Drug sensitivity and apoptosis in tamoxifen resistant breast cancer.

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Acknowledgements.

Thanks to everyone in the Tenovus centre for cancer research at the the Welsh School of Pharmacy, who were there with practical help and advice when it was needed most.

Thanks also to the Pharmacy dept. at Velindre hospital, Cardiff, for providing many of the drugs used in this study.

Finally, thanks to my family and friends, who have given me all the help and support I could ever need.

Drug sensitivity and apoptosis in tamoxifen resistant breast cancer.

Tamoxifen has long been used in the treatment of hormone responsive breast cancer. However, tumours frequently develop resistance within 2-5 years of treatment, characterised by the return of tumour growth.

The epidermal growth factor receptor (EGFR) is an important contributing factor in allowing formerly tamoxifen sensitive tumours to grow in the presence of tamoxifen. High levels of EGFR in many tumours correlate with a poor prognosis and an increased resistance to cytotoxic drugs. It was the initial aim of this study to ascertain whether the increased EGFR signalling associated with tamoxifen resistance results in a phenotype more resistant to cytotoxic drugs, and to study the underlying mechanisms that may cause this.

Rather than observing the expected increase in resistance to cytotoxic drugs upon the development of tamoxifen resistance, a greatly increased sensitivity to the radiomimetic drug bleomycin was observed. Inhibition of EGFR in either the tamoxifen sensitive or resistant cells had very little effect on bleomycin sensitivity,

The rate of uptake of various drugs was measured, and found to be identical between tamoxifen sensitive cells and their tamoxifen resistant derivatives. Microarray analysis of mRNA levels of drug efflux proteins also showed no significant decrease in drug efflux pump gene expression, with two efflux pump genes (MRP3 and MRP4) showing increased expression.

Tamoxifen resistant cells displayed greatly increased sensitivity to the apoptosis inducer camptothecin, and showed a significant increase in the levels of activated caspases present. Immunocytochemistry revealed a significant downregulation of the anti-apoptotic protein bcl-2.. Sensitivity to bleomycin was also measured and was found to inversely correlate to bcl-2 status.

Finally bcl-2 levels were modulated using oestrogens and antioestrogens, and with an siRNA directed against the oestrogen receptor. The effect on bleomycin sensitivity was examined. Reduction of bcl-2 expression by either method had no effect on bleomycin sensitivity

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Abbreviations used in this thesis

AIBC1	- Amplified in breast cancer 1
ABC	- ATP binding cassette.
ABCB	- ATP binding cassette (subfamily B)
ABCC	- ATP binding cassette (subfamily C)
ABCG	- ATP binding cassette (subfamily G)
ADAM	- A Disintegrin And Metalloprotease-like (protein)
AF-1	- Activation function 1 (of the oestrogen receptor)
AF-2	- Activation function 2 (of the oestrogen receptor)
ATM	- Ataxia-telangiectasia mutated.
ATP	- Adenosine triphosphate
BASC	- BRCA1-associated genome surveillance complex
BCRP	- Breast cancer resistance protein
BH	- Bcl-2 homology (domain)
BH1	- Bcl-2 homology 1 (domain)
BH2	- Blc-2 homology 2 (domain)
BH3	- Bcl-2 homology 3 (domain)
BH4	- Bcl-2 homology 4 (domain)
BRCA1	- Breast cancer susceptibility gene 1
BRCA2	- Breast cancer susceptibility gene 2
CAD	- Caspase-activated deoxyribonuclease
cAMP-	- Cyclic adenosine monophosphate
CASP	- Comet assay software project
Cbl	- Casitas b-lineage lymphoma
CCRF	- Cytochrome-C releasing factor
cDNA	- Complementary Deoxy-Ribonucleaic Acid
C/EBPβ	- CCAAT enhancer binding protein
CFTR	- Cystic fibrosis transmembrane conductance regulator
CREB	- Cyclic adenosine monophosphate binding-element
dATP	- Deoxyadenosine triphosphate
DBD	- Deoxyribonucleic acid binding domain
DD	- Death domain
DED	- Death effector domain
DISC	- Death-inducing signalling complex
DNA	- Deoxyribonucleaic acid
DNA-PK	- Deoxyribonucleaic acid dependent protein kinase
DMSO	- Dimethyl sulphoxide
DSB	- Double stranded break (in DNA)
E2F1	- E2F transcription factor 1
EDTA	- Ethylenediaminetetraacetic acid
EGF	- Epidermal growth factor
EGFR	- Epidermal growth factor receptor
ER	- Estrogen receptor / oestrogen receptor
ERα	- Estrogen receptor / oestrogen receptor α
ERβ	- Estrogen receptor / oestrogen receptor β
ERE	- Estrogen/oestrogen response element
Ets	- Erythroblast transformation specific
FADD	- Fas-associated protein with death domain
FISH	- Forced *in-situ* hybridisation

