars represent protein levels after TPA treatment (0.2μM), closed bars after treatment with TPA and genistein. Results, which are presented as fold changes established by laser densitometry compared to incubates in which TPA was omitted, are the mean±SEM of 3 independent experiments. Asterisks indicate that values are significantly different from incubates with TPA only ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of Fos western blot analysis see section 2.11.

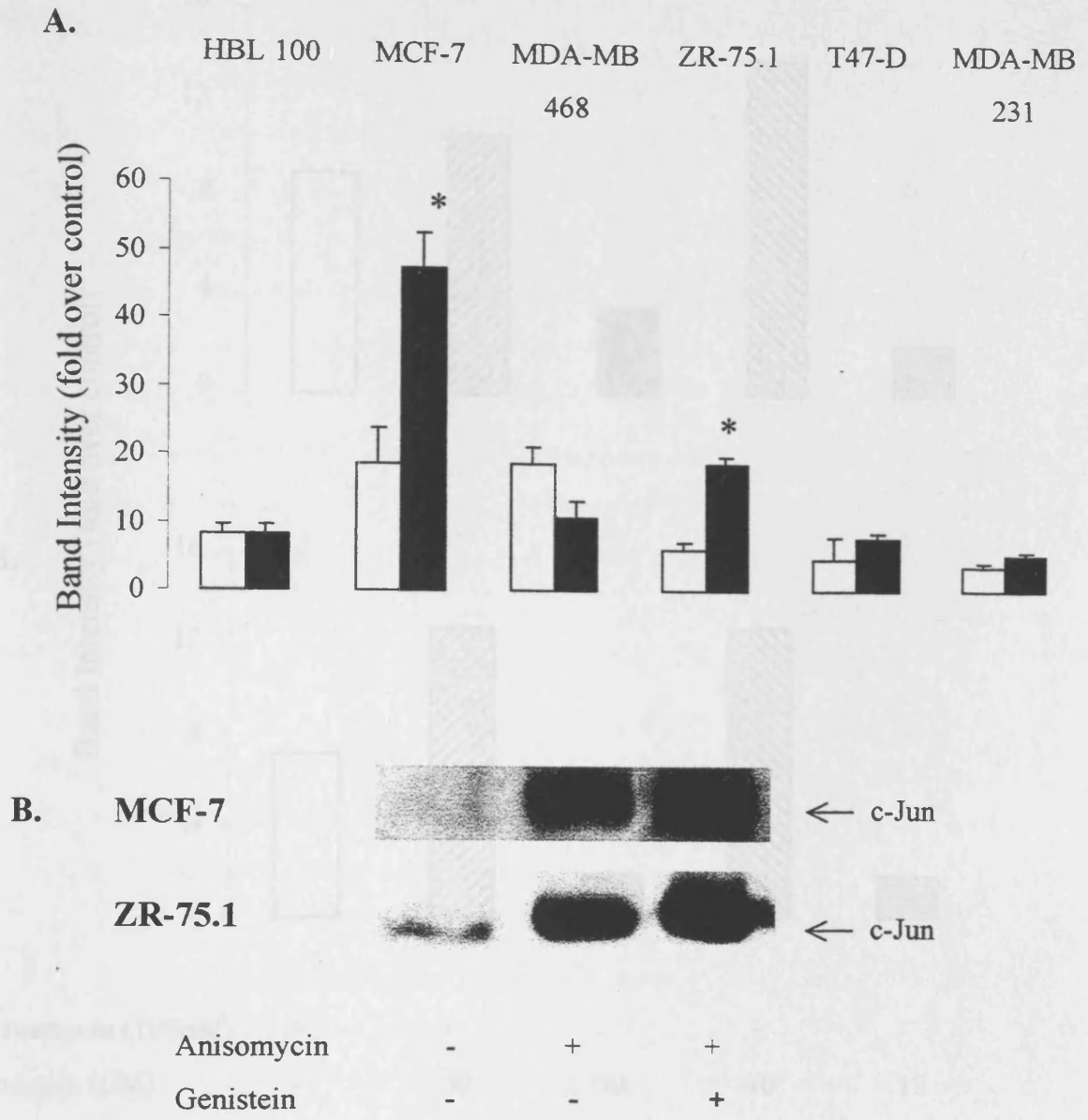


Fig. 4.9. Effect of genistein (50 μM) on anisomycin-induced c-Jun levels in (A): HBL 100, MCF-7, MDA-MB 468, ZR-75.1, T47-D and MDA MB 231 as reflected by densitometric analysis of western blots and (B) MCF-7 and ZR-75.1 cells as shown by representative western blots. Open bars represent protein levels after anisomycin treatment (100nM), closed bars after treatment with anisomycin and genistein. Results in A are presented as fold changes in band density established by laser densitometry compared to control incubates and are the mean±SEM of 3 independent experiments. Asterisks indicate that values are significantly different from incubates with anisomycin only ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of Jun western blot analysis see section 2.11.

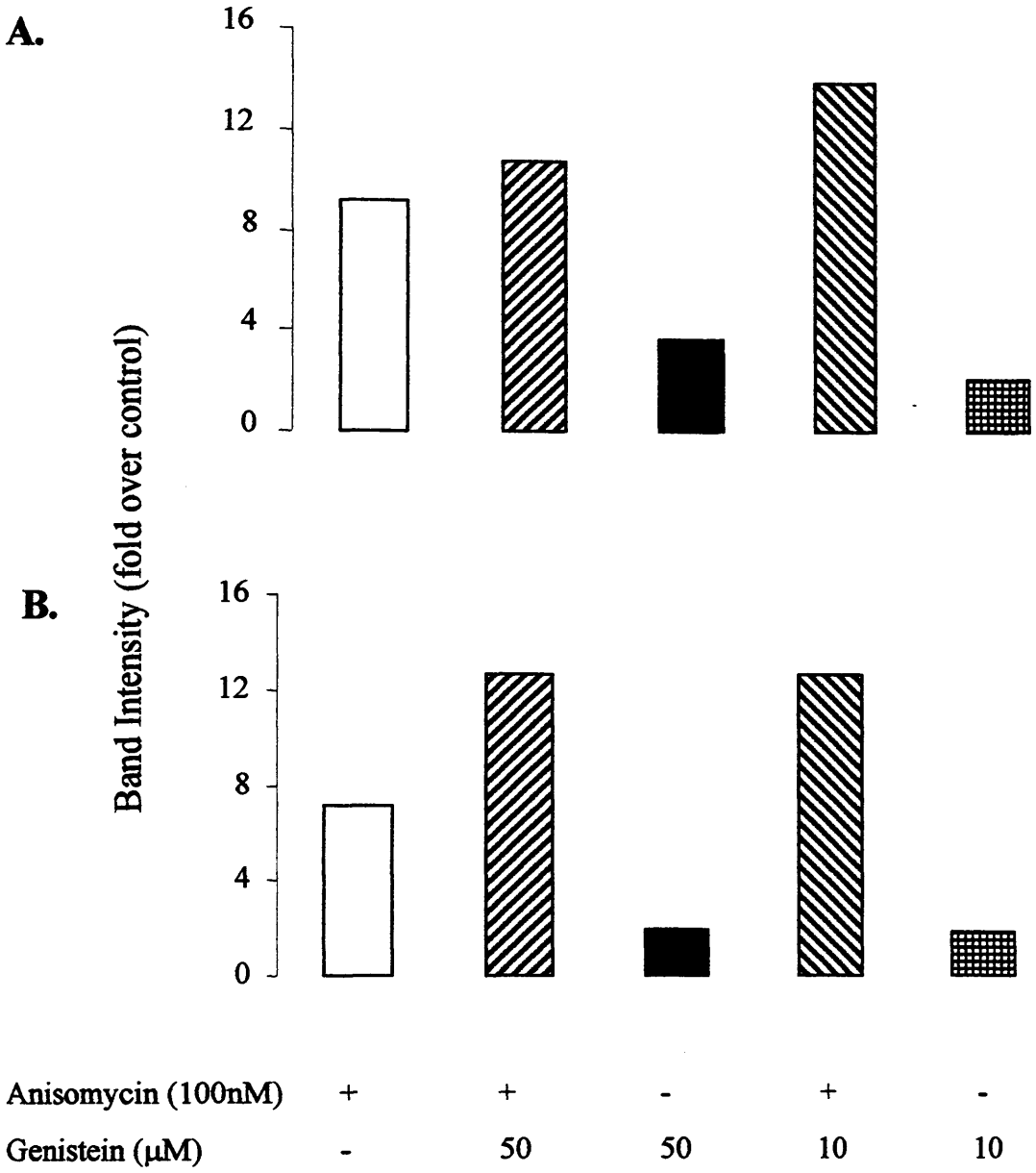


Fig. 4.10. Effect of genistein (50 μM) on c-Jun protein levels in (A) MCF-7 and (B) ZR-75.1 cells reflected by densitometric analysis of western blots.

Results, which are presented as fold changes in band density established by laser densitometry compared to control incubates, are the mean of 2 independent experiments. For details of Jun western blot analysis see section 2.11.

The possibility of a synergistic-type effect between genistein and anisomycin in their ability to stimulate c-Jun protein levels in MCF-7 and ZR-75.1 cell lines as observed in figure 4.10 was examined further. Investigations were initiated to assess whether this effect could be replicated when cells were treated with genistein combined with a different stimulus of the stress activated MAPK pathway, or whether this effect was limited to anisomycin. In the following experiments, expression of c-Jun was stimulated by incubation of cells with sorbitol (300μM) for 30 minutes following pre-treatment with genistein (50μM) for 30 minutes.

Results in figure 4.11 show sorbitol did not appear to upregulate expression of c-Jun, even when repeated over a different time course up to 5 hours (results not shown). This made it difficult to observe any synergistic-like effect between genistein and sorbitol in ZR-75.1 cells, although a four-fold increase in c-Jun protein was observed in MCF-7 cells, after treatment with sorbitol together with genistein.

Anisomycin proved to be a better stimulus to detect expression of c-Jun when compared to sorbitol. As sorbitol did not stimulate c-Jun in MCF-7 cells, further experiments were conducted using anisomycin.

4.5. The effect of genistein on c-Jun phosphorylation

Experiments were extended to investigate the effect of genistein and/or anisomycin on c-Jun phosphorylation in both MCF-7 and ZR-75.1 cells. The experiments were designed to test the hypothesis that an increase in levels of c-Jun protein after treatment with genistein and anisomycin was caused by an upregulation of JNK activity, which phosphorylates c-Jun. MCF-7 and ZR-75.1 cells were incubated with genistein (50μM) for 30 minutes followed by treatment with anisomycin for a further 30 minutes and the samples were assayed for JNK activity.

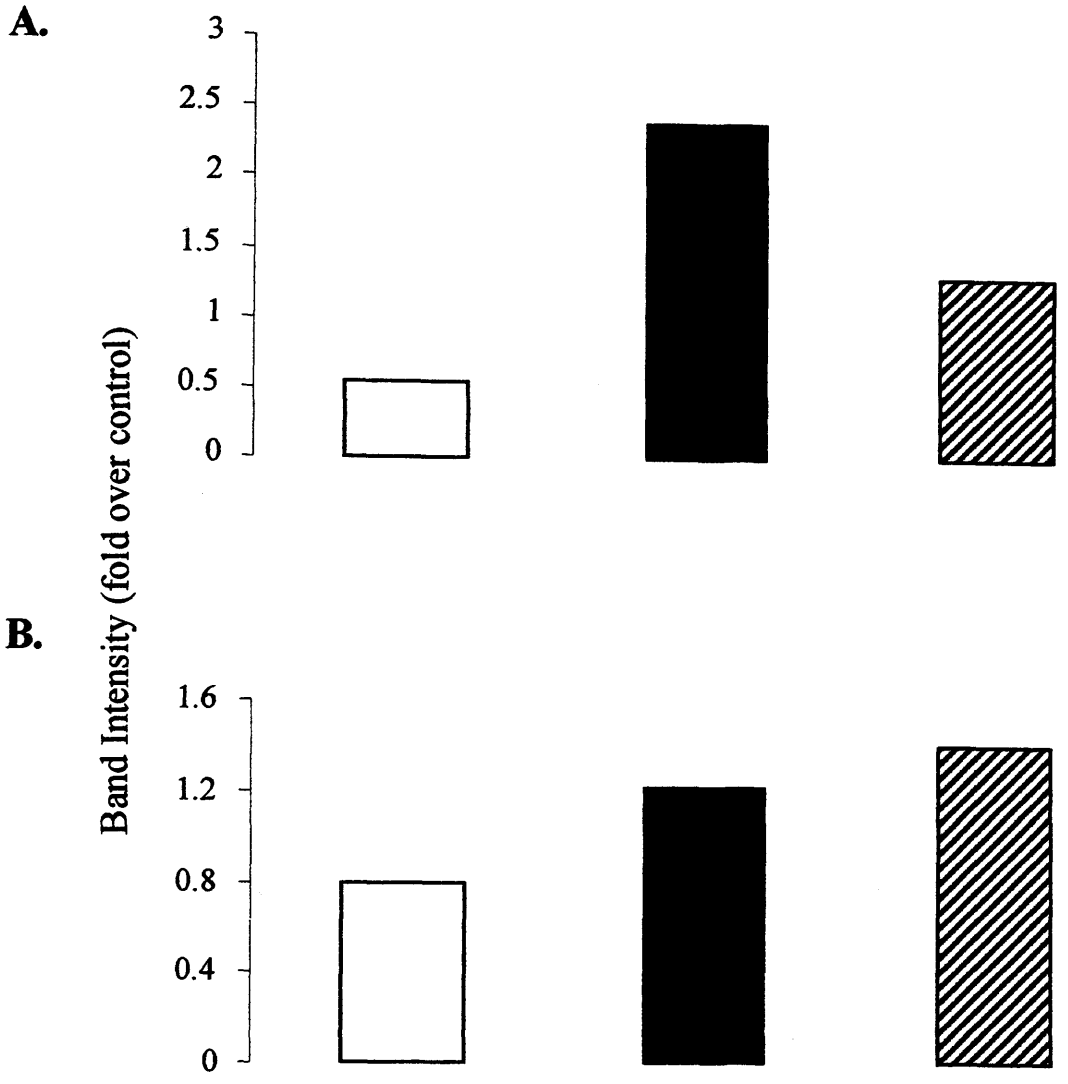


Fig. 4.11. Effect of genistein (50 μM) on sorbitol-induced c-Jun protein levels in (A) MCF-7 and (B) ZR-75.1 cells reflected by densitometric analysis of western blots.

Open bars represent protein levels after sorbitol treatment (300μM), closed bars treatment with sorbitol and genistein and hatched bars treatment with genistein alone. Results, which are presented as fold changes in band density established by laser densitometry compared to control incubates, are the mean of 2 independent experiments. For details of Jun western blot analysis see section 2.11.

In MCF-7 cells, a three-fold increase in c-Jun phosphorylation (JNK activity) was observed after treatment with anisomycin (figure 4.12). When cells were treated with genistein in conjunction with anisomycin, levels of c-Jun phosphorylation were increased by 43% over anisomycin alone, however, genistein alone had no effect. In ZR-75.1 cells, a three-fold increase in c-Jun phosphorylation was observed after treatment with anisomycin (figure 4.13), and similarly to MCF-7 cells, genistein on its own had no effect.

4.6. The effect of genistein on the MAPK signalling cascade

Changes in AP-1 transcription factor activity may also be mediated by alterations in cell signalling cascades, by directly affecting the activity of MAPK, for example ERKs that directly affect c-Fos, or JNKs that affect levels of c-Jun.

The growth-inhibitory effects of genistein were examined further with respect to the ERKs, as induction of AP-1 activity and c-Fos protein expression were both inhibited by genistein in T47-D and MDA-MB 468 cells. In order to investigate the effect on ERKs, cells were treated with genistein (50μM) for 30 minutes followed by TPA (0.2μM) for 15 minutes (as pre-determined by a time course between 5 and 60 minutes; results not shown) and the effect of genistein on TPA-induced ERK 1 and 2 phosphorylation was studied using Western blot analysis.

4.6.1. The effect of genistein on ERK 1 and 2 phosphorylation

TPA induced ERK 1 and 2 phosphorylation in MCF-7, ZR 75.1 and T47-D cells, but not in HBL 100, MDA-MB 468 and MDA-MB 231 cells (figure 4.14). Genistein significantly inhibited TPA-induced ERK phosphorylation in ZR-75.1 and T47-D cells, by 70 % and 47 %, respectively. Genistein failed to affect ERK phosphorylation in HBL 100, MCF-7, MDA-MB 468 and MDA-MB 231 cells.