FITC	- Fluorescein isothiocyanate
FLICE	- FADD-like interleukin1 converting enzyme
FLIP	- FLICE inhibitory protein
FSH	- Follicle-stimulating hormone
GrB	- Granzyme B
GDP	- Guanosine diphosphate
GTP	- Guanosine triphosphate
HMG-1	- High motility group (protein) 1
HRAS	- Harvey rat sarcoma viral oncogene homolog
HRR	- Homologous recombinational repair
HSP90	- Heat-shock protein (90kD)
hTdp1	- Human tyosyl-DNA phosphodiesterase
IAP	- Inhibitor of apoptosis protein
ICAD	- Inhibitor of caspase-activated deoxyribonuclease
IL-3	- Interleukin-3
Jak2	- Janus kinase 2
JNK	- C-jun N-terminal kinase
LBD	- Ligand binding domain
LH	- Luteinising hormone
LHRH	- Luteinising hormone releasing hormone
LMPA	- Low melting point agarose
LMP1	- Latent membrane protein (of Epstein-Barr virus)
MAP Kinase	- Mitogen activated protein kinase
MDM2	- Mouse double minute 2 (human homolog of)
MDR	- Multidrug resistance
MEKK4	- Mitogen activated protein kinase kinase kinase 4
MNU	- Methylnitrosourea
MRP	- Multidrug resistance protein
mRNA	- Messenger ribonucleic acid
NBD	- Nucleotide binding domain
NHEJ	- Non-homologous end joining
NSCLC	- Non-small cell lung cancer
OTM	- Olive tail moment
PCNA	- Proliferating cell nuclear antigen
PAK2	- p21 activated kinase 2
p14arf	- p14 alternate reading frame
PBS	- Phosphate-buffered saline
Pgp	- P-glycoprotein
PIP2	- Phosphatidyl inositol-4,5-bisphosphate
PIP3	- Phosphatidyl inositol-3,4,5-triphosphate
PKB	- Protein kinase B
PKC	- Protein kinase C
PLC-γ	- Phospholipase C gamma
PNK	- Polynucleotide kinase
PTB	- Phosphotyrosine binding (domain)
PTEN	- Phosphatase and tensin homolog
RNA	- Ribonucleaic acid
RPA	- Replication protein A
RPMI	- Roswell park memorial institute
RTK	- Receptor tyrosine kinase

SCGE	- Single cell gel electrophoresis
SDS-PAGE	- Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS	- Sodium dodecyl sulphate
SERM	- Selective oestrogen (estrogen) receptor modulator
SH2	- Src homology 2 (domain)
siRNA	- Small interfering ribonucleaic acid
SOS	- Son of sevenless
SRC	- Steroid receptor cofactor
TGF-α	- Transforming growth factor alpha
TKB	- Tyrosine kinase binding (domain)
TNF	- Tumour necrosis factor
TNFR-1	- Tumour necrosis factor receptor - 1
TRAIL	- TNF-related apoptosis inducing ligand
ZPR1	- Zinc finger protein 1

Chapter 1
Introduction.

1.1 Breast Cancer.

Breast cancer is the unchecked and rapid growth of a population of cells within the breast tissue. The tumour that results from this uncontrolled division may be termed either benign or malignant. Benign tumours can grow to a considerable size, but are unable to spread to neighbouring tissues and thus can be easily removed without spread or reoccurrence. Malignant tumours however are able to spread to neighbouring tissues and thus represent a serious clinical problem, causing more widespread damage and being more difficult to eradicate. In epithelial tissue a tumour is usually considered malignant if it is able to penetrate the basal lamina. Breast cancers frequently occur in the cells making up the ducts of the breast (ductal carcinomas) and in the cells that line the lobes of the breast (lobular carcinomas). In some rarer cancers cancerous cells can grow inside lymph vessels (inflammatory breast cancer) or underneath the nipple (Paget's disease).

1.1.1 Incidence.

Breast cancer is the most frequently diagnosed cancer in women in the United Kingdom, responsible for 30% of all cancers. Around 41,000 British women are diagnosed with breast cancer annually (Data from Office of National Statistics). The disease also affects men, albiet to a much lesser extent, with male breast cancer responsible for around 1% of all breast cancers in the UK (Ying et al., 2005). There is a higher incidence of breast cancer in the western world than in Africa and Asia, although women from Africa and Asia who migrate to western countries experience an incidence of breast cancer equal to that of native westerners after several generations (Ziegler et al., 1993). This indicates that the higher incidence of breast cancer in the west is most likely caused by lifestyle rather than genetic factors.

1.1.2 Risk Factors.

Aside from being a woman, and leading a western lifestyle, numerous other factors have been proven to contribute to the risk of breast cancer development.

1.1.2.1 Age and menstruation.

As with many other cancer types, age is a very important factor concerned with the risk of developing breast cancer, with incidence increasing steadily with age from puberty until the menopause, where it remains steady for several years before steadily increasing until around 80 years, where the rate of incidence begins to decrease. The ages at which menstruation begins and ends (Thomas, 1984) are important risk factors for breast cancer, with early menarche and/or late menopause resulting in increased breast cancer risk, most likely due to increased exposure to oestrogen during an increased number of cumulative menstrual cycles (Chavez-MacGregor et al., 2005).

1.1.2.2 Reproductive history.

It has long been established that pregnancy at a young age protects against breast cancer, with women who become pregnant before the age of 18 displaying 40% of the breast cancer risk of nulliparous women. (MacMahon et al., 1970). A number of possible explanations have been proposed for this effect, with the most widely held opinion being that the differentiation of the breast tissue during pregnancy causes a decrease in the number of epithelial cells liable to become cancerous (so called stem 1 cells). Examination of breast tissue from postmenopausal parous and non-parous women has confirmed a decrease in stem 1 cells in the breasts of parous women, with an increase in stem 2 cells, which are epithelial but refractory to transformation (Russo et al., 2006; Russo et al., 2005). Studies in rats have shown that mimicking pregnancy using appropriate doses of oestradiol and progesterone has a protective effect against breast carcinogenesis induced by methylnitrosourea (MNU) (Guzman et al., 1999). The risk of developing breast cancer also decreases with each full term pregnancy.

1.1.2.3 Family history.

Around 10% of breast cancers are attributed to a family history of the disease. To date, two genes have been discovered which confer an increased susceptibility to breast cancer, namely breast cancer susceptibility gene 1 (BRCA1) (Hall et al., 1990) and breast cancer susceptibility gene 2 (BRCA2).(Wooster et al., 1994), both of which code for large proteins involved in the maintenance of genomic integrity through interactions with a wide variety of proteins. BRCA1 is a nuclear protein, activated by phosphorylation and capable of contributing to a wide range of cellular processes, including cell cycling, proliferation, several specific DNA repair pathways, centromere function, transcriptional regulation and apoptosis (Rosen et al., 2003). The product of the BRCA2 gene is slightly larger than BRCA1 and also maintains genomic integrity through interactions with molecules concerned with cell cycling, DNA repair and apoptosis such as RAD51 and p53 (Marmorstein et al., 1998). Consistent with this, a particular pathway involved with the repair of DNA is known to be impaired by a lack of BRCA2 (Xia et al., 2001). Like BRCA1, BRCA2 is also thought to have a transcriptional activation function (Milner et al., 1997). Mutations in these genes can result in a lifetime risk of breast cancer of up to 80% to the carrier, depending on the site and nature of the mutation. Mutations in BRCA genes can also cause a very large increase in the risk of developing ovarian cancer (Pal et al., 2005) as well as a smaller increased risk of a variety of cancers, most notably cancers of the stomach and pancreas (Friedenson, 2005). Mutations in BRCA1 account for approximately 40% of familial breast cancer cases (Miki et al., 1994) with mutations in BRCA2 responsible for slightly smaller fraction (35-40%) (Wooster et al., 1995). Of the 20-25% of remaining familial cases, increased breast cancer risk can be inherited by mutations in a wide range other genes such as PTEN, p53 and ATM (Dumitrescu & Cotarla, 2005). The fact that a small percentage of cases of heritable breast cancer are not currently explained by mutations in existing genes has led to speculation of