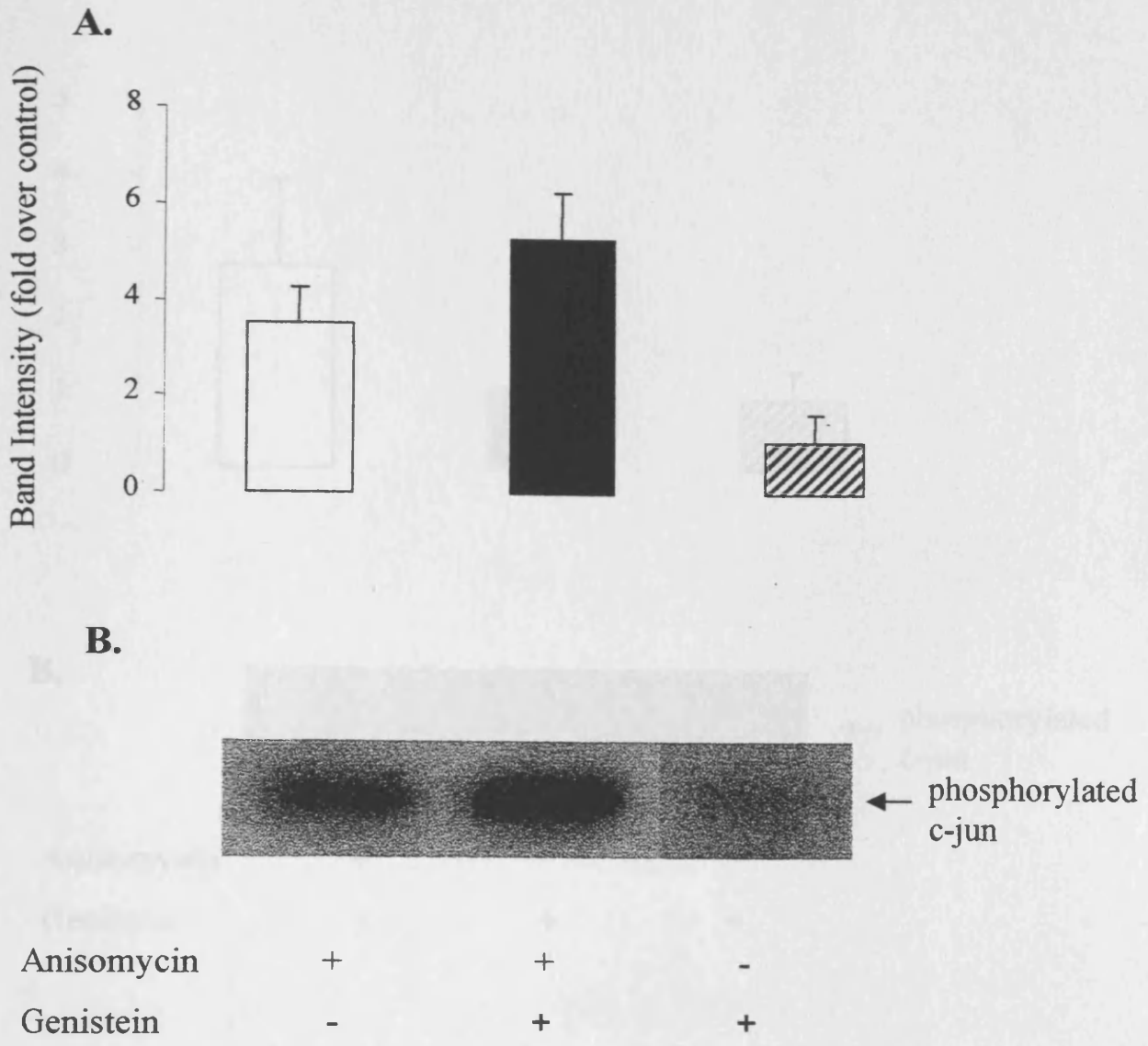


Fig. 4.12. Effect of genistein (50 μM) on anisomycin-induced c-Jun phosphorylation in MCF-7 cells reflected by (A) densitometric analysis of autoradiography blots and (B) representative autoradiography.

Open bar represents phosphorylation after anisomycin treatment (100nM), closed bar treatment with anisomycin and genistein and hatched bar treatment with genistein alone. Results in A are presented as fold changes in band density established by laser densitometry compared to control incubates. For details of JNK kinase assay see section 2.12.

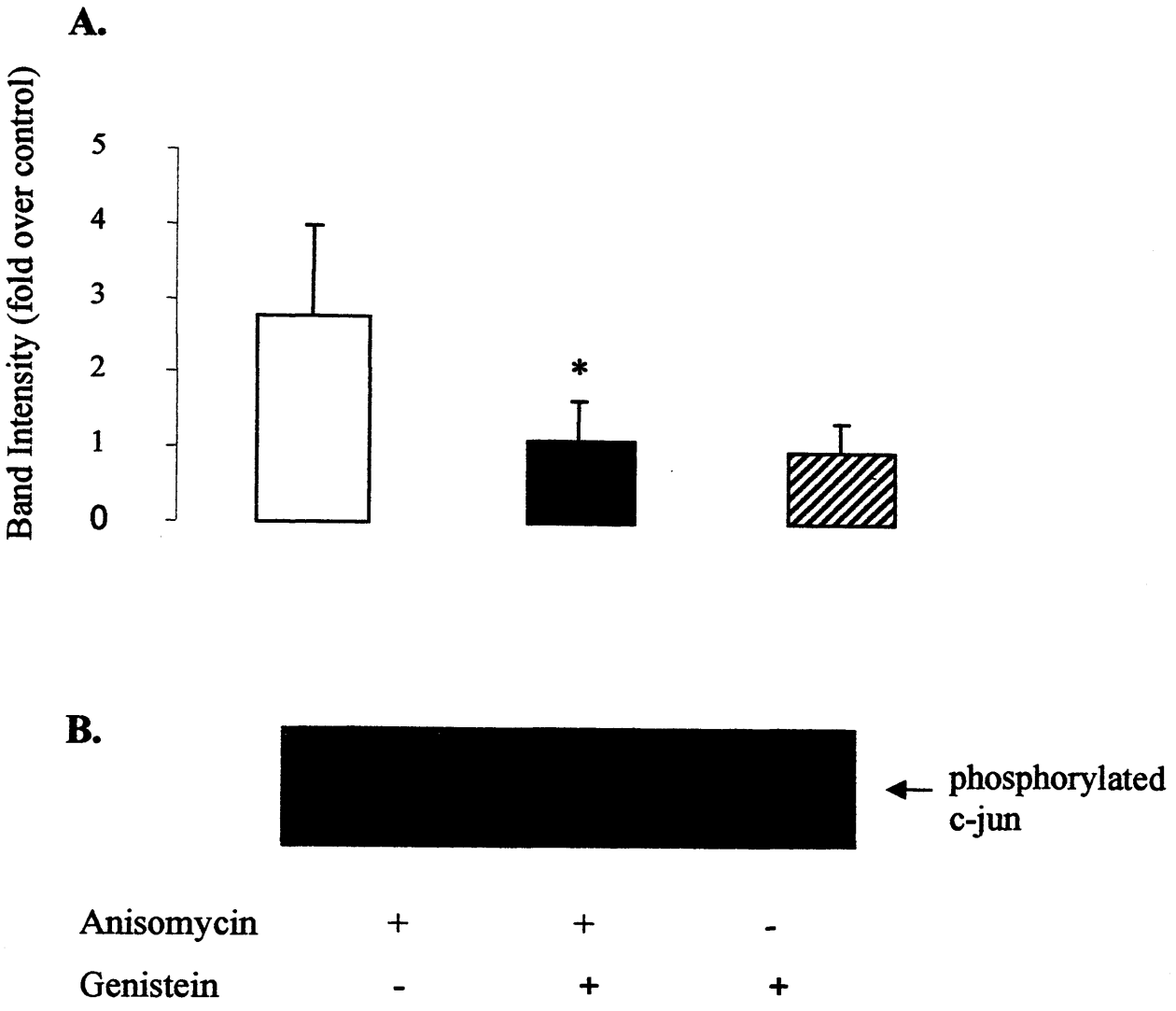


Fig. 4.13. Effect of genistein (50 μM) on anisomycin-induced c-Jun phosphorylation in ZR-75 cells reflected by (A) densitometric analysis of autoradiography blots and (B) representative autoradiography.

Open bar represents phosphorylation after anisomycin treatment (100nM), closed bar treatment with anisomycin and genistein and hatched bar genistein alone. Results in A are presented as fold changes in band density established by laser densitometry compared to control incubates. Asterisks indicate that values are significantly different from incubates with anisomycin only ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of JNK kinase assay see section 2.12.

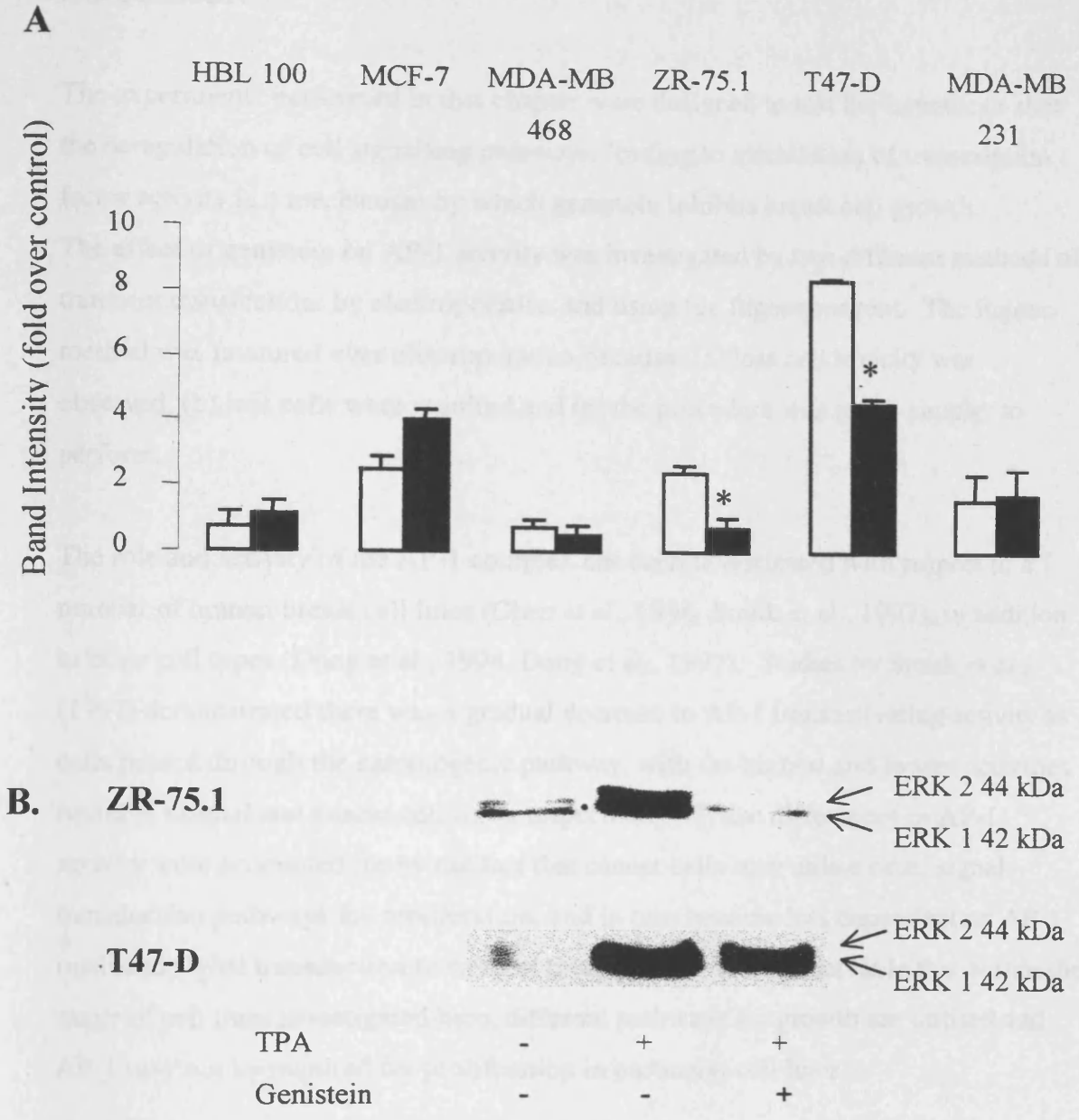


Fig. 4.14. Effect of genistein (50 μM) on TPA-induced ERK phosphorylation in (A): HBL 100, MCF-7, MDA-MB 468, ZR-75.1, T47-D and MDA MB 231 as reflected by densitometric analysis of western blots and (B) ZR-75.1 and T47-D cells as shown by representative western blots.

Open bars represent phosphorylation after TPA treatment (0.2μM), closed bars phosphorylation after treatment with TPA and genistein. Results, which are presented as fold changes established by laser densitometry compared to incubates in which TPA was omitted, are the mean±SEM of 3 independent experiments. Genistein on its own did not affect ERK phosphorylation. Asterisks indicate that values are significantly different from incubates with TPA only ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of ERK western blot analysis see section 2.10.

DISCUSSION

The experiments performed in this chapter were designed to test the hypothesis that the deregulation of cell signalling pathways, leading to modulation of transcription factor activity is a mechanism by which genistein inhibits breast cell growth. The effect of genistein on AP-1 activity was investigated by two different methods of transient transfection: by electroporation and using the fugene reagent. The fugene method was favoured over electroporation because: (a) less cell toxicity was observed, (b) less cells were required and (c) the procedure was much simpler to perform.

The role and activity of the AP-1 complex has been investigated with respect to a number of human breast cell lines (Chen et al., 1996, Smith et al., 1997), in addition to other cell types (Dong et al., 1994, Dong et al., 1997). Studies by Smith *et al.*, (1997) demonstrated there was a gradual decrease in AP-1 transactivating activity as cells passed through the carcinogenic pathway, with the highest and lowest activities found in normal and cancer cell lines, respectively. These differences in AP-1 activity were accounted for by the fact that cancer cells may utilise other signal transduction pathways for proliferation, and in turn become less dependent on AP-1 mediated signal transduction to support their growth. It is conceivable that within the range of cell lines investigated here, different pathways for growth are utilised and AP-1 may not be required for proliferation in particular cell lines.

There are a number of possible explanations regarding how genistein may inhibit AP-1 activity in breast cells. Firstly, it may interfere with DNA binding, secondly, it may exert its effect via inhibiting components of the AP-1 complex and thirdly, it may interfere with the MAPK cell signalling cascade and inhibit the activity of specific components.

Investigation into the effect of genistein on levels of AP-1 DNA-binding activity was performed by gel shift assay. Studies by Smith *et al.*, (1997) observed high and low

basal AP-1 DNA-binding activity in normal and immortal HME cells and breast cancer cells lines, respectively. It is difficult to compare basal levels of AP-1 DNA binding activity between the cell lines as the experiments were not performed simultaneously. Genistein, (50μM), when added alone appeared to induce levels of AP-1 DNA binding activity in T47-D and MDA-MB 468 cells. When genistein was added in conjunction with TPA, AP-1 DNA-binding was significantly inhibited in T47-D and ZR-75.1 cells.

In these experiments, ZR-75.1 cells exhibited high transactivating, but low DNA-binding activity. A study by Chen *et al.* (1996), observed a lack of correlation between levels of AP-1 binding and transactivation in a number of breast cancer cells. However, in their study, levels of AP-1 transactivation and binding correlated well in ZR-75.1 cells. This discrepancy may be due to differences between cells, such as passage number, or the use of different methods to examine AP-1 activity.

Therefore, although it can be suggested that genistein may exert its inhibitory effect on AP-1 activity by interfering with the DNA-binding activity in T47-D and MDA-MB 468 cells, the inhibitory effect on DNA binding in ZR-75.1 cells is more difficult to interpret.

The inhibitory effect on AP-1 activity by genistein may also occur via modulation of components of the AP-1 protein complex. Deregulation of cell growth may occur by the inability of AP-1 proteins to form a stable complex due to the overexpression of target proteins leading to apoptosis, or by interfering with transcription factors implicated in the progression of the cell cycle (Angel and Karin, 1991). Induction of c-Fos expression by agents such as growth factors and phorbol esters have been shown to be associated with cell growth and proliferation (Foletta, 1996), and is characterised by a rapid accumulation of c-fos mRNA. At 50μM, genistein inhibited levels of c-Fos protein in T47-D and MDA-MB 468 cells, consistent with the work of Schultze-Mosgau *et al.* (1998) who observed inhibition of c-fos mRNA by genistein at 50μM in MDA-MB 468 cells. The inhibition of c-Fos also correlates with

inhibition (albeit not significant) of AP-1 activity seen in T47-D and MDA-MB 468 cells. It is therefore possible that these two breast cell lines may utilise the MAPK pathway to regulate their growth in a similar fashion, with genistein modulating proliferation at the transcription factor level.

The other major component of the AP-1 transcription factor, c-Jun, is known to be regulated by phosphorylation by JNK on Ser-63/73 (Wisdom et al., 1999). Mitogen-induced AP-1 activity can be enhanced via c-Jun phosphorylation, and this phosphorylation in the c-Jun activation domain can lead to the transactivation of c-Jun through an autoregulatory loop (Angel and Karin, 1991).

The results obtained in this study show an upregulation of c-Jun protein levels in both MCF-7 and ZR-75.1 cells after treatment with genistein and anisomycin, with no effect seen in the remaining four cell lines. This result is compatible with the observation that genistein appeared to increase, rather than decrease TPA-induced AP-1 activity in ZR-75.1 cells, however, no correlation was seen in MCF-7 cells.

An explanation for the increase in c-Jun protein levels in MCF-7 and ZR-75.1 cells may be connected with the extent of c-Jun phosphorylation. Phosphorylation of c-Jun can either lead to an increase in AP-1 activity via an autoregulatory loop (Angel and Karin, 1991), or it may lead to an accumulation of c-Jun protein. Hyper-phosphorylated c-jun may not be able to participate in the formation of a competent AP-1 transcription factor (Davies, 1999). It is also possible that genistein may interfere with degradation of c-Jun protein, which is in turn, associated with increased phosphorylation by JNK (Davies, 1999). If genistein interferes with phosphorylation, and therefore c-Jun degradation, it may result in an accumulation of protein, due to the increased half-life of the protein.

Further experiments were performed, to test the hypothesis that treatment of MCF-7 and ZR-75.1 cells with genistein and anisomycin leads to an increase in c-Jun phosphorylation. Interestingly, genistein alone appeared to inhibit, rather than

induce, phosphorylation of c-Jun in both cell lines, but when MCF-7 cells were treated with both genistein and anisomycin, c-Jun phosphorylation increased. Therefore, it can be suggested that in MCF-7 cells, the increase in c-jun phosphorylation by genistein results in an increase in c-Jun protein levels, with no consequences on AP-1 activity. In ZR-75.1 cells, the inhibition of phosphorylation by genistein results in an accumulation of protein which coincides with an increase in protein and AP-1 activity. These results show functional consequences of the contrasting changes elicited by genistein with respect to c-Jun protein on the one hand and JNK activity on the other are difficult to predict.