the existence of a BRCA3 gene on chromosome 13q21 (Thompson et al., 2002).

1.1.2.4 Diet and alcohol.

A number of lifestyle factors are known to affect lifetime breast cancer risk. After the menopause, obese women have an increased risk of developing breast cancer (Carmichael & Bates, 2004). Significant (20kg) weight gain after the age of 18 is also an established risk factor for the development of post menopausal breast cancer (Huang et al., 1997b). It is thought that this increased risk is due to increased oestrogen production within the peripheral adipocytes, and therefore increased exposure of obese women to oestrogens (Stoll, 2000).

Numerous studies have also identified a moderate increase in breast cancer risk associated with alcohol consumption (Smith-Warner et al., 1998). This is thought to be due to alcohol causing increased levels of circulating oestrogens (Singletary & Gapstur, 2001) and the oestrogenic effect of ethanol directly stimulating tumour growth (Fan et al., 2000).

1.1.2.5 Male breast cancer risk factors.

Most risk factors for female breast cancer which can be sensibly applied to men also constitute risk factors for the development of male breast cancer. Factors such as family history, possession of mutated BRCA2, and obesity all can contribute to an increased risk of male breast cancer. Additional risk factors for men include treatment with antiandrogens (for example for Hodgkin's disease) and suffering from Klinefelter's syndrome (Ying et al., 2005).

1.1.3 Oestrogens and breast cancer.

The observation in 1896 that oophorectomy was an effective treatment for metastatic breast cancer in premenopausal women (Beatson, 1896) firmly established the direct relationship between the ovaries and breast cancer progression. At the time the nature of the relationship was

unknown, and the existence of hormones was yet to be proved. Ten years later it was discovered that the ovaries secrete two distinct substances responsible for menstruation and implantation which were later identified as oestrogen and progesterone (Marshall & Jolley, 1906). The discovery of the oestrogen receptor (ER) (Jensen & Jacobson, 1960) initiated the era of molecular studies that revealed that steroid hormones, including oestrogens diffuse into cells and illicit their effect not directly, but via interactions with specific receptors. The oestrogen receptor was shown to act as a transcription factor (O'Malley et al., 1968), and subsequently, a minimal oestrogen response element (ERE) –GTCANNNTGACC- was identified (Klein-Hitpass et al., 1986). A second ER has since been identified (Mosselman et al., 1996), which is usually termed ERβ to distinguish it from the older, conventional ER, which is now referred to as ERα.

The known risk factors for the development of breast cancer affirm the strong relationship between oestrogen signalling and breast cancer. During the development of a breast tumour, oestrogen signalling can be increased in a number of ways. Increased expression of aromatase, one of the enzymes involved in oestrogen biosynthesis, has been detected in breast tissue surrounding breast tumours (Bulun et al., 1993). Breast tumours also frequently overexpress oestrogen receptors compared with normal breast epithelia thus increasing their responsiveness to normal physiological levels of oestrogen.

Three naturally occurring oestrogens account for the vast majority of oestrogen signalling in women, oestradiol, oestriol and oestrone. Of these three endogenous oestrogens, oestradiol displays the strongest affinity for both ERα and Erβ, and oestrone the weakest (Kuiper et al., 1997). Oestrogen biosynthesis is catalysed by aromatase cytochrome P450 (P450arom- the product of the *CYP19* gene), which catalyses the formation of the phenolic A-ring present in oestrogens from C_{19} androgenic substrates such as testosterone and androstenedione (Simpson & Davis, 2001). In premenopausal women, oestrogens act as classical endocrine hormones,

the majority of oestrogen biosynthesis occurs in the ovaries, and circulating levels of oestrogens elicit their effect not only in epithelial breast tissue, but also contribute to the regulation of a variety of other tissues, including urinogenital tissues, the maintenance of bone density and the metabolism of lipids (in particular cholesterol) and carbohydrates (Hamadeh et al., 2005; Liu et al., 2004). The level of circulating oestrogens in premenopausal woman changes throughout the menstrual cycle, and is regulated by the action of the gonadotrophins lutenising hormone (LH) and follicle-stimulating hormone (FSH). Oestrogen biosynthesis also occurs away from the ovaries, with the *CYP450* gene being expressed, and therefore aromatase activity being detected in a number of tissues including adipose tissue, and bone. Following the menopause, gonadal oestrogen biosynthesis ceases, and extra-gonadal oestrogen biosynthesis is responsible for the production of oestrogens (Simpson et al., 2000).

1.1.4 Oestrogen receptor structure and function.

1.1.4.1 ER Structure.

In common with other nuclear receptors, both ER isoforms consist of five separate domains. (fig 1.1). The N-terminal A/B domain, contains AF-1, a transcriptional activator function which is ligand independent and may be activated by growth factor signalling cascades. This domain is poorly conserved between ER isoforms. The C domain, which contains the DNA binding domain (DBD) and contributes to dimerisation, and the E domain which contains the ligand binding domain (LBD) and also the second (ligand dependent) activation function (AF-2) are better conserved between the ER isoforms. They show 96% amino acid identity in the DBDs, and 53% identity in the LBDs respectively (Weihua et al., 2003). This suggests that while the two receptors may vary in their affinity for various ligands, they bind to and regulate similar regions of DNA. The D and F regions display no conservation between ER isoforms at all. Region D is considered a linker between the DBD and LBD and is known to

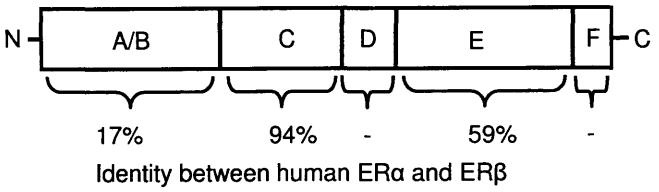


Figure 1.1 Structure of human oestrogen receptor. The main structural regions of both isoforms of the ER display varying amounts of identity between the two isoforms ERα and ERβ. The A/B region contains the constitutively active AF-1 activation function, The C region contains the DNA binding domain and the E region contains the ligand binding domain.

contain the site of Hsp90 binding, while region F can be considered a c-terminal extension of the LBD (Ruff et al., 2000).

1.1.4.2 Ligand binding.

Oestrogen and other ER ligands are able to diffuse through the cell membrane and nuclear envelope and can therefore activate ERs which are primarily located in the nucleus. In common with other members of the steroid receptor superfamily, in the absence of a suitable ligand, ER forms an inactive oligomer in complex with a number of other proteins including immunophilins and heat shock proteins (Pratt & Toft, 1997). The presence of oestrogen causes dissociation of ER from the complex and the dimerisation of ER (Kumar & Chambon, 1988), which enables receptor phosphorylation and a protein conformational change to occur. Heterodimers of ERα and Erβ can be formed as well as homodimers of either ERα or ERβ (Pace et al., 1997). Dimerisation results in the formation of a hydrophobic cleft which is vital for the recruitment of co-activators which allow contact with the general transcriptional machinery. Co-activators promote transcription by facilitating the assembly of the pre-initiation complex, and by re-arranging chromatin to allow access of RNA polymerase to DNA. Members of the steroid receptor coactivator family (SRC-1) are frequently recruited in this role, and a member of the SRC-1 family, AIBC1 has been shown to be upregulated in a significant proportion of breast tumours (Anzick et al., 1997).