Genistein has been reported to stimulate levels of JNK in human epidermal carcinoma A431 cells (Croisy-Delcey et al., 1997). If this stimulatory effect also occurs in breast cancer cell lines, it may be a mechanism by which c-Jun and thus AP-1 activity is upregulated, for example, in ZR-75.1 cells. Unfortunately, due to the time scale of this project the effect of genistein on JNK in breast cell lines was not examined, however this area of work remains a very important area of investigation and may provide more information on the effect of genistein at the levels of c-Jun.

The induction of c-*fos* and c-*jun* genes may be an early key event in cell signalling cascades leading to apoptosis (Satomi et al., 1999), however, under some circumstances in cultured fibroblasts and hepatoblasts, c-Jun can promote proliferation instead. c-Jun has been implicated in the progression of the cell cycle and experiments performed in fibroblasts showed that c-Jun is required for progression through G1, due in part to its ability to directly regulate cyclin D1 expression (Wisdom et al., 1999). It is important to note, however, that the effect of c-Jun on cellular responses strongly depends on the cell type and the milieu of other regulatory signals the cell is receiving (Leppa and Bohmann, 1999). It is conceivable that the upregulation of c-Jun elicited by genistein in MCF-7 and ZR-75.1 cells may contribute to increased levels of apoptosis, and is a mechanism by which genistein decreases cell numbers and inhibits growth.

MAP kinases are mediators of signal transduction from the cell surface to the nucleus and the transcription factor AP-1 has been identified as a target of MAP kinase cell signalling pathways (Karin, 1995). MAP kinase signalling pathways influence AP-1 activity by both increasing the abundance of AP-1 components and by stimulating their activity directly, and further experiments were performed to observe the effect of genistein on the activity of ERKs.

In T47-D cells, the decrease of ERK phosphorylation (figure.4.14) was compatible with the attenuation of both c-Fos protein levels (figure 4.8) and AP-1 activity (figure 4.4). These findings suggest that in these cells genistein may inhibit growth by interfering with MAPK signalling, which in turn abrogates AP-1 transcription *via* attenuation of c-Fos expression. In MDA-MB 468 cells, the observed reduction of c-Fos protein expression by genistein (figure 4.8), is compatible with the effect of genistein on c-fos transcription reported previously (Schultze-Mosgau et al., 1998), but there appears to be no effect on ERK phosphorylation.

The effects of genistein on MAPK cell signalling pathways which influence the activity of the AP-1 transcription factor, are important when the mechanisms by which genistein inhibits growth and proliferation is elucidated. Genistein appears to differentially modulate cell signalling pathways between cell lines, with pathways in the HBL 100 and MDA-MB 231 cells relatively unaffected by genistein, in that there was no inhibition of AP-1 activity or DNA binding, no effect on AP-1 components and no effect on ERK phosphorylation. Therefore, genistein does not appear to mediate growth-inhibition by interference with cell signalling pathways in these cell lines and a different mechanism must be involved.

In T47-D cells, genistein exerts its growth inhibitory effects by downregulating levels of ERK phosphorylation, resulting in an decrease in levels of c-Fos protein, which in turn leads to an decrease in AP-1 DNA binding and AP-1 activity. This pattern of results is similar for MDA-MB 468 cells, although no inhibition of ERK

phosphorylation was observed. Therefore, in these two cell lines it is clear that modulation of MAPK by genistein contributes to its growth inhibitory actions.

In ZR-75.1 cells, treatment with genistein resulted in an increase in AP-1 activity; it inhibited both c-Jun phosphorylation and protein levels and also inhibited ERK phosphorylation. These results are difficult to interpret, but it can be suggested that although genistein modulates components of signal transduction pathways to some extent, this is not the major mechanism by which it inhibits growth of ZR-75.1 cells. Cell signalling in MCF-7 cells was somewhat affected by genistein, in that an upregulation of levels of c-jun phosphorylation and protein was observed, however, this effect was not translated into an effect on AP-1 activity, so again, genistein must mediate its inhibitory effects on cell growth via a different mechanism.

To conclude, the effects elicited by genistein on cell signalling pathways resulting in modulation of AP-1 activity are inconsistent between the cell types, and the mechanisms by which genistein inhibits cell growth differ from cell to cell.

CHAPTER 5: THE EFFECT OF GENISTEIN ON THE CELL CYCLE AND APOPTOSIS.

INTRODUCTION

Because of the fundamental importance of functional cell cycle machinery in cell proliferation and differentiation, proteins that regulate the cell cycle are potential targets for tumourigenesis (Landberg and Roos, 1997). Such proteins may also be targets for therapeutic agents used to inhibit the growth of cancer cells. The anti-cancer effects of several drugs are mediated by cell cycle arrest and involve modulation of the action of cyclins and CDKs, which regulate cell cycle progression (Balabhadrapathruni et al., 2000). Genistein has been reported to induce cell cycle arrest at both G1/S (Kuzumaki et al., 1998) and G2/M phase transitions (Matsukawa and Aoike 1993, Pagliacci et al., 1994, Choi et al., 1998, Davis et al., 1998, Salti et al., 2000). The molecular basis for cell cycle arrest by genistein has not been determined fully, however, both inhibition of cyclin B1 protein and induction of the CDKI p21 have been implicated as possible mechanisms by which deregulation occurs (Choi et al., 1998).

Apoptosis is an important path through which chemopreventive agents can inhibit the growth of cancer cells. Some of the molecular mechanisms implicated in apoptosis have been elucidated and the induction of apoptosis is known to be partly mediated by several genes, such as p53, Bax, Bcl-2 and $p21^{WAF1/CIP1}$ (Li et al., 1999a).

Genistein has been reported to induce apoptosis by upregulation of p21 in MCF-7 and MDA-MB 231 cells (Pagliacci et al., 1994, Choi et al., 1998), in H460 cells (Lian et al., 1999) and also in mouse fibroblast cells (Kuzumaki et al., 1998). Activation of $p21^{WAF1/CIP1}$ can occur through p53 dependent or independent pathways, and elevated levels of p21 have been associated with a

decrease in CDK activity in damaged cells destined for apoptosis (El-Deiry et al., 1993).

Results presented in chapters 3 and 4 have provided evidence that the growth-inhibitory effect of genistein on six different breast cell lines does not occur by identical mechanisms. In addition to interfering with cell signalling pathways and modulation of transcription factors, genistein may also mediate its growth-inhibitory effect through deregulation of the cell cycle.

The experiments described in this chapter were designed to investigate the effect of genistein on the cell cycle and apoptosis. The hypothesis tested was that genistein arrests cell cycle progression and induces apoptosis similarly in all cell lines.

RESULTS

5.1. The effect of genistein on cell cycle regulation

5.1.1. Analysis of the cell cycle by flow-cytometry

Experiments were performed to test the hypothesis that the concentration of genistein required to inhibit cell growth (10μM), would induce cell cycle arrest in all cell lines to a similar extent. The effect of genistein (1 and 10μM) on the cell cycle after 96 hours was examined using a flow-cytometer, based on a method by Ormerod, (1993). Genistein blocked cell cycle progression in the G2/M phase of the cell cycle in HBL 100, MCF-7, T47-D, MDA-MB 468 and MDA-MB 231 cells grown under conditions of reduced serum (table 5.1 and figure 5.1). ZR- 75.1 cells proved to be insensitive to cell cycle arrest by genistein, however a slight increase in cell numbers was seen in the G1 phase.

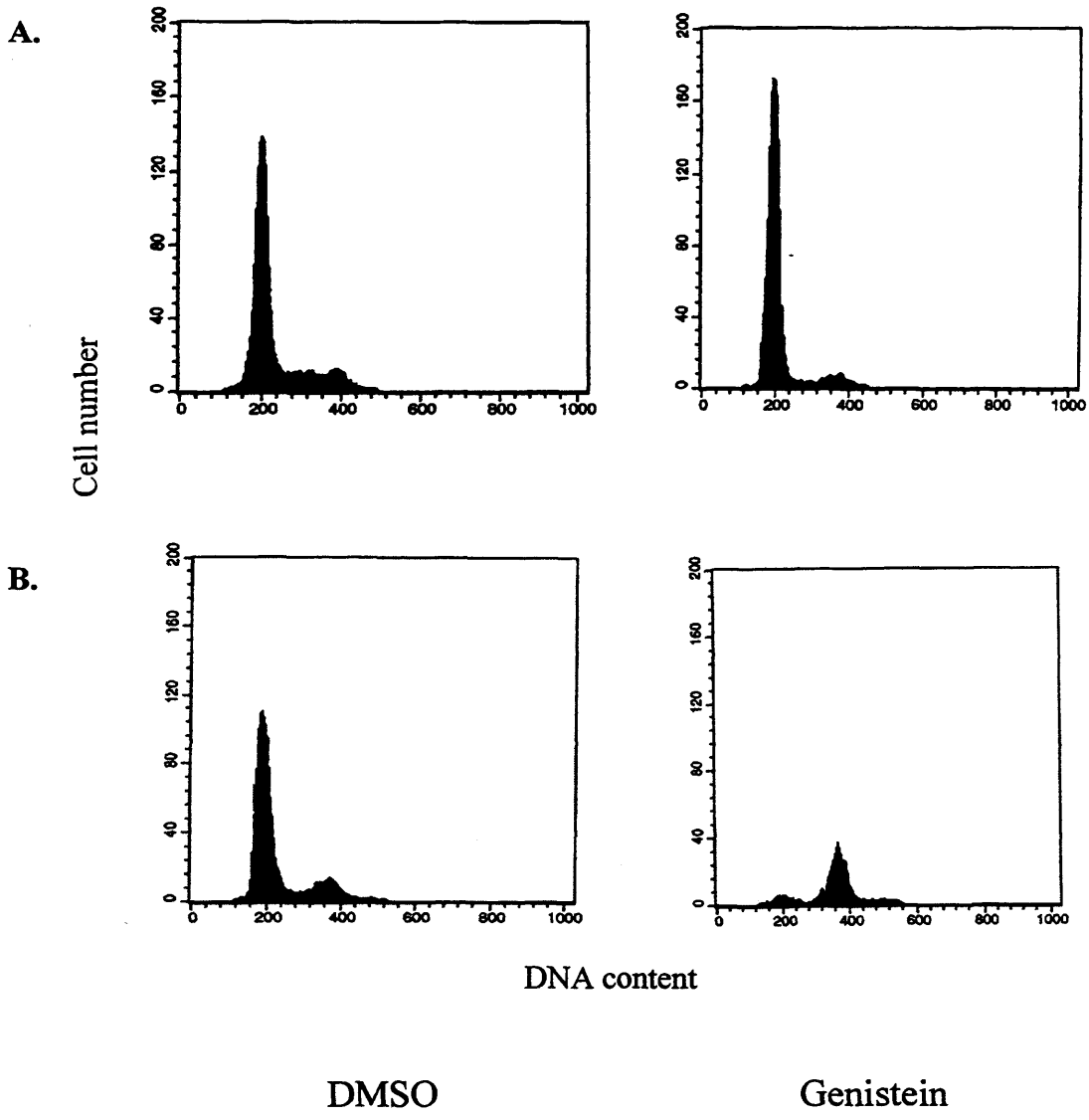


Fig. 5.1. Effect of genistein (10μM) on the cell cycle in (A) ZR-75.1 and (B) MDA-MB 468 cells by DNA profile.

DNA profiles of cell cycle distribution are presented in cells cultured in medium supplemented with 2% FCS, after treatment with either DMSO (control) or genistein (10μM) for 96 hours. For details of cell cycle analysis see section 2.13.

Table 5.1. Effect of genistein (1 and 10 μM) for 96 hours on cell cycle distribution.

Cell type	[Genistein]	Cell cycle distribution[1]		
		G1	S	G2/M
HBL 100	0[2]	61.8 ± 3.1	13.8 ± 3.6	20.2 ± 3.1
	1	61.6 ± 3.1	13.4 ± 2.5	22.1 ± 3.1
	10	34.4 ± 16.5*	13.7 ± 2.1	45.5 ± 13.8*
MCF-7	0	78.1 ± 7.7	9.6 ± 2.9	8.5 ± 3.1
	1	81.8 ± 4.9	7.8 ± 3.7	8.5 ± 3.5
	10	79.2 ± 2.4	9.2 ± 2.4	22.0 ± 4.2*
MDA-MB 468	0	72.0 ± 6.0	11.1 ± 2.8	13.7 ± 6.1
	1	69.8 ± 7.4	13.5 ± 3.6	13.1 ± 5.7
	10	62.2 ± 13.0	11.2 ± 2.8	43.3 ± 18.6*
ZR-75.1	0	77.9 ± 8.8	8.8 ± 4.9	9.9 ± 2.6
	1	77.6 ± 8.8	9.5 ± 6.5	9.7 ± 4.3
	10	86.2 ± 5.3	4.8 ± 2.4	7.7 ± 2.3
T47-D	0	76.7 ± 2.5	8.8 ± 1.4	11.5 ± 4.2
	1	76.7 ± 3.1	8.1 ± 3.4	10.4 ± 2.1
	10	65.8 ± 10.5*	7.3 ± 1.4	20.9 ± 8.3*
MDA-MB 231	0	74.6 ± 2.9	6.3 ± 2.3	17.3 ± 1.7
	1	76.7 ± 2.6	6.0 ± 1.9	15.4 ± 2.1
	10	63.3 ± 25.6*	9.2 ± 4.0	25.9 ± 6.0*

[1] Results are the mean±SEM of four separate experiments performed in media supplemented with 2% stripped FCS, each conducted in duplicate.

[2] 0μM represents DMSO control.

* Significantly different from control cells ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test).

grown in medium supplemented with 10% FCS were less susceptible to the growth-inhibitory effects of genistein (10μM). Experiments were performed culturing HBL 100 and T47-D cells in medium supplemented with 10% FCS, and treated with genistein for 96 hours as before. Results obtained showed that both HBL 100 and T47-D grown in this media were insensitive to the cell cycle arresting effects of genistein (data not shown).

5.1.2. The effect of genistein on cyclin B1 protein levels

The ability of genistein to induce cell cycle arrest at the G2/M phase transition in MCF-7 and MDA-MB 468 breast cells has been linked to its ability to inhibit levels of cyclin B1 protein (Choi et al., 1998, Balabhadrapathruni et al., 2000). The following experiments were performed to investigate whether the inhibitory effect on cyclin B1 protein by genistein occurred in all breast cells when grown under the conditions used for cell cycle arrest experiments.

Cells were treated with genistein (10μM) for 96 hours, after which nuclear protein was extracted, subjected to SDS PAGE (based on a method by Sambrook *et al.*, 1989) and the level of protein detected using an antibody specific for cyclin B1. Satisfactory results were obtained from preliminary experiments in which whole cell lysates were used, but it was decided to use nuclear protein extracts to give the best possible chance of clean and clear western blots.

The effect of genistein on levels of cyclin B1 protein is shown in figure 5.2. Genistein did not inhibit levels of cyclin B1 protein. Compared to control cells, genistein appeared to increase cyclin B1 protein by 50% after 24 hours in MCF-7 cells, with no effect seen at 48, 72 or 96 hours. Similarly, in MDA-MB 468 cells, genistein increased levels of cyclin B1 by 65% after 48 hours.

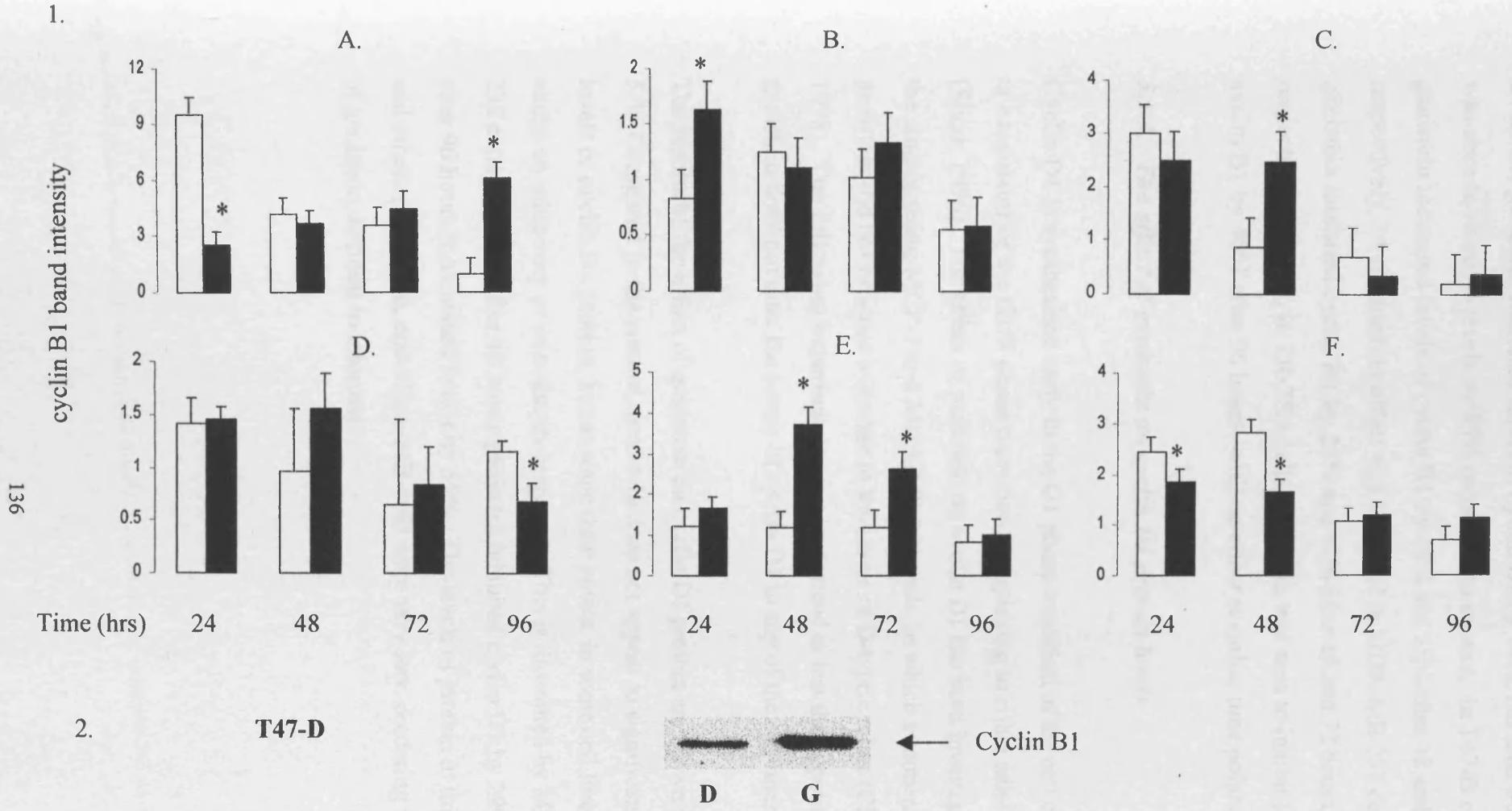


Fig. 5.2. Effect of genistein (10μM) on cyclin B1 protein in (A) HBL 100, (B) MCF-7 (C) MDA-MB 468, (D) ZR-75.1, (E) T47-D and (F) MDA-MB 231 breast cells after 24, 48, 72 and 96 hours.