This ligand dependent activation of ER is promoted through the AF-2 (activation function 2) domain and is complemented by a second activation function (termed AF-1) which is not dependent on the presence of a ligand and therefore following ER phosphorylation is constitutively active (Moras & Gronemeyer, 1998). Both these factors are thought to be required for the successful promotion of transcription by ER, although the exact contribution made to the recruitment of cofactors by each factor can vary according to a number of factors including cell type (Merot et al., 2004).

As well as directly binding to EREs, and promoting transcription, ER can also mediate changes in gene expression through interaction with other transcription factors. ER has been shown to increase the activity of the AP-1 (Pfahl, 1993) and the Sp1 transcription factors (Duan et al., 1998) and to inhibit the activity of NF-κB (Stein & Yang, 1995).

The effects of ER activation are not restricted to the direct regulation of genes or transcription factors. ER has been shown to directly activate several protein kinase cascades independent of transcriptional regulation, including the Src-Shc-Ras-Raf-MEK-1-ERK pathway (Migliaccio et al., 1996) and the PI3K/AKT pathway (Castoria et al., 2001). These non-genomic effects mediated via ER appear to be ligand specific, with certain ligands eliciting a mild effect on the transcriptional activity of ER, but a strong effect on protein kinase activity and *vice versa* (Wessler et al., 2005). The activation of the ERK and PI3K/AKT pathways by ER has been shown to be dependent on plasma membrane oestrogen receptors (Chen et al., 1999; Levin, 1999), which are products of the same transcript as nuclear ER, but localised to calveolae, small organelles on the cell surface which facilitate the congregation of signalling molecules (Kim et al., 1999). The genomic and non genomic effects of ER on intracellular signalling pathways are summarised in fig 1.2.

1.1.5 Blocking ER signalling as a therapeutic strategy for the treatment of breast cancer

As has been discussed, the growth of many breast cancers is driven by an increase in oestrogen signalling. Increased oestrogen signalling can be achieved in a number of ways, through increased production of enzymes responsible for the production of oestrogen, through increased sensitivity of cells to oestrogen by ER overexpression, and by increased availability of the cofactors which help ER to modulate gene expression. Therapies that target and attempt to block oestrogen signalling are thus frequently used to slow down or prevent relapse in the 70% of

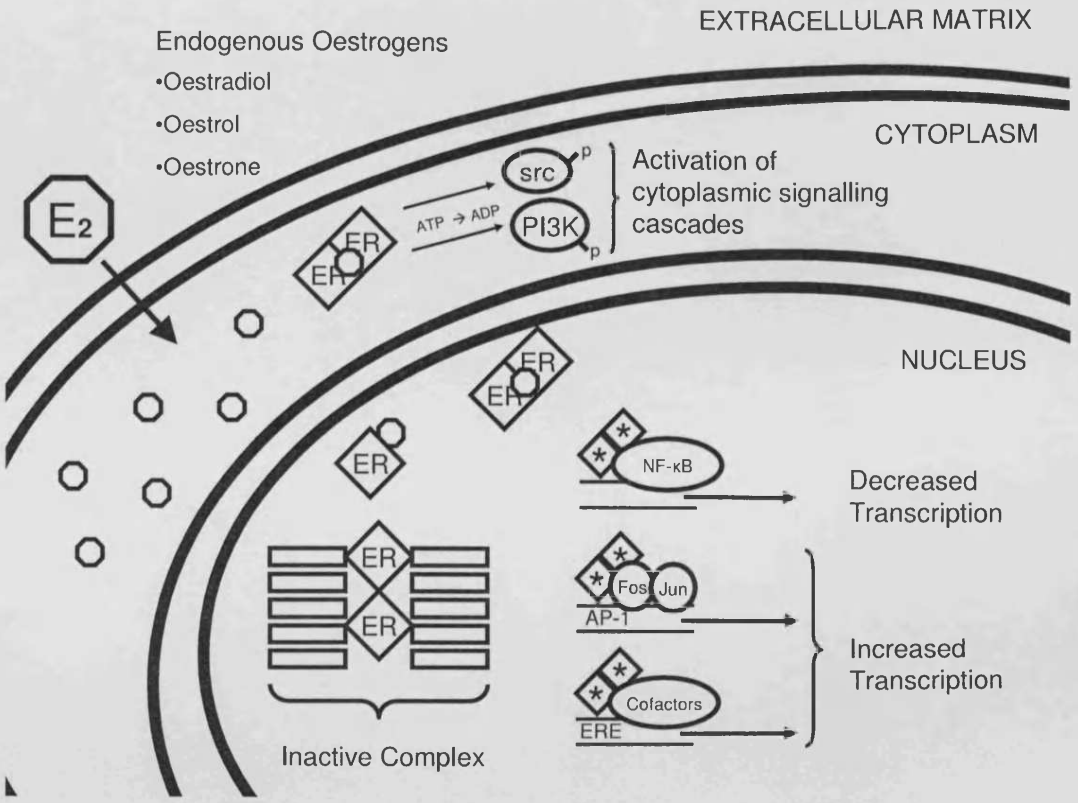


Figure 1.2. Activation of intracellular signalling pathways by ER. ER can modulate signalling either through direct phosphorylation of signalling molecules, such as Src and PI3K, or through the activation or repression of transcription of target genes.

cases of breast cancer which are oestrogen responsive, following initial treatment with radiation or surgery.

1.1.5.1 Oestrogen deprivation.

There are now more sophisticated methods of depriving breast cancers of oestrogens than the removal of the ovaries first described by Beatson over a century ago. In pre menopausal women, analogues of Luteinising hormone releasing hormone (LHRH inhibitors) which prevent the release of LH and therefore reduce gonadal oestrogen synthesis are used to reduce oestrogen levels and thus reduce the rate of tumour growth (Sharma et al., 2005). In post-menopausal women, where the ovaries do not contribute to circulating levels of oestrogen, oestrogen deprivation strategies are targeted towards the inhibition of aromatase activity, as this enzyme is responsible for oestrogen synthesis in local tumour sites and in adipose tissue where the majority of post-menopausal oestrogen synthesis occurs. There are two main classes of aromatase inhibitors. Non-steroidal aromatase inhibitors, such as aminoglutethimide and anastrazole that inhibit oestrogen production through inhibition of the P450 domain of the aromatase enzyme, and steroid-derived aromatase inhibitors, such as formestane and exemestane that inhibit aromatase through interactions with the steroid binding site (Joensuu et al., 2005). Aromatase inhibitors are occasionally used to treat pre-menopausal women who have undergone oophorectomy, to further reduce levels of circulating oestrogen (Osborne & Schiff, 2005).

1.1.5.2 Inhibition of ER.

An alternative to reducing the amount of oestrogen produced is to inhibit the ability of ER to respond to the existing oestrogen signal. A large number of small molecules exist which can bind to the ligand binding site of ER and modulate the oestrogen signal. The numerous combinations of ligand affinities, ER isoforms, and ER co-activators across tissue types means that most small molecule 'inhibitors' of ER can exhibit both oestrogenic and anti-oestrogenic characteristics depending on their location.

For this reason most small molecule inhibitors of ER are termed selective oestrogen receptor modulators (SERMs). SERMs are used in the treatment of a variety of conditions outside of breast cancer, including the use of clomiphene for the treatment of infertility (Macklon et al., 2006), and raloxifene for the prevention of osteoporosis (Ohmichi et al., 2005).