Open bars represent protein levels after treatment with control (DMSO), closed bars after treatment with genistein. Results, which are presented as fold changes established by laser densitometry compared to incubates omitting DMSO, are the mean±SEM of 3 independent experiments. Asterisks indicate that values are significantly different from incubates with DMSO only ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of Western blot analysis see section 2.14.

In other cell lines, genistein exhibited varying effects. In HBL 100 cells after 24 hours, genistein inhibited levels of cyclin B1 by 74%, but after 96 hours was seen to increase levels by 85% compared to control. In T47-D cells, genistein increased levels of cyclin B1 by 69% and 55% after 48 and 72 hours, respectively. A contrasting effect was observed in MDA-MB 231 cells, where genistein inhibited cyclin B1 by 25% and 42% after 48 and 72 hours, respectively. Lastly, in ZR-75.1 cells, genistein was seen to inhibit levels of cyclin B1 by 40% after 96 hours, with no effect at earlier time points.

5.1.3. The effect of genistein on cyclin D1 protein levels

Cyclin D1 is synthesised early in the G1 phase transition of the cell cycle and is a regulator of the G1/S phase transition, complexing to either cdk4 or cdk6 (Sherr, 1996). The effect of genistein on cyclin D1 has been investigated in the studies using MCF-7 and MDA-MB 231 cells, in which treatment with genistein did not produce a change in the levels of D-type cyclins (Choi et al., 1998). The following experiments were performed to test the hypothesis that genistein does not alter the levels of cyclin D1 in any of the cell lines.

The results of the effect of genistein on cyclin D1 protein are shown in figure 5.3. Compared to the control, genistein does not appear to significantly effect levels of cyclin D1 protein, but at some time points, in some cell lines, it elicits an inhibitory or stimulatory response. This is illustrated by MDA-MB 231 cells in which after 48 hours genistein inhibited cyclin D1 by 29%, but after 96 hours it increased levels by 55%. The levels of protein in this cell line and others (HBL 100, and MDA-MB 468) were very low, rendering the effects of genistein difficult to interpret.

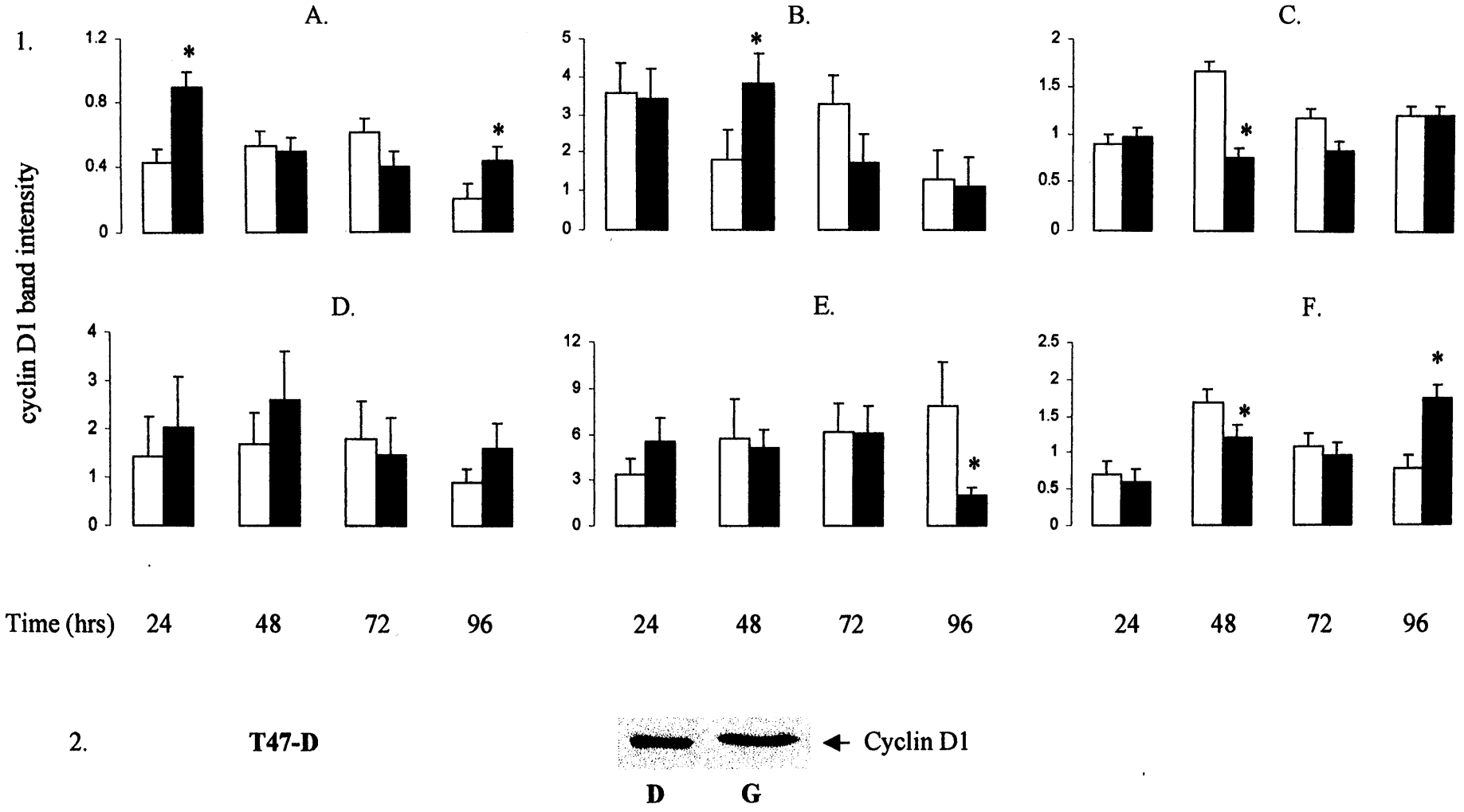


Fig. 5.3. Effect of genistein (10μM) on cyclin D1 protein in (A) HBL 100, (B) MCF-7 (C) MDA-MB 468, (D) ZR-75.1, (E) T47-D and (F) MDA-MB 231 breast cells after 24, 48, 72 and 96 hours.

Open bars represent protein levels after treatment with control (DMSO), closed bars after treatment with genistein. Results, which are presented as fold changes established by laser densitometry compared to incubates omitting DMSO, are the mean±SEM of 3 independent experiments. Asterisks indicate that values are significantly different from incubates with DMSO only ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of Western blot analysis see section 2.14.

DMSO has been shown to elicit varying effects on the levels of both cyclin B1 and D1 protein in these experiments. A 9-fold increase in cyclin B1 protein over controls was seen in HBL 100 cells treated with DMSO and an 8-fold increase in cyclin D1 protein was seen in T47-D cells when observing levels of cyclin D1. These increases in protein by DMSO can mask the actual effect of genistein and it renders the identification of the real effect of genistein difficult to recognise.

5.2. Genistein-induced apoptosis

Genistein has been reported to induce apoptosis in a number of cancer cell lines, including breast (Shao et al., 1998, Li et al., 1999a, Li et al., 1999b, Balabhadrapathruni et al., 2000), head and neck squamous cell carcinoma (Alhasan et al., 1999), colon (Salti et al., 2000) and prostate (Davis et al., 1998) by different mechanisms at varying concentrations. In breast cancer cells, the induction of apoptosis by genistein has been illustrated by cleavage of PARP and the activation of caspase-3 (Li et al., 1999b).

The activation of caspases in apoptosis results in nuclear, plasma-membrane and mitochondrial changes, and caspase-3 is thought to be partially or totally responsible for the proteolysis of substrates, such as PARP, which contain a common D- -D motif (Cohen, 1997). Other mechanisms by which genistein induces apoptosis include induction of p21 (Shao et al., 1998) and alteration of expression of anti-or pro-apoptopic genes, such as Bcl-2 and Bax, respectively (Li et al., 1999a, Li et al., 1999b, Nakagawa et al., 2000).

The following experiments were designed to test the hypothesis that genistein induces apoptosis to a similar extent in all breast cell lines and that apoptosis is a mechanism by which genistein elicits its growth-inhibitory effect.

5.2.1 The effect of genistein on PS externalisation

PS externalisation can be measured using a vascular protein called Annexin V, which binds to PS on the external membrane of apoptopic cells, allowing levels of apoptosis to be quantitated. The cells were cultured in medium containing 10% FCS and treated with genistein (10 μM) for 96 hours. Significant levels of programmed cell death were only observed in MDA-MB 468 cells (figures 5.4 and 5.5), with the percentage of apoptopic cells rising from 5% in control cells (DMSO) to 19.5 % in genistein treated cells (figure 5.5). In the other 5 breast cell types, genistein did not cause changes indicative of apoptosis, although it did increase the necrotic cell population slightly in HBL 100, T47- D and ZR-75.1 cells (table 5.2).

Results in chapter 3 show that genistein inhibited cell growth in MDA-MB 468 cells when cultured in medium containing either 10% FCS or 2% FCS, and the growth-inhibitory effect was slightly more pronounced when grown in the latter. Further experiments were performed to test the hypothesis that genistein induces apoptosis to the same extent in MDA-MB 468 cells grown in medium containing 2% FCS.

The results shown in figure 5.5 show genistein (10μM) significantly induced apoptosis in MDA-MB 468 cells cultured in medium containing 2% FCS, whilst also significantly increasing the necrotic cell population.

5.2.2. The effect of genistein on PARP cleavage in MDA-MB 468 cells

Cleavage of PARP protein is an early indicator of apoptosis (Germain et al., 1999) and is characterised by concomitant degradation of the full size molecule (116 kDa) and the accumulation of an 85 kDa product.

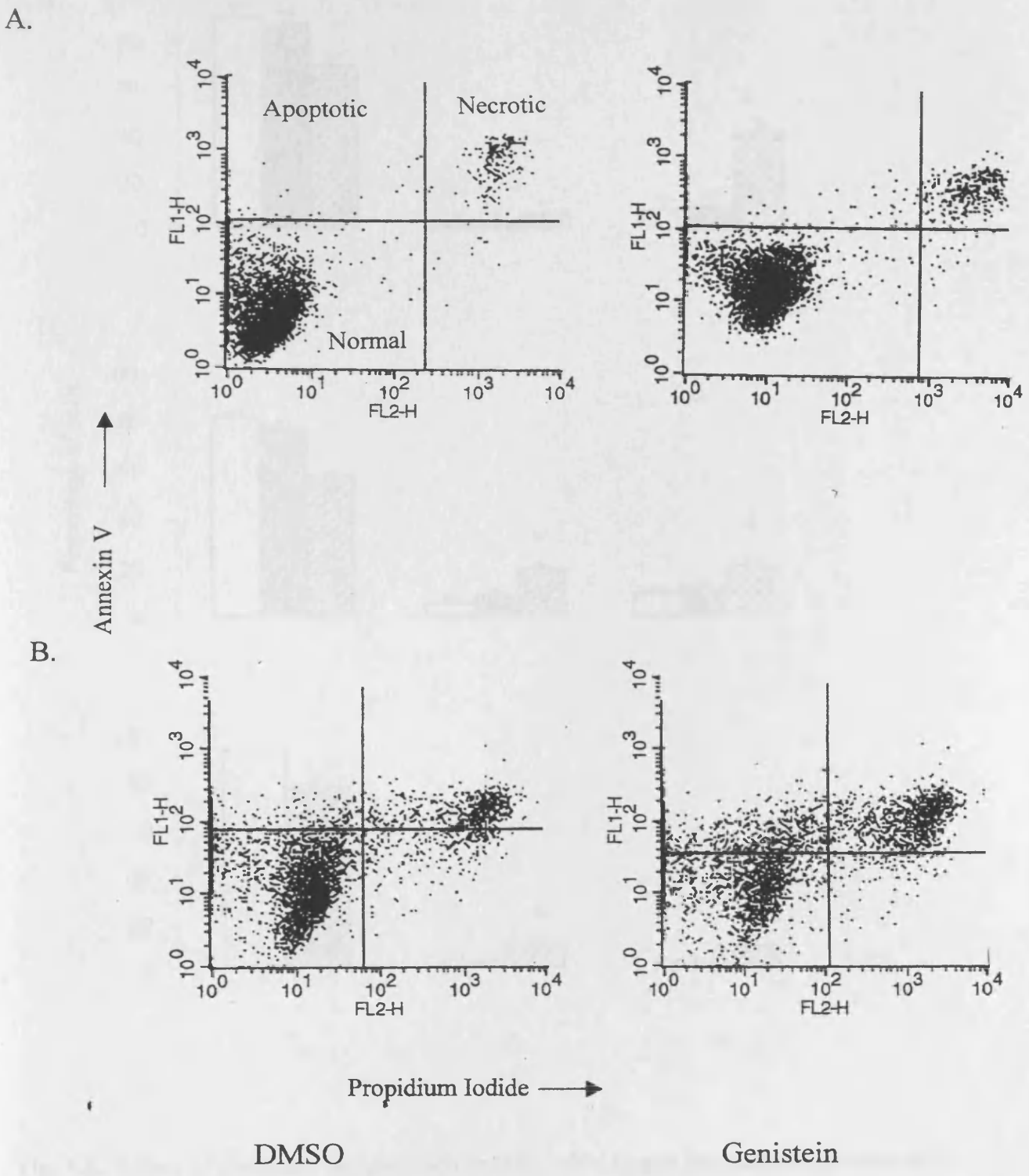


Fig. 5.4. The effect of genistein (10μM) on apoptosis in (A) HBL 100 and (B) MDA-MB 468 cells shown by annexin staining.

Results, presented as a single experiment for each cell line treated with either DMSO or genistein (10μM) for 96 hours, are depicted by annexin diagrams. For details of annexin staining see section 2.15.

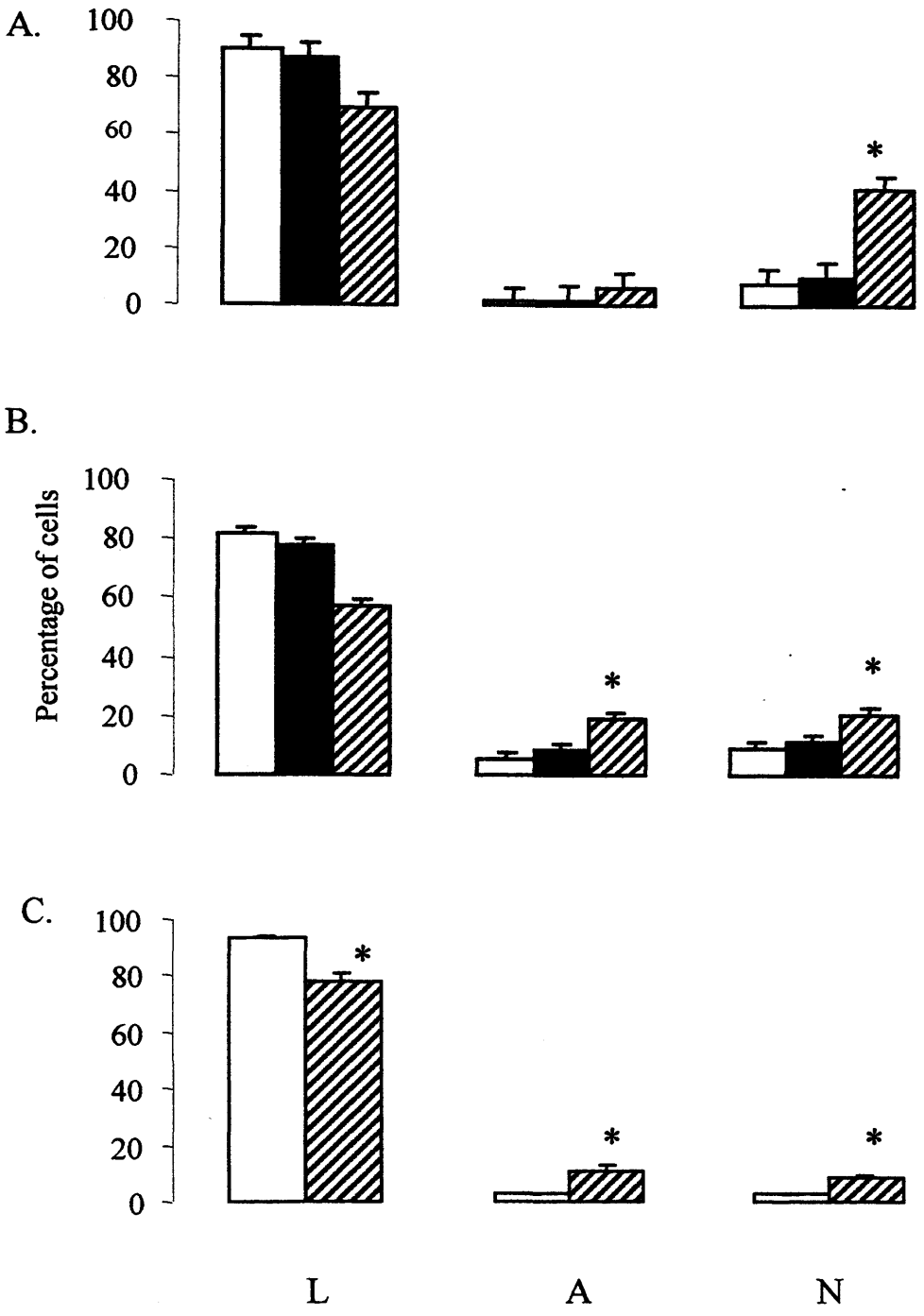


Fig. 5.5. Effect of genistein on apoptosis in HBL 100 (A) and MDA-MB 468 cells (B,C) grown in medium containing 10% FCS (A,B) or 2% FCS (C).

Results are presented as a percentage of live (L), apoptopic (A) and necrotic (N) cells after 96 hr. Open bars represent DMSO control, closed bars genistein 1μM and hatched bars genistein 10 μM. Results are the mean±SEM of 4 independent experiments. Asterisks indicate that values are significantly different from DMSO controls ($p=<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of annexin staining see section 2.15.

Table 5.2. Effect of genistein (1 and 10 μM) on percentage apoptosis in breast cell lines grown in medium containing 10% FCS.

Cell type	[Genistein]	Percentage apoptosis[1]		
		Live	Apoptopic	Necrotic
MCF-7	0	89.7 ± 0.6	4.7 ± 0.3	5.2 ± 0.4
	1	94.3 ± 0.5	1.6 ± 0.2	3.7 ± 0.5
	10	80.4 ± 0.3	3.9 ± 0.2	15.2 ± 0.4*
T47-D	0	90.4 ± 1.8	3.1 ± 1.5	6.6 ± 1.4
	1	91.8 ± 2.0	1.2 ± 0.4	6.2 ± 1.6
	10	94.8 ± 0.5	1.1 ± 0.2	3.4 ± 0.4
ZR-75.1	0	63.0 ± 0.3	6.4 ± 0.7	20.8 ± 0.1
	1	82.4 ± 0.8	5.5 ± 0.2	8.0± 0.3
	10	89.0 ± 0.5*	1.4 ± 0.2	6.7 ± 0.4
MDA-MB 231	0	92.1 ± 2.8	2.6 ± 0.9	4.6 ± 1.4
	1	91.9 ± 2.9	2.5 ± 1.4	4.7 ± 1.6
	10	87.5 ± 1.9	3.5 ± 0.7	7.9 ± 1.3

[1] Results are the mean±SEM of four separate experiments, conducted in media supplemented with 10% FCS..

[2] 0μM represents DMSO control.

* Significantly different from control cells ($P<0.05$, two-way ANOVA, Fischer's LSD posthoc test).

In order to confirm the apoptosis observed by annexin staining, the effect of genistein on PARP protein was examined. MDA-MB 468 cells were treated with genistein (10 μM) for 72 and 96 hours, after which cells were harvested and subjected to SDS PAGE, and PARP protein was detected by a specific antibody. A representative blot is shown in figure 5.6, which shows genistein did not induce PARP protein degradation in cells treated for 72 hours, compared to a DMSO control. However, after 96 hours, there was a decrease in PARP protein band intensity of 38 % in cells exposed to genistein, as compared to control cells.

5.2.3. The effect of genistein on caspase-3 activity in MDA-MB 468 cells

The execution phase of apoptosis generally involves the activation of a class of cysteine proteases known as caspases (Bratton et al., 2000). The activation of the effector caspase, caspase 3 (CPP32), has been classed as an early marker of apoptosis (Darmon et al., 1995). Effector caspases are known to cleave a number of structural and regulatory proteins, for example, PARP, lamins and cytokeratins, and are directly responsible for a number of the morphological features associated with apoptosis (Bratton et al., 2000). Caspase-3 is thought to be partially or totally responsible for the cleavage of PARP (Darmon et al, 1995) and genistein has been reported to induce the activity of caspase-3 in breast cells (Li et al., 1999b) and also in testicular TM4 cells (Kumi-Diaka and Butler, 2000).

The apoptosis induced by genistein in MDA-MB 468 cells, as seen by both annexin staining and PARP cleavage, were further confirmed by changes in caspase-3 activity, based on a method by MacFarlane *et al.*, 1997. MDA-MB 468 cells were treated with genistein (10 μM) for 72 and 96 hours, after which they were harvested and the cell extracts were added to Z-DEVD.afc, a synthetic tetrapeptide which acts as a substrate for caspase-3. The samples

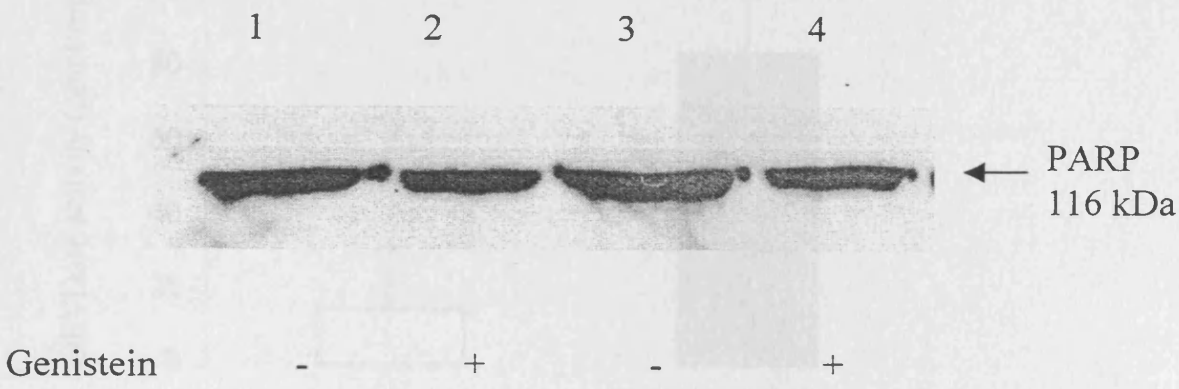


Fig. 5.6. The effect of genistein (10μM) on PARP degredation in MDA-MB 468 cells cultured in medium containing 10% FCS after 72 and 96 hours.

The result is presented as a western blot, representative of a single experiment. Cells were treated with genistein for 72 hours in lanes 1 and 2 and for 96 hours in lanes 3 and 4. Cells without DMSO (untreated), responded as those shown here after treatment with DMSO for 72 hours. For details of western blot analysis see section 2.5.3

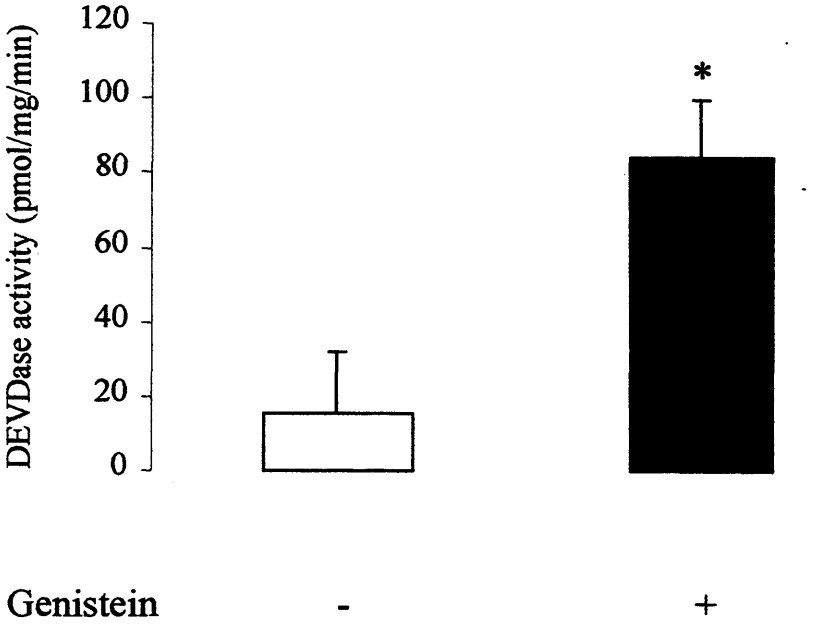


Fig. 5.7. Effect of genistein (10μM) on caspase-3 activity in MDA-MB 468 cells cultured in medium containing 10% FCS.

Cells were incubated for 96 hours. Results are presented as amount of DEVDase activity. Cells without DMSO responded as those shown here with DMSO. Open bars represent treatment with DMSO and closed bars treatment with genistein 10μM. Values are the mean ±SEM of three independent experiments. Asterisks indicate that values are significantly different from control ($p=<0.05$, two-way ANOVA, Fischer's LSD posthoc test). For details of enzyme assay see section 2.17.

were assayed using fluorescence for the liberation of 7-amino-4-trifluromethylcoumarin, as a measure of caspase-3 like activity. An 8-fold increase in activity was observed in MDA-MB 468 cells treated with genistein for 96 hours compared to controls (figure 5.7).

DISCUSSION

The chemopreventive and anti-neoplastic effects of several agents, including genistein, are mediated in part by cell cycle arrest and modulation of the action of cyclins and CDKIs (Balabhadrapathruni et al., 2000). The experiments carried out in this chapter were designed to explore the effect of genistein on the cell cycle and to test the hypothesis that genistein elicits a similar effect in all of the cell lines tested.

The effect of genistein on the cell cycle is well documented, with studies reported in a number of different cell lines. Genistein (50μM) has been reported to induce cell cycle arrest at G2/M phase after 96 hours in both HN4 cells, a head and neck squamous cell carcinoma cell line (Alhasan et al., 1999) and PC3 and LNCaP prostate cells (Davis et al., 1998). Genistein (30μM) also induced cell cycle arrest at G2/M phase in H460 cells, a non-small-cell lung cancer cell line (Lian et al., 1998) and HGC-27 cells, which are derived from a human gastric cancer (Matsukawa and Aoike, 1993). In contrast, in studies using murine BALB/c 3T3 fibroblasts and B16-F1 melanoma cell lines, it was reported that genistein (180μM) induced cell cycle arrest at the G1/S phase transition (Kuzumaki et al., 1998).

The effect of genistein on cell cycle arrest has also been studied in breast cell lines, with cell cycle arrest reported in MCF-7 (Constantinou et al., 1998, Shao et al., 1998, Nakagawa et al., 2000), MDA-MB 468 (Balabhadrapathruni et al., 2000) and MDA-MB 231 cells (Choi et al., 1998, Nakagawa et al., 2000) at

designed to test the hypothesis that genistein induces cell cycle arrest in all cells at the G2/M phase transition.

The results in table 5.1 show that genistein (10μM) significantly arrested cells at the G2/M phase in five of the six cell lines, with the exception of ZR-75.1 cells, in which only a slight increase in G1 cell population was observed. The data obtained from cell cycle analysis is not inconsistent with the effect of genistein on cell growth (see chapter 3). This correlation suggests a mechanism for the growth-inhibitory action of genistein is its deregulation of the cell cycle, resulting in an accumulation of cells at the G2/M phase transition of the cycle. The ZR-75.1 cells, however, were insensitive to this effect of genistein; therefore the growth inhibition in these cells must occur by a different mechanism.
Further experiments were performed, using HBL 100 and T47-D cells grown in media containing 10% FCS, in an attempt to correlate induction of cell cycle arrest with growth inhibition (see chapter 3). The results gained show that both cell lines were insensitive to the cell cycle arresting effect of genistein under these conditions. The results correlated well in both cell lines as genistein elicited no growth-inhibitory effects and had no effect on cell cycle progression.

The ability of genistein to cause cell cycle arrest suggests that it may interfere with cyclins, cyclin dependent kinases or their inhibitors. To explore the mechanisms by which genistein interferes with cell cycle progression, the effect on cyclins B1 and D1 were investigated. Cyclin B1, complexed with cdc2, accumulates during the late S- and early G2-phase of the cell cycle allows entry of cells into M phase and is then rapidly degraded at the end of mitosis, allowing the cells to divide (Sherr, 1996). In contrast, cyclin D1, complexed with either cdk4 or cdk6 is necessary for progression of cells from G1- into S- phase of the cell cycle. As breast cell cycle arrest by genistein

occurs at the G2/M phase transition, experiments were designed to test the hypothesis that genistein inhibits levels of cyclin B1 protein, but has no effect on levels of cyclin D1. The effect of genistein on cyclin B1 has been reported in MCF-7 and MDA-MB 231 cells, with an inhibition of both cyclin B1 protein and mRNA, in addition to immunoprecipitated cdc2, which was observed in cells after treatment with 100μM genistein (Choi et al., 1998).

From the results shown in figures 5.2 and 5.3 genistein appeared to have little or no effect on the levels of cyclin B1 and D1 activity in any of the six cell lines examined, although previous studies with MCF-7 and MDA-MB 468 cells showed significant inhibition of cyclin B1 (Choi et al., 1998). This is not surprising, as the concentration of genistein used here was 10-fold lower than that used in the previously described experiment. Genistein has also been reported to elicit a bi-phasic response on levels of cyclin B1 in MDA-MB 468 cells, with both an increase and decrease in protein levels observed after treatment with 25μM and 100μM respectively (Balabhadrapathruni et al., 2000). Therefore, the absence of an effect of genistein at 10μM is not unexpected and cell cycle arrest must occur by a mechanism other than inhibition of cyclin B1 activity.

Levels of cyclin D1 protein have been reported to remain unchanged, even after exposure to genistein (100μM) for 72 hours and in addition, genistein has also been reported to have no effect on cdk4 and cdk6 activity (Choi et al., 1998). The effect of genistein on levels of cyclin D1 protein shown in figure 5.3 proved negligible and it is consistent with published studies (Choi et al., 1998, Balabhadrapathruni et al., 2000). These results suggest cyclin D1 does not play a role in the cell cycle arrest elicited by genistein.

Cell cycle arrest at G1 in both normal and malignant cells has been associated with the induction of p21 (Choi et al., 1998). Increasing the level of p21

enhances its association with CDK-cyclin complexes, resulting in decreased CDK activity, which in turn inhibits the phosphorylation of specific endogenous substrates such as pRB (Shao et al., 1998). Genistein has been reported to induce levels of p21 in a number of breast cell lines, including MDA-MB 231 (Shao et al., 1998, Choi et al., 1998, Li et al., 1999b), MDA-MB 435 (Li et al., 1999a) and MCF-7 cells (Shao et al., 1998, Choi et al., 1998).

Experiments were carried out to investigate the levels of p21 after treatment with genistein in all cell lines, but three different monoclonal antibodies all proved unable to detect proteins of the correct size and thus the experiments were abandoned. It would have been useful to determine whether elevated levels of p21 could be observed in all genistein-treated cells lines, or just in those in which apoptosis occurred.

After determining the effect of genistein on cell cycle proteins and the cell cycle itself, experiments were designed to investigate the ability of genistein to induce apoptosis. Genistein has been reported to induce apoptosis in a number of cell lines by a variety of mechanisms. An induction of levels of p21 has not only been observed in breast cancer cell lines, but also in prostate cancer cells (Davis et al., 1998), non small cell lung cancer cells (Lian et al., 1998, Lian et al, 1999), mouse fibroblasts and melanoma cells (Kuzumaki et al., 1998). Genistein has been reported to induce apoptosis in breast cells by altering the expression of apoptosis-regulating genes. This has been shown to occur in H460 cells in a study by Lian *et al.* (1999), where genistein upregulated levels of Bax, but had no effect on levels of Bcl-2 protein. The expression of Bcl-2 in MDA-MB 231 cells was down regulated after treatment with genistein (30μM), whilst levels of Bax were seen to increase (Li et al., 1999b). An identical effect was observed by Li *et al* (1999a) when studying the effect of gene expression in genistein-treated MDA-MB 435 cells. A down-regulation in levels of Bcl-2 was also evident in MCF-7 cells treated with genistein

(150μM) (Constantinou et al., 1998), in addition to an increase in Bcl-2 phosphorylation, which may inhibit in its ability to interfere with apoptosis.

The results shown in figure 5.5 show that genistein induced significant levels of apoptosis in MDA-MB 468 cells after 96 hours, as borne out by measurement of annexin staining, which is consistent with other published studies. In addition, culturing MDA-MB 468 cells in medium containing charcoal stripped serum did not affect the ability of genistein to induce apoptosis. In the other breast cell types, genistein did not cause changes indicative of apoptosis, although it increased the necrotic cell population slightly in HBL 100, MCF-7 and MDA-MB 231 cells.
Further experiments were performed to confirm genistein-induced apoptosis in MDA-MB 468 cells. Analysis of PARP protein by western blot showed a decrease in PARP protein band intensity in cells exposed to genistein, as compared to control cells, furthermore, genistein increased caspase-3 like enzyme activity 8-fold over control cells. These results provide strong evidence that apoptosis was induced in genistein-treated MDA-MB 468 cells, even at a low concentration and that the disappearance of PARP was probably due to the activity of caspase-3.

To conclude, it is apparent that the growth-inhibitory actions of genistein are mediated, in part, by the deregulation of the cell cycle and also by the induction of apoptosis. It is clear that although genistein appears to mediate a number of effects which may contribute to its growth-inhibitory activity regarding cell cycle progression and apoptosis, it does not affect all breast cells similarly and the mechanisms by which it inhibits growth varies between cell lines. The results in this chapter, however, do not provide any evidence for the mechanisms by which genistein induces apoptosis, therefore, further investigation using MDA-MB 468 cells will be important in the elucidation of such mechanisms.