1.1.6 Tamoxifen.

Tamoxifen, a non-steroidal triphenylethylene derivative is a SERM and is primarily used as a preventative adjuvant endocrine therapy for ER positive breast cancer following radiotherapy or surgery, and has been in use for over 20 years. As with oestrogen, tamoxifen binds directly to the LBD of ER in breast cancer cells and promotes conformational changes. Unlike activation by oestrogen however, the conformational change also impairs AF-2 activity, decreases co-activator binding and increases co-repressor binding, therefore acting as an antioestrogen. Significantly, tamoxifen often allows AF-1 responses and can thus stimulate the transcription of some oestrogen responsive genes (Jackson et al., 1997) which rely on AF1 activity (Tzukerman et al., 1994). The ability to act as either antagonist or agonist dependent on species or tissue type can be observed in the way tamoxifen treatment reduces the incidence of contralateral breast cancer, and reduces the rate of incidence of breast cancer in high-risk women (in both cases acting as an antagonist), but also maintains bone density and increases the risk of endometrial cancer (Assikis et al., 1996) (in both cases acting as an ER agonist). In spite of this increased risk of endometrial cancer, the large incidence of breast cancer, along with the well established reduction in tumour recurrence and mortality of 47% and 26% respectively (Nicholson & Johnston, 2005) mean that tamoxifen remains a widely used drug for the prevention and treatment of breast cancer.

1.1.6.1 Tamoxifen resistance.

While the majority of breast tumours display an initial response to treatment with tamoxifen, in nearly all cases, following several years of

treatment, tumour progression occurs in the presence of tamoxifen, resulting in relapse and poor patient prognosis.

In nearly all cases of tamoxifen resistance, changes in non-hormonal growth factor signals have been observed to contribute to the ability of formerly tamoxifen sensitive tumours to progress in the presence of tamoxifen. The epidermal growth factor receptor (EGFR) has been identified as a key mediator of tamoxifen resistant tumour growth. The expression of EGFR mRNA in tamoxifen sensitive breast cancer cells has been demonstrated to enable growth in the presence of tamoxifen (van Agthoven et al., 1992), as has the expression of erbB2, a closely related member of the EGFR family of tyrosine kinase receptors (Benz et al., 1993) (the structure and function of EGFR and other EGFR family members are discussed in detail in section 1.2). Examination of clinical tumours has also revealed that increased levels of EGFR and other erbB family members are associated with an increased likelihood of tamoxifen resistance, and a decrease in survival rate (Nicholson et al., 1993). Inhibitors of EGFR signalling, such as gefitinib, have been demonstrated to strongly inhibit the growth of tamoxifen resistant cells in culture (Knowlden et al., 2003), but following around six months of treatment, growth continues even in the absence of a functioning EGFR signalling pathway (Jones et al., 2004). Methods of EGFR inhibition and their therapeutic value in the treatment of a variety of cancers is discussed in detail in section 1.2.3. It has been shown that cells resistant to the growth inhibitory effects of both tamoxifen and gefitinib utilise insulin-like growth factor-I receptor to activate intracellular pathways required for growth and proliferation (Jones et al., 2004).

More recent research has revealed that EGFR signalling does not simply replace ER signalling during the development of tamoxifen resistance but remains an important contributor to cell signalling throughout. It is known that levels of ER remain fairly constant throughout the development of tamoxifen resistance (Brunner et al., 1993), and that cells resistant to one antioestrogen often display sensitivity to other

antioestrogens both in the clinical setting (Howell et al., 2002) and in cell culture models (Coopman et al., 1994). This indicates that ER still contributes to the regulation of proliferation in the presence of tamoxifen. Consistent with this, it has been shown that as well as stimulating growth and proliferation though the direct activation of downstream mediators, stimulation of EGFR and other erbB family can result in activation of the AF-1 activity of ER thorough phosphorylation at serine 118 (Britton et al., 2006). Studies with inhibitors have shown that a functional MAPK pathway is required for this transactivation to occur. (Bunone et al., 1996). This activated ER can regulate the transcription of the several EGFR ligands, including amphiregulin (the ligands of the EGFR family are discussed in detail in 1.2.1) and thus an autocrine loop is established which serves to generate proliferative signals (Knowlden et al., 2003).

Tamoxifen resistant cells have also been shown to exhibit a number of other changes in intracellular signalling which may contribute to tumour development and therefore result in a poor prognosis. Cell culture models of tamoxifen resistant tumours have been shown to display increased Src activity, leading to increased invasiveness and motility (Hiscox et al., 2005) and increased Akt activity (Jordan et al., 2004).

1.2. Epidermal Growth Factor Receptor (EGFR).

1.2.1 The Epidermal Growth Factor Receptor (EGFR).

The epidermal growth factor