CHAPTER 6: GENERAL DISCUSSION.

Recently, the isoflavone genistein has received a great deal of attention because of the discovery that it has been shown to inhibit PTK activity *in vitro* (Akiyama et al., 1987). As tyrosine phosphorylation plays a crucial role in both cell proliferation and transformation, genistein has been investigated extensively with respect to its possible cancer chemopreventive properties.

The results presented in this study show that growth of six human-derived breast cell lines was inhibited by genistein at 10 μM. This growth inhibition was consistent with many other published studies (see chapter 3). A number of differences between the breast cell lines in terms of their sensitivity towards genistein were apparent (see table 6.1). Firstly, the enhancement of breast cell growth by genistein (1 μM), seen in MCF-7 cells, was not a generic feature of human-derived breast cells. Secondly, some breast cell types, exemplified by HBL 100 and T-47 D cells, were only sensitive to the growth inhibition exerted by genistein under conditions of low serum concentration in the culture medium. Thirdly, only ZR-75.1 cells were insensitive to the cell cycle arresting effect of genistein. Fourthly, an induction of apoptosis accompanied exposure to growth-inhibitory concentrations of genistein in MDA-MB 468 cells, but not in other breast cell types (see chapter 5). Lastly, the breast cells lines differed considerably in terms of responsiveness of pivotal mitogenic signalling steps and transcription factor activity towards genistein (see chapter 4).

The observation that genistein in the 10^{-6} M range promoted the growth of MCF-7 cells is consistent with that reported by Martin *et al.* (1978) and Wang *et al.* (1996), in which growth stimulation was observed at concentrations of 200nM and between 10-100nM, respectively. Such stimulation of breast cell growth by genistein has raised the concern that physiologically achievable concentrations of genistein, such as 1-3μM, might support - rather than counteract - the progression of breast cancer in women with pre-malignant changes. This type of effect would severely confound any potential benefit of genistein consumption and emphasizes the importance of fully characterising phyto-oestrogens in terms of biological activity, the balance

between agonistic and antagonistic properties, natural potency and long and short term effects (Whitten et al., 1995).

American scientists have recently raised clinical concerns about the consumption of soya (figure 6.1). They report that while soya may help to reduce cholesterol, it may also lead to health problems in animals including thyroid disorders and altering the sexual development of foetuses. Isoflavones can act as alternative substrates for the enzyme thyroid peroxidase (Divi et al, 1996), but although other flavanoids act as better substrates for this enzyme (Divi et al 1997), it is clear that further investigation is required into the potential beneficial and adverse effects of genistein before it can used as a therapeutic agent.

The growth-stimulatory effect of genistein has been attributed, at least in part, to its ability to modulate growth via the oestrogen receptor (Hsieh et al., 1998). This study, however, provides no supporting evidence to indicate that oestrogen receptors are involved, and further investigation into blocking the effects of oestrogen receptors, for example by antisense experiments, is required. However, a comparison between the cell lines regarding oestrogen receptor status (see table 3.3) rendered the possibility unlikely that the growth promotion caused by genistein specifically in MCF-7 cells was intrinsically linked to expression of either oestrogen receptor-α or -β or both.

In addition, results from this study show the growth-inhibitory action of genistein does not appear to be mediated through either oestrogen or EGF-receptors. It does not mean that these receptors are not important in breast cell growth, just that genistein may utilise other pathways in order to modulate cell proliferation.

The notion that divergent, oestrogen receptor-independent mechanisms, such as interference with the progression of the cell cycle, induction of apoptosis and modulation of cell signalling cascades, mediate the growth-arresting efficacy of genistein in the different breast cell lines is supported by three pieces of evidence (see chapters 4 and 5).

Soya alert over cancer and brain damage link

Special report: what's wrong with our food?

by Antony Barnett, public affairs editor
Observer

Sunday August 13, 2000

A health warning was sounded last night over the dangers of eating soya after two senior American government scientists revealed that chemicals in the product could increase the risk of breast cancer in women, brain damage in men and abnormalities in infants.

The disclosure, which sent shockwaves through the multi-billion dollar food industry, came after the scientists decided to break ranks with colleagues in the US Food and Drug Administration and oppose its decision last year to approve a health claim that soya reduced the risk of heart disease. They wrote an internal protest letter warning of 28 studies revealing toxic effects of soya.

In an interview with The Observer, one of the scientists, Daniel Doerge, an expert on soya, said: 'We have very real worries that this health claim will be used by the industry as an endorsement of much wider health benefits to soya beyond the heart. Research has shown a clear link between soya and the potential for adverse effects in humans.'

BSE and other health scares related to meat have led to rocketing sales of soya-related products in Britain. But it is not just vegetarian foods such as tofu that use soya. It is a key ingredient in products from meat sausages and fish fingers to salad creams and breakfast cereals.

The concerns of Doerge and fellow FDA researcher Daniel Sheehan focus on chemicals in soya known as isoflavones which have effects similar to the female hormone oestrogen.

While these chemicals may help to prevent a range of conditions including high cholesterol, they also lead to health problems in animals including altering sexual development of foetuses and causing thyroid disorders. Although soy is thought to protect against breast cancer, some studies show that chemicals in soya may increase the chances of breast cancer which uses oestrogen-type hormones for growth.

Their letter to the FDA seen by The Observer states: 'There is abundant evidence that some of the isoflavones found in soy demonstrate toxicity in oestrogen sensitive tissues and in the thyroid. Additionally, the adverse effects in humans occur in several tissues.

'During pregnancy in humans, isoflavones per se could be a risk factor for abnormal brain and reproductive tract development.'

This will frighten mothers who increasingly use soya milk for babies. Doerge said: 'They are exposing their children to chemicals which we know have adverse effects in animals. It's like doing a large uncontrolled and unmonitored experiment on infants.'

The soya industry insists that most research shows the health benefits of soya outweigh risks and that adverse effects seen in animals do not apply to humans.

Richard Barnes, European director of the US Soy Bean Association, said: 'Millions of people around the world have been eating soya for years and have shown no signs of abnormalities or disorders.'

Fig. 6.1. Soya alert over cancer and brain damage link report. Taken from the Observer, Sunday 13th August 2000.

Firstly, genistein caused apoptosis in MDA-MB 468 cells, but not in any of the other five cell types studied, even though it caused similar growth arrest in these cell lines to that seen in MDA-MB 468 cells. Secondly, genistein significantly arrested cells in G2 in five of the six cell lines, but not ZR-75.1 cells, in which growth arrest was accompanied by a slight increase in the G1 cell population. Lastly, the cell lines differed in their susceptibility towards genistein-induced inhibition of elements of TPA-induced cell signalling: AP-1 activity was inhibited only in MDA-MB 468 and T47-D cells, ERK activation only in ZR 75.1 and T47-D cells, and c-Fos levels only in MDA- MB 468 and T47-D cells (table 6.1).

Over the last few years it has become clear that perturbation of signalling pathways is a frequent event during the acquisition of a transformed phenotype (Franks et al., 1998). The role of both MAPK cell signalling pathways and the AP-1 transcription factor complex are important in cell growth and differentiation, and it is conceivable that genistein may elicit its growth-inhibitory effects via modulation of such cellular processes. Modulation of cell signalling cascades by genistein in this study can be demonstrated in T47-D cells, where it is suggested that genistein inhibits growth by interfering with MAPK signalling, which in turn abrogates AP-1 transcription *via* attenuation of c-Fos expression.

Genistein appeared to have a negligible effect on AP-1 components in cell lines, other than T47-D, however an increase in c-Jun protein levels was observed in MCF-7 and ZR-75.1 cells. This induction of c-Jun was compatible with the observation that genistein appeared to increase, rather than decrease AP-1 transcription factor activity in ZR-75.1 cells. Nevertheless, the increase in c-Jun protein was accompanied not by an increase, but rather by a decrease, in anisomycin-induced JNK activity. So, the functional consequences of the contrasting changes elicited by genistein with respect to c-Jun protein on the one hand and JNK activity on the other are difficult to predict. The observations in MCF-7 and ZR-75.1 cells show that under certain conditions, such as concentration of agent and cell type, genistein can elicit activatory signals, and some of these signals might contribute to the growth-arresting ability of genistein.

In a study by Davies *et al.* (1999), it was reported that genistein attenuated the activation of the NFκB transcription factor in human prostate cells, by inhibiting phosphorylation of IκBα and thereby blocking the nuclear translocation of NFκB, thus preventing DNA binding and NFκB activation. These findings provide a mechanism by which genistein-induced inactivation of NFκB could lead to an induction of apoptosis. It is possible that genistein may also inhibit NFκB activation in human breast cells, which may provide a mechanism by which apoptosis is induced in MDA-MB 468 cells. Investigation into the effect of genistein on NFκB activation in breast cells, in addition to those results obtained for AP-1, may provide a useful insight into the effect of genistein on transcription factors important in the regulation of cell growth.

The capacity of genistein to inhibit mitogen-stimulated growth of mammalian cells in culture has been presumed to be due to the inhibition of tyrosine kinases (Kim et al., 1998). These kinases include EGF–R kinase, pp60 $^{v\text{-}src}$ and pp110$^{gag\text{-}fes}$ kinases (Akiyama et al., 1987), cdk2 and cdc2 kinases (Choi et al., 1998) and p56/p53^{lyn} kinase, which inhibits phosphorylation on Tyr15 on CDK1 (Kaufmann, 1998).

A study by Kim *et al.* (1998), questioned whether altered tyrosine phosphorylation by genistein was due to a direct effect on tyrosine kinases. They showed that genistein did not affect either EGF-stimulated EGF-R autophosphorylation, or EGF-stimulated tyrosine phosphorylation of intracellular target proteins such as PKC, at concentrations which inhibited cell proliferation. In addition, *in vivo* data obtained from the rat mammary gland regarding the effect of genistein on tyrosine kinase activity did not support the *in vitro* reports that genistein inhibits PTK activity, but rather that genistein directly increased EGF-R expression, resulting in enhanced mammary gland differentiation (Lamartiniere and Wang, 1999). These studies concluded that altered tyrosine phosphorylation was not a direct effect of genistein, but was related to indirect effects mediated by other mechanisms, such as those mediated *via* the oestrogen receptor. Therefore, more investigations are required into

the action of genistein regarding tyrosine kinase phosphorylation and elucidating its mechanisms of action.

The ability of genistein to arrest cell cycle progression in five cell lines may be due to its activation of Chk2 kinase. The activation of this kinase by genistein was correlated with cell cycle arrest at the G2/M phase in melanoma cells (Darbon et al., 2000). It is conceivable that if ZR-75.1 cells are deficient in this kinase, which is involved in the replication and DNA-damage checkpoints, it may provide an explanation why cell cycle progression was not arrested by genistein in this cell line.

The overall interpretation of the results across the 6 cell types is confounded by the finding that for some cells, certain signalling events were resistant to induction by the phorbol ester TPA. TPA failed to elicit AP-1 activation in MDA-MB 231 and MCF-7 cells, and ERK phosphorylation in HBL 100 and MDA-MB 468 cells in a reproducible manner. Irrespective of this insensitivity towards TPA, susceptibility towards the effects of genistein varied substantially between the different cell types. Nevertheless, some observations can be rationalised in terms of mechanistic connectivity or complementarity, as previously shown in T47-D cells. Table 6.1 summarises the effects of genistein on growth, apoptosis, cell cycle distribution, AP-1 activity, ERK phosphorylation and c-Fos and c-Jun protein levels in the cell lines studied.

Undoubtedly the concentrations of genistein which have been reported here, and by others, to elicit growth arrest and cause changes in signalling, are higher than the 3-4 μM reported in the plasma of individuals who consume large amounts of soya (Adlercreutz et al., 1995). Nevertheless, as genistein and related isoflavanoid molecules have recently been considered as potential chemopreventive or chemotherapeutic agents in their own right and not only as constituents of the food matrix, it is conceivable that they could be administered clinically at much higher doses than those associated with dietary polyphenol consumption, thus potentially achieving target tissue concentrations in the 10^{-5} - 10^{-4} M range.

In summary, the findings in this study suggest that whilst genistein at higher concentrations consistently arrests the growth of breast cells, susceptibility to genistein-induced growth promotion at low concentrations is not a generic feature of cells derived from this tissue. Furthermore, the results obtained are consistent with the notion that induction of apoptosis, G2 cell cycle arrest, inhibition of components of MAPK signalling, c-Fos protein expression and AP-1 binding and/or transactivation may contribute to the growth-inhibitory effect of genistein in some types of breast cells. None of these effects of genistein constitute a predominant mode of growth-arresting action, as they do not occur consistently in all breast cell lines. Instead, the relative importance of the individual components of the heterogeneous mechanisms associated with growth modulation by this isoflavone are probably breast cell type-specific.

The exposure of humans to soy phyto-oestrogens goes back nearly five millennia (Barnes, 1995a) and today, those countries, in which large quantities of soy are consumed, report a lower incidence of many chronic diseases, such as breast, lung and prostate cancer and leukaemia (Messina et al., 1994). A novel mechanism for the action of genistein based on enhancement of TGF-β has been proposed (Kim et al., 1998), which may provide a link between the beneficial effects of genistein in diseases, such as atherosclerosis and inflammatory bowel disease.

Several clinical trials are underway to determine whether phyto-oestrogens are therapeutic or have secondary preventive effects for patients with existing breast cancer (Barnes, 1998), that will provide us with important results for further investigation into the effects of soy isoflavones. It is very difficult to make a statement regarding the mechanism of action of genistein, since a large array of potential mechanisms have been observed in this study and our knowledge of their effect on breast cancer is far from complete. However, the knowledge of cell-type specific effects of genistein as shown here, may at some point in the future, contribute to the reduction in breast cancer in the Western world.

	Cell type					
	HBL 100	MCF7	MDA 468	ZR-75	T47-D	MDA 231
Growth inhibition[a]	-[b]	+	+	+	-	+
Apoptosis[a]	-	-	+	-	-	-
G2/M arrest[c]	+	+	+	-	+	+
G1 arrest[c]	-	-	-	+	-	-
AP-1 transactivation (+TPA)[d]	↑	-	↑	↑	↑	-
AP-1 transactivation (+TPA and genistein)[e]	-	-	↓	↑	↓	
c-fos levels (+TPA and genistein)[e]	-	-	↓	-	↓	-
c-jun levels (+anisomycin and genistein)[e]	-	↑	-	↑	-	-
ERK 1/2 phosphorylation (+TPA)[d]	-	↑	-	↑	↑	-
ERK 1/2 phosphorylation (+TPA and genistein)[e]	-	-	-	↓	↓	-

[a]Effect of genistein (10 μM) in cells maintained in 10 % FCS. [b]- = no effect; + = effect; ↑ =upregulation; ↓ -= downregulation. [c]Effect of genistein (10 μM) in cells maintained in 2 % charcoal stripped FCS. [d]Effect of TPA alone. [e]Effect of genistein (50 μM) after stimulation by TPA or anisomycin. For details see results chapters and general discussion.

Table 6.1. Effects of genistein on breast-derived cell types.

CHAPTER 7: REFERENCES.

Adlercreutz, C.H.T., Goldin, B.R., Gorbach, S.L., Hockerstedt, A.V., Watanabe, S., Hamalainen, E.K., Markkanen, M.H., Makela, T.H., Wahala, K.T., Hase, T.A., and Fotsis, T. (1995). Soybean phyto-oestrogen intake and cancer risk. *J Nutr* **125**, 757S-770S.

Adolph, W.H. and Kiang, P.C. (1920). The nutritive value of soybean products. *China Med Journal* **34**, 268-270.

Afifi, A. A. and Azen, S.P. (1979). The Analysis of Variance. In: Statistical analysis: a computer orientated approach. A.A. Afifi and S.P. Azen eds. (London: Academic Press Inc., London), pp198-273.

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* **262**, 5592-5595.

Alhasan, S.A., Pietrasczkiwicz, H., Alonso, M.D., Ensley, J., and Sarkar, F.H. (1999). Genistein-induced cell cycle arrest and apoptosis in a head and neck squamous cell carcinoma cell line. *Nutr Cancer* **34**, 12-19.

Anderson, J.J.B., Anthony, M., Messina, M., and Garner, S.C. (1999). Effects of phyto-oestrogens on tissues. *Nutr Res Rev* **12**, 75-116.

Angel P and Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochim Biophys Acta* **1072**, 129-157.

Antonsson, B. and Martinou, J. (2000). The Bcl-2 protein family. *Exp Cell Res* **256**, 50-57.

Balabhadrapathruni, S., Thomas, T.J., Yurkow, E.J., Amenta, P.S., and Thomas, T. (2000). Effects of genistein and structurally related phytoestrogens on cell cycle kinetics and apoptosis in MDA-MB 468 human breast cancer cells. *Oncol Rep* **7**, 3-12.

Baquet, C., R. (1999). Breast cancer epidemiology: an overview. *Am J Manag Care* **5**, S367-S370.

Barnes, S. (1995a). Evolution of the health benefits of soy isoflavones. *Proc Soc Exp Biol Med* **217**, 386-392.

Barnes, S. (1995b). Effect of genistein on *in vitro* and *in vivo* models of cancer. *J Nutr* **125**, 777s-783s.

Barnes, S. (1998). Phyto-oestrogens and breast cancer. *Bailliere's Clinical Endocrinology and Metabolism.* **12**, 559-579.

Barnes, S. and Peterson, T.G. (1995). Biochemical targets of the isoflavone genistein in tumour cell lines. *Proc Soc Exp Biol Med* **208**, 103-108.

Bradford, M.M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* **7(72)**, 248-254.

Bratton, S.B., MacFarlane, M., Cain, K., and Cohen, G.M. (2000). Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. *Exp Cell Res* **256**, 27-33.

Brinkley, B.R., Beall, P.T., Wible, L.J., Mace, M.L., Turner, D.S., and Callieau, R.M. (1980). Variations in cell form and cytoskeleton in human breast carcinoma cells *in vitro*. *Cancer Res* **40**, 3118-3129.

Brooks, S.C., Locke, E.R., and Soule, H.D. (1973). Oestrogen receptor in a human cell line (MCF-7) from breast carcinoma. *J Biol Chem* **248**, 6251-6253.

Caelles, C., Gonzalez-Sancho, J.M., and Munoz, A. (1997). Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes Dev* **11**, 3351-3364.

Callieau, R., Young, R., Olive, M., and Reeves, W.J. Jr. (1974). Breast tumour cell lines from pleural effusions. *J Natl Cancer Inst* **53(3)**, 661-674.

Casanova, M., You, L., Gaido, K.W., Archibeque-Engle, S., Janszen, D.B., and d'A Heck, H. (1999). Developmental effects of dietary phyto-oestrogens in sprauge-dawley rats and interactions of genistein and daidzein with rat oestrogen receptors α and β *in vitro*. *Toxicol Sci* **51**, 236-244.

Chen, T.K., Smith, L.M., Gebhardt, D.K., Birrer, M.J., and Brown, P.H. (1996). Activation and inhibition of the AP-1 complex in human breast cancer cells. *Mol Carcinogenesis* **15**, 215-226.

Choi, Y.H., Zhang, L., Lee, H.L., and Park, K. (1998). Genistein-induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells. *Int J Oncol* **13**, 391-396.

Choi, Y.H., Lee, W.H., Park, K-Y., and Zhang, L. (2000). P53-independent induction of p21, reduction of cyclin B1 and G2/M arrest by the isoflavone genistein in human prostate carcinoma cells. *Jpn J Cancer Res* **91**, 164-173.

Cohen, G.M. (1997). Caspases: the executioners of apoptosis. *Biochem J* **326**, 1-16 (review).

Coldham, N.G., Howells, L.C., Santi, A., Montesissa, C., Langlais, C., King, L.J., MacPherson, D.D., and Sauer, M.J. (1999). Biotransformation of genistein in the rat:

elucidation of metabolite structure by product ion mass fragmentology. *J Steroid Biochem Mol Biol* **70**, 169-184.

Coldham, N.G. and Sauer, M.J. (2000). Pharmacokinetics of [^{14}C]genistein in the rat: gender-related differences, potential mechanisms of biological action, and implications for human health. *Toxicol Appl Pharmacol* **164**, 206-215.

Constantinou, A.I., Kamath, N., and Murley, J.S. (1998). Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle, and induces apoptosis of human breast adenocarcinoma MCF-7 cells. *Eur J Cancer* **34**, 1927-1934.

Cotran, R.S., Kumar, V., and Robbins, S.L. (1994). Neoplasia. In: Pathological basis of disease. S.L. Robbins, ed. (Philadelphia: WB Saunders), pp. 241-303.

Coward, L., Barnes, N.C., Setchell, K.D.R., and Barnes, S. (1993). Genistein, daidzein, and their beta-glycoside conjugates: anti-tumour isoflavones in soybean foods from American and Asian diets. *J Agric Food Chem* **41**, 1961-1967.

Croisy-Delcey, M., Croisy, A., Mousset, S., Letourneur, M., Bisagni, E., Jacquemin-Sablon, A., and Pierre, J. (1997). Genistein analouges: effects on epidermal growth factor receptor tyrosine kinase and on stress-activated pathways. *Biomed Pharmacother* **51**, 286-294.

Darbon, J., Penary, M., Escalas, N., Casagrande, F., Goubin-Gramatica, F., and Ducommun, B. (2000). Distinct Chk2 activation pathways are triggered by genistein and DNA-damaging agents in human melanoma cells. *J Biol Chem* **275**, 15363-15369.

Darmon, A.J., Nicholson, D.W., and Bleakley, R.C. (1995). Activation of the apoptotic protease CPP32 by cytotoxic T-cell derived granzyme. *Nature* **377**, 446-448.

Davies, J.N., Kucuk, O., and Sarkar, F.H. (1999). Genistein inhibits NFkB activation in prostate cancer cells. *Nutr Cancer* **35**, 167-174.

Davis, J.N., Singh, B., Bhuiyan, M., and Sarkar, F.H. (1998). Genistein-induced upregulation of $p21^{WAF1/CIP1}$, downregulation of cyclin B, and induction of apoptosis in prostate cancer cells. *Nutr Cancer* **32**, 123-131.

Davis, J.N., Muqim, N., Bhuiyan, M., Kucuk, O., Pienta, K.J., and Sarkar, F.H. (2000). Inhibition of prostate specific antigen expression by genistein in prostate cancer cells. *Int J Oncol* **16**, 1091-1097.

Davies, R.J. (1999). Signal transduction by the c-Jun N-terminal kinase. Biochemical Society Symposium *64*, 1-12.

Deng, T. and Karin, M. (1994). c-Fos transcriptional activity stimulated by H-ras-activated protein kinase distinct from JNK and ERK. *Nature* **371**, 171-175.

Dickson, R.B. and Lippman, M.E. (1995). Growth factors in breast cancer. *Endocr Rev* **16**, 559-589.

Dickson, R.B., Gottardis, M.M., Merlino, G.T. (1991). Molecular insights into breast cancer from transgenic mouse models. *Bioessays* **13(11)**, 591-596.

Divi, R.L. and Doerge, D.R. (1996). Inhibition of thyroid peroxidase by dietary isoflavaniods. *Chem Res Toxicology* **9**, 16-23.

Divi, R.L., Chang, H.C. and Doerge, D.R. (1997). Anti-thyroid isoflavones from soybean; isolation, characterisation and mechanisms of action. *Biochem Pharmacol* **54**, 1087-1096.

Dixon-Shanies, D. and Shaikh, N. (1999). Growth inhibition of human breast cancer cells by herbs and phyto-oestrogens. *Oncol Rep* **6**, 1383-1387.

Dong, Z., Birrer, M.J., Watts, R.G., Matrisian, L.M., and Colburn, N.H. (1994). Blocking of tumour promotor-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc Natl Acad Sci USA* **91**, 609-613.

Dong, Z., Ma, W., Huang, C., and Yang, C.S. (1997). Inhibition of tumour promotor-induced activator protein 1 activation and cell transformation by tea polyphenols, epigallocatechin gallate, and theaflavins. *Cancer Res* **57**, 4414-4419.

Dotzlaw, H., Leygue, E., Watson, P., and Murphy, L.C. (1996). Expression of oestrogen receptor β in human breast tumours. *J Clin Endocrinol Metab* **82**, 2371-2374.

Eissa, S., Labib, R., Khalifa, A., Swelam, N., Khalil, F., and ElShenawy, A.M. (1999). Regulators of apoptosis in human breast cancer. *Clin Biochem* **32**, 321-326.

El-Deiry, W.S., Takashi, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumour suppression. *Cell* **75**, 817-825.

Engel, L.W., Young, N.A., Tralka, T.S., Lippma, M.E., O'Brien S.J., and Joyce, M.J. (1978). Establishment and characterisation of three new continuous cell clines derived from human breast carcinomas. *Cancer Res* **38(10)**, 3352-3364.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J. (1997). Human oestrogen receptor β gene structure, chromosomal localisation and expression pattern. *J Clin Endocrinol Metab* **82**, 4258-4265.

Enmark, E. and Gustafsson, J. (1998). Oestrogen receptor β - a novel receptor opens up new possibilities for cancer diagnosis and treatment. Endocrine-Related Cancer *5*, 213-222.

Fernandez, P.L., Jares, P., Rey, M.J., Campo, E., and Cardesa, A. (1998). Cell cycle regulators and their abnormalities in breast cancer. *J Clin Path Mol Path* **51**, 305-309.

Fioravanti, L., Cappelletti, V., Miodini, P., Ronchi, E., Brivio, M., and DiFronzo, G. (1998). Genistein in the control of breast cancer cell growth: insights into the mechanism of action *in vitro*. *Cancer Lett* **130**, 143-152.

Foletta, V.C. (1996). Transcription factor AP-1, and the role of Fra-2. *Immunol Cell Biol* **74**, 121-133.

Fotsis, T., Pepper, M., Adlercreutz, H., Fleischmann, G., Hase, T., Montesano, R., and Schweigerer, L. (1993). Genistein, a dietary-derived inhibitor on *in vitro* angiogenesis. *Proc Natl Acad Sci USA* **90**, 2690-2694.

Franks, L.M and Teich, N.M. (1998). Introduction to the Cellular and Molecular Biology of Cancer. L.M. Franks and N.M. Teich, eds. (Bath: Oxford University Press).

Freathy C., Brown, D.B., Roberts, R.A and Cain, K. (2000). Transforming growth factor beta (1) induces apoptosis in rat FaO hepatoma cells via cytochrome c release and oligomerization of Apaf-1 to form a approximate 700-kd apoptosome caspase processing complex. *Hepatology* **32(4)**, 750-760.

Fritz, W.A., Coward, L., Wang, J., and Lamartiniere, C.A. (1998). Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. *Carcinogenesis* **19**, 2151-2158.

Fuqua, S.A.W., Schiff, R., Parra, I., Freidrichs, W.E., Su, J., McKee, D.D., Slentz-kesler, K., Moore, L.B., Willson, T.M., and Moore, J.T. (1999). Expression of wild-type oestrogen receptor β and variant isoforms in human breast cancer. *Cancer Res* **59**, 5425-5428.

Gaffney, E.V., Pigott, D.A., and Grimaldi, M.A. (1979). Human serum and the growth of human mammary cells. *J Natl Cancer Inst* **63(4)**, 913-918.

Germain, M., Affart, E.B., D'Amours, D., Dixit, V.M., Salvesen, G.S., and Poirier, G.G. (1999). Cleavage of automodified poly (ADP-ribose) polymerase during apoptosis. *J Biol Chem* **274**, 28379-28384.

Gescher, A., Pastorino, U., Plummer, S.M., and Manson, M.M. (1998). Suppression of tumour development by substances derived from the diet - mechanisms and clinical implications. *Br J Clin Pharmacol* **45**, 1-12.

Giguere, V., Tremblay, A., and Tremblay, G.B. (1998). Oestrogen receptor β: Re-evaluation of oestrogen and anti-oestrogen signalling. *Steroids* **63**, 335-339.

Heinonen, S., Wahala, K., and Adlercreutz, H. (1999). Identification of isoflavone metabolites dihydrodaidzein, dihydrogenistein, 6'-OH-O-dma, and cis-4-OH-equol in human urine by gas chromotagraphy-mass spectroscopy using authentic reference compounds. *Anal Biochem* **274**, 211-219.

Hsieh, C.Y., Santell, R.C., Haslam, S.Z., and Helferich, W.G. (1998). Oestrogenic effects of genistein on the growth of oestrogen receptor- positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. *Cancer Res* **58**, 3833-3838.

Ichijo, H. (1999). From receptors to stress-activated MAP kinases. *Oncogene* **18**, 6087-6093.

Ingram, D., Sanders, K., Kolybaba, M., and Lopez, D. (1997). Case-control study of phyto-oestrogens and breast cancer. *Lancet* **350**, 990-994.

Johnson, G.L. and Vaillancourt, R.R. (1994). Sequential protein kinase reactions controlling cell growth and differentiation. *Curr Opin Cell Biol* **6**, 230-238.

Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270**, 16483-16486.

Karin, M. (1996). The regulation of AP-1 activity by mitogen-activated protein kinases. *Phil. Trans. R. Soc. Lond.* **351**, 127-134.

Karin, M. and Hunter, T. (1995). Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr Opin Cell Biol* **5**, 747-757.

Katzenellenbogen, B.S. (1996). Oestrogen Receptors: bioactivites and interactions with cell signalling pathways. *Biol Reprod* **54**, 287-293.

Kaufmann, W.K. (1998). Human topoisomerase II functions, tyrosine phosphorylation and cell cycle checkpoints. *Proc Soc Exp Biol Med* **217**, 327-334.

Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-257.

Kienhuis, C.B.M., Slyser, M., de Goeij, C.C.J., Koenders, P.G., and Benraad, T.J. (1993). Epidermal growth factor receptor levels increase but epidermal growth factor ligand levels decrease in mouse mammary tumours during progression from hormone dependence to hormone independence. *Breast Cancer Res Treat* **26**, 289-295.

Kieran, M.W., Katz, S., Vail, B., Zon,L.I., and Mayer, B.J. (1999). Concentration-dependent positive and negative regulation of a MAP kinase by a MAP kinase kinase. *Oncogene* **18**, 6647-6657.

Kim, H., Peterson, T.G., and Barnes, S. (1998). Mechanisms of action of the soy isoflavone genistein: emerging role for its effects via transforming growth factor β signalling pathways. *Am J Clin Nutr* **68,** 1418S-1425S.

King, R.A. and Bursill, D.B. (1998). Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am J Clin Nutr* **67**, 867-872.

Kuiper, G.J.M., Carlsson, B., Grandien, K., Enmaek, E., Haggblad, J., Nilsson, S., and Gustafsson, J. (1997). Comparison of the ligand binding specificity and trancsript tissue distribution of oestrogen receptors α and β. *Endocrinol* **138**, 863-870.

Kuiper, G.J.M., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. (1996). Cloning of a novel oestrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* **93**, 5925-5930.

Kuiper, G.G.J.M., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., vanderSaag, P.T., vanderBurg, P., and Gustafsson, J.A. (1998). Interaction of oestrogenic chemicals and phyto-oestrogens with oestrogen receptor beta. *Endocrinol* **139**, 4252-4263.

Kumi-Diaka, J. and Butler A. (2000). Caspase-3 protease activation during the process of genistein-induced apoptosis in TM4 testicular cells. *Biol Cell* **92**, 115-124.

Kuzumaki, t., Kobayashi, T., and Ishikawa, K. (1998). Genistein induces p21 expression and blocks the G1 to S phase transition in mouse fibroblast and melanoma cells. *Biochem Biophys Res Commun* **251**, 291-295.

Lamartiniere, C.A., Moore, J., Holland, M., and Barnes, S. (1995). Neonatal genistein chemoprevents mammary cancer. *Proc Soci Exp Biol Med* **208**, 120-123.

Lamartiniere, C.A. and Wang, J. (1999). Genistein: breast cancer protection and *in vivo* mechanisms of action. *J Med Food* **2**, 151-157.

Lamartiniere, C.A., Britton Murrill, W., Manzolillo, P.A., Zhang, J., Barnes, S., Zhang, X., Wei, H., and Brown, N.M. (1998). Genistein alters the ontogeny of mammary gland development and protects against chemically-induced mammary cancer in rats. *Proc Soci Exp Biol Med* **217**, 358-364.

Lamp, S.J., Findlay, D.M., Moseley, J.M. and Martin, T.J. (1981). Calcitonin induction of persistent activated state of adenylate cyclase in human breast T47-D cells. *J Biol Chem* **23**, 12269-12274.

Landberg, G. and Roos, G. (1997). The cell cycle in breast cancer. *APMIS* **105**, 575-589.

Lange, C.A., Richer, J.K., Shen, T., and Horwitz, K.B. (1998). Convergence of progesterone and epidermal growth factor signalling in breast cancer. *J Biol Chem* **273**, 31308-31316.

LeBail, J.C., Varnat, F., Nicolas, J.C., and Habrioux, G. (1998). Oestrogenic and antiproliferative activities on MCF-7 human breast cancer cells by flavonoids. *Cancer Lett* **130**, 209-216.

Lee, S.K., Song, L., Mata-Greenwood, E., Kelloff, G.J., Steele, V., and Pezzuto, J.M. (1999). Modulation of *in vitro* biomarkers of the carcinogenic process by chemopreventive agents. *Anticancer Res* **19**, 35-44.

Lee, S.F. and Lin, J. (1997). Inhibitory effects of phytopolyphenols on TPA-induced transformation, PKC activation, and c-jun expression in mouse fibroblast cells. *Nutr Cancer* **28**, 177-183.

Leppa, S. and Bohmann, D. (1999). Diverse functions of JNK signalling and c-Jun in stress response and apoptosis. *Oncogene* **18**, 6158-6162.

Leygue, E., Dotzlaw, H., Watson, P.H., and Murphy, L.C. (1998). Altered oestrogen receptor alpha and beta messenger RNA expression during human breast tumourigenesis. *Cancer Res* **58**, 3197-3201.

Leygue, E., Dotzlaw, H., Watson, P., and Murphy, L.C. (1999). Expression of oestrogen receptor β 1, β 2, and β 5 messenger RNAs in human breast tissue. *Cancer Res* **59**, 1175-1179.

Li, Y.W., Bhuiyan, M., and Sarkar, F.H. (1999a). Induction of apoptosis and inhibition of c-erbB-2 in MDA-MB-435 cells by genistein. *Int J Oncol* **15**, 525-533.

Li, Y., Upadhyay, S., Bhuiyan, M., and Sarkar, F.H. (1999b). Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. *Oncogene* **18**, 3166-3172.

Lian, F., Bhuiyan, M., Li, Y.W., Wall, N., Kraut, M., and Sarkar, F.H. (1998). Genistein induced G2/M arrest, p21(waf1) upregulation, and apoptosis in a non-small-cell lung cancer cell line. *Nutr Cancer* **31**, 184-191.

Lian, F., Li, Y., Bhuiyan, M., and Sarkar, F.H. (1999). P53 independent apoptosis induced by genistein in lung cancer cells. *Nutr Cancer* **33(2)**, 125-131.

MacFarlane, M., Cain, K., Sun, X., Alnemri, E S., and Cohen, G. M. (1997). Processing /activation of at least four interleukin 1β - converting enzyme-like proteases during the execution phase of apoptosis in human monocytic tumour cells. *J Cell Biol* **137**, 469-479.

Martin, P.M., Horwitz, K.B., Ryan, D.S., and McGuire, W.L. (1978). Phyto-oestrogen interaction with oestrogen receptors in human breast cancer cells. *Endocrinol* **103**, 1860-1867.

Matsukawa, Y. and Aoike, A. (1993). Genistein arrests cell cycle progression at G_2-M. *Cancer Res* **53**, 1328-1331.

McIntyre, B.S. and Sylvester, P.W. (1998). Genistein and erbstatin inhibition of normal mammary epithelial cell proliferation is associated with EGF-receptor down-regulation. *Cell Prolif* **31**, 35-46.

Messina, M.J., Persky, V., Setchell, K.D.R., and Barnes, S. (1994). Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data. *Nutr Cancer* **21**, 113-131.

Michalides, R.J.A.M. (1999). Cell cycle regulators: mechanisms and their role in aetiology, prognosis, and treatment of cancer. *J Clin Path* **52**, 555-568.

Miodini, P., Fioravanti, L., Di Fronzo, G., and Cappelletti, V. (1999). The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function. *Br J Cancer* **80**, 1150-1155.

Mosselman, S., Polman, J., and Dijkema, R. (1996). ERβ Identification and charaterisation of a novel human oestrogen receptor. *FEBS Lett* **392**, 49-53.

Nagata, C. and Shimizu, H. (1996). Risk factors for breast cancer: findings from comparative studies on Japanese in Japan, Japanese and Whites in the United States. *Gann Monograph Cancer Res* **44**, 51-57.

Nagel, S., vom Saal, F.S., and Welshons, W.V. (1998). The effective free fraction of oestradiol and xenoestrogens in human serum measured by whole cell uptake assays: physiology of delivery modifies oestrogenic activity. *Proc Soc Exp Biol Med* **217**, 300-308.

Nakagawa, H., Yamamoto, D., Kiyozuka, Y., Tsuts, K., Uemura, Y., Hioki, K., Ttsutsui, Y., and Tsubura, A. (2000). Effects of genistein and synergistic action in combination with eicosapentaenoic acid on the growth of breast cancer cell lines. *J Cancer Res Clin Oncol* **126(8)**, 448-454.

Nakatani, K., Thompson, D.A., Barthel, A., Sakaue, H., Liu, W., Weigel, R.J., and Roth, R.A. (1999). Up-regulation of Akt3 in oestrogen receptor dependent-deficient breast cancers and androgen-independent prostate cancer lines. *J Biol Chem* **274**, 21528-21532.

Nguyen, L.Q. and Jameson, J.L. (1998). The cell cycle. In: Principles of Molecular Medicine. J.L. Jameson, ed. (Totowa, NJ. Humana Press Inc.), pp. 65-73.

Ormerod, M.G. (1990). In: Flow Cytometry. A Practical Approach. D. Rickwood, B.D. Hames eds, (Oxford Univ Press), pp75.

Paech, K., Webb, P., Kuiper, G.J.M., Nilsson, S., Gustafsson, J., Kushner, P.J., and Scanlan, T.S. (1997). Differential ligand activation of oestrogen receptors ERα and ERβ at AP-1 sites. *Science* **277**, 1508-1510.

Pagliacci, M.C., Smacchia, G., Migliorati, G., Grignani, F., Riccardi, C., and Nicoletti, I. (1994). Growth-inhibitory effects of the natural phyto-oestrogen genistein in MCF-7 human breast cancer cells. *Eur J Cancer* **30**, 1675-1682.

Pennie, W.D., Aldridge, T.C., and Brooks, A.N. (1998). Differential activation by xenoestrogens of ER-α and ER-β when linked to different response elements. *J Endocrinol* **158**, R11-R14.

Peterson, D.N., Tkalcevic, G.T., Koza-Taylor, P.H., Tin, T.G., and Brown, T.A. (1998). Identification of oestrogen receptor beta-2, a functional variant of oestrogen receptor beta expressed in normal rat tissues. *Endocrinol* **139**, 1082-1092.

Peterson, T.G. (1995). Evaluation of the biochemical targets of genistein in tumour cells. *J Nutr* **125**, 784S-789S.

Peterson,T. G. and Barnes, S. (1993). Genistein and biochanin A inhibit the growth of human prostate cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* **22**, 335-345.

Peterson, T.G. and Barnes, S. (1996). Genistein inhibits both oestrogen and growth factor stimulated proliferation of human breast cancer cells. *Cell Growth Differ* **7**, 1345-1351.

Peterson, T.G., Coward, L., Kirk, M., Falany, C.N., and Barnes, S. (1996). The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A. *Carcinogenesis* **17**, 1861-1869.

Peterson, T.G., Ji, G., Kirk, M., Coward, L., Falnay, C.N., and Barnes, S. (1998). Metabolism of the isoflavones genistein and biochanin A in human breast cancer cell lines. *Am J Clin Nutr* **68(suppl)**, 1505S-1511S.

Pitot, H. (1989). Progression: the terminal stage in carcinogenesis. *Jpn J Cancer Res* **80**, 599-607.

Reinli, K. and Block, G. (1996). Phyto-oestrogen content of foods- a compendium of literature values. *Nutr Cancer* **26**, 123-148.

Robinson, M.J. and Cobb, M.H. (1997). Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* **9**, 180-186.

Sainsbury, J.R.C., Sherbert, G.V., Farndon, J.R., and Harris, A.L (1985). Epidermal-growth-factor receptors and oestrogen receptors in human breast cancer. *Lancet Feburary* **16**, 364-366.

Salti G.I., Grewal, S., Mehta, R.R., Das Gupta, T.K., Boddie Jr, A.W., and Constantinou, A.I. (2000). Genistein induces apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells. *Eur J Cancer* **36**, 796-802.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). SDS-Polyacrylamide gel electrophoresis of proteins. In Molecular Cloning: A Laboratory Manual. J. Sambrook, E. F. Fritsch, and T. Maniatis eds (Cold Spring Harbor Laboratory Press), pp18.47.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463.

Santell, R.C., Kieu, N., and Helferich, W.G. (2000). Genistein inhibits growth of oestrogen-independent human breast cancer cells in culture but not in athymic mice. *J Nutr* **130**, 1665-1669.

Satomi, Y., Miyamoto, S., and Gould, M.N. (1999). Induction of AP-1 activity by perillyl alcohol in breast cancer cells. *Carcinogenesis* **20**, 1957-1961.

Saunders, P.T.K. (1998). Oestrogen receptor beta (ER-β). Reviews in Reproduction. **3**, 164-171.

Schultze-Mosgau, M., Dale, I.L., Gant, T.W., Chipman, J.K., Kerr, D.J., and Gescher, A. (1998). Regulation of *c-fos* transcription by chemopreventive isoflavanoids and lignans in MDA-MB-468 breast cancer cells. *Eur J Cancer* **34**, 1425-1431.

Setchell, K.D.R., Borriello, S.P., Hulme, P., Kirk, D.N., and Axelson, M. (1984). Non-steriodal oestrogens of dietary origin: possible roles in hormone-dependent disease. *Am J Clinl Nutr* **40**, 569-578.

Setchell, K.D.R. (1998). Phyto-oestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am J Clinl Nutr* **68**, 1333S-1346S.

Shao, Z., Alpaugh, M.L., Fontana, J.A., and Barsky, S.H. (1998). Genistein inhibits proliferation similarly in oestrogen receptor-positive and negative human breast carcinoma cell lines characterized by p21(WAF1/CIP1) induction, G2/M arrest, and apoptosis. *J Cell Biochem* **69**, 44-54.

Sherr, C.J. (1996). Cancer cell cycles. *Science* **274**, 1672-1677.

Smith, L.M., Birrer, M.J., Stampfer, M.R., and Brown, P.H. (1997). Breast cancer cells have lower activating protein 1 transcription factor activity than normal mammary epithelial cells. *Cancer Res* **57**, 3046-3054.

Snedecor, G.W. and Cochran, W.G. (1980). Analysis of variance. In: Statistical Methods. G.W. Snedecor and W.G. Cochran eds (Ames: The Iowa State University Press), pp215-237.

Soule, H.D., Vazguez, J., Long, A., Albert, S., and Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* **51(5)**, 1409-1416.

van Agthoven, T., van Agthoven, T.L.A., Portengen, H., Foekens, J.A., and Lambert, C.J. (1992). Ectopic expression of epidermal growth receptors induces hormone independence in ZR-75.1 human breast cancer cells. *Cancer Res* **52**, 5082-5088.

Vladusic, E.A., Hornby, A.E., Guerra-Vladusic, F.K., and Lupu, R. (1998). Expression of oestrogen receptor β messenger RNA variant in breast cancer. *Cancer Res* ***58***, 210-214.
Wahala, K., Salakka, A., and Adlercreutz, H. (1998). Synthesis of novel mammalian metabolites of the isoflavanoid phyto-oestrogens daidzein and genistein. *Proc Soc Exp Biol Med* **217**, 293-299.

Walker, R.A., Jones, J.L., Chappell, S., Walsh, T., and Shaw, J.A. (1997). Molecular pathology of breast cancer and its application to clinical management. *Cancer Metastasis Rev* **16**, 5-27.

Wang, T.T.Y., Sathamoorthy, N and Phang, J.M. (1996). Molecular effects of genistein on oestrogen receptor mediated pathways. *Carcinogenesis* **17**, 271-275.

Wang, Y., Yaping, E., Zhang, X., Lebwohl, M., DeLeo, V., and Wei, H. (1998). Inhibition of ultraviolet B (UVB)-induced *c-fos* and *c-jun* expression *in vivo* by a tyrosine kinase inhibitor genistein. *Carcinogenesis* **19**, 649-654.

Watanabe, T., Inoue, S., Ogawa, S., Ishii, Y., Hiroi, H., Ikeda, K., Orimo, A., and Muramatsu, M. (1997). Agonistic effect of tamoxifen is dependent on cell type, ERE-promoter context and oestrogen receptor subtype: functional difference between oestrogen receptors α and β. *Biochem Biophys Res Commun* **236**, 140-145.

Wattenberg, L.W. (1995). An overview of chemoprevention: current status and future prospects. *Proc Soc Exper Biol Med.* **216**, 133-141.

Wei, H., Wei, L., Frenkel, K., Bowen, R., and Barnes, S. (1993). Inhibition of tumour promotor-induced hydrogen peroxide formation *in vitro* and *in vivo* by genistein. *Nutr Cancer* **20**, 1-12.

Wei, H., Bowen, R., Cai, Q., Barnes, S., and Wang, Y. (1995). Antioxidant and antipromotional effects of the soybean isoflavone genistein. *Proc Soc Exp Biol Med* **208**, 124-130.

Welsch, P.L., Scubert, E.L., and King, M.C. (1998). Inherited breast cancer: an emerging picture. *Clin Genetics* **54**, 447-458.

Welshons, W.V., Nagel, S.C., and vom Saal, F.S. (1999). Protein binding interations: approaches to quantifying 'free' and 'bound' fractions of genistein and other xenobiotic oestrogens in serum. *J Med Food* **2**, 135-137.

Whitten, P.L., Lewis, C., Russell, E., and Naftolin, F. (1995). Potential adverse effects of phyto-oestrogens. *J Nutr* **125**, 771S-776S.

Wisdom, R., Jonhson, R.S., and Moore, C. (1999). c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. *EMBO J* **18**, 188-197.

Wiseman, H. (1998). Oestrogen metabolism and breast cancer risk. *Biochem Soc Trans* **27**, 299-304.

Xing, C. and Imagawa, W. (1999). Altered MAP kinase (ERK1,2) regulation in primary cultures of mammary tumour cells: elevated basal activity and sustained response to EGF. *Carcinogenesis* **20**, 1201-1208.

Yamishita, Y., Kawada, S., and Nakano, H. (1990). Induction of mammalian topoisomerase II dependent DNA cleavage by non-intercalative flavanoids, genistein and orobol. *Biochem Pharmacol* **39**, 737-744.

Yan, C. and Han, R. (1998). Genistein suppresses adhesion-induced protein tyrosine phosphorylation and invasion of B16-BL6 melanoma cells. *Cancer Lett* **129**, 117-124.

Yasuda, T., Mizunama, S., Kano, Y., Saito, K., and Oshawa, K. (1996). Urinary and billiary metabolites of genistein in rats. *Biol Pharm Bull* **19(3)**, 413-417.

Zava, D.T. and Duwe, G. (1997). Oestrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells *in vitro*. *Nutr Cancer* **27**, 31-40.

Zava, D.T., Blen, M., and Duwe, G. (1997). Oestrogenic activity of natural and synthetic oestrogens in human breast cancer cells in culture. *Environ Health Perspect* **105**, 637-645.

Zhou, Y. and Lee, A.S. (1998). Mechanism for the suppression of the mammalian stress response by genistein, an anti-cancer phyto-oestrogen from soy. *J Natl Cancer Inst* **90**, 381-388.

Zi, X. and Agarwal, R. (1999). Modulation of mitogen-activated protein kinase activation and cell cycle regulators by the potent skin cancer preventive agent silymarin. *Biochem Biophys Res Commun* **263**, 528-536.

APPENDIX

PRIMER SEQUENCES

Oestrogen receptor β first round primers

Forward: 5' - TCA CTT CTG CGC TGT CTG CAG CG –3'

Reverse: 3' - CTT GGG TCG CTG TGA CCA GA –5'

Oestrogen receptor β second round primers

Forward: 5'- GCC CAA GAG AAG TGG CGG CCA GC -3'

Reverse: 3'- AAA TCA AGT AGT TGC CAG GAG C -5'

AP-1 oligonucleotide sequence

Forward: 5' - CGC TTG ATG AGT CAG CCG GAA - 3'

Reverse: 3' - GCG AAC TAC TCA GTC GGC CTT - 5'

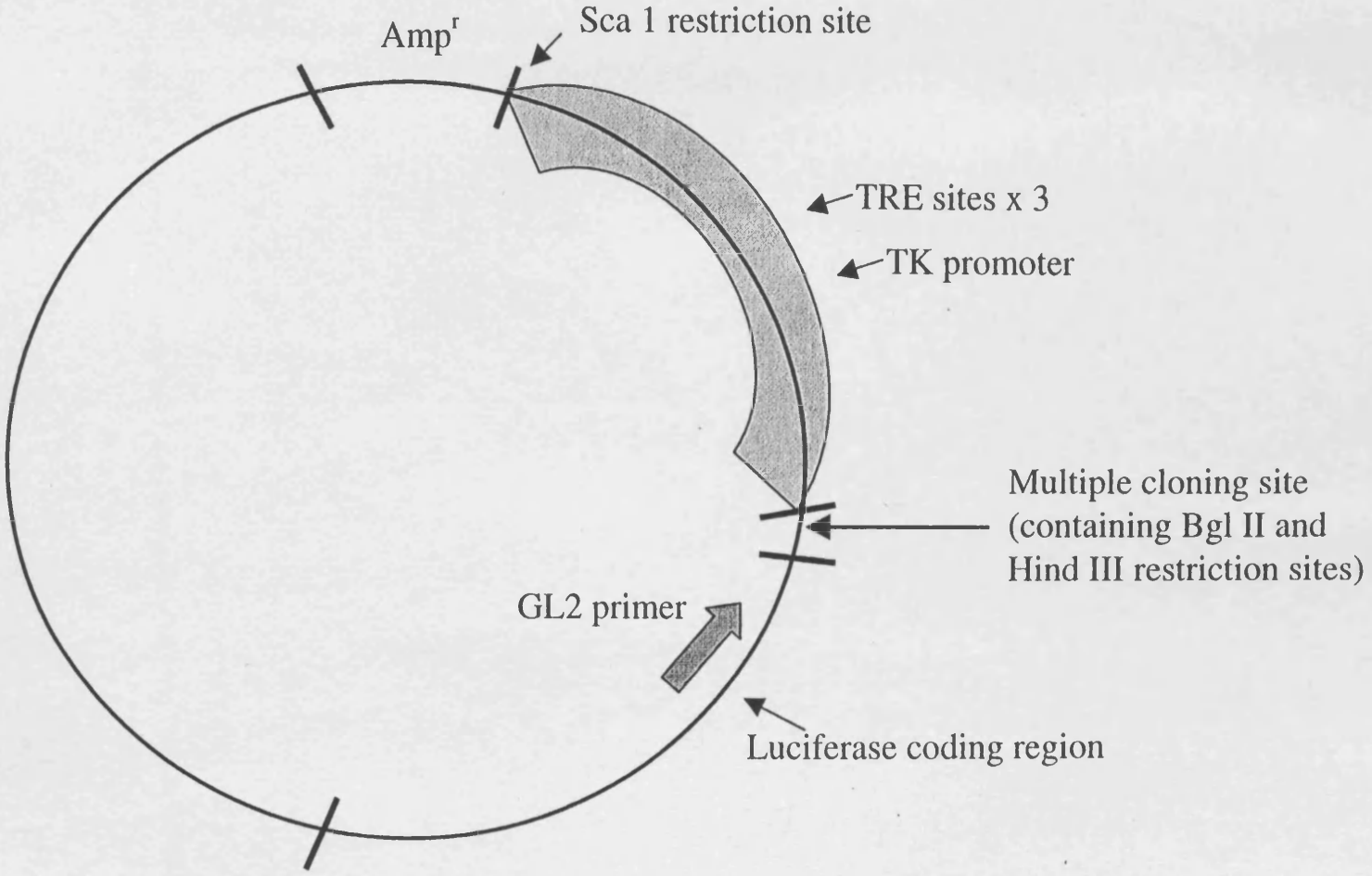
NFκB oligonucleotide sequence

Forward: 5' - AGT TGA GGC GAC TTT CCC AGG C - 3'

Reverse: 3' - TCA ACT CCC CTG AAA GGG TCC G - 5'

GL2 primer sequence (counter clockwise)

5' – CTT TAT GTT TTT GGC GTC TTC CA – 3'



Simple circle map of TRE reporter construct (5.3 kB) showing Sca I, Bgl II and Hind III restriction sites, position and direction of GL2 primer and site of PBLCAT2 insertion (1.1kB) into a pGL2 basic vector (4.2kB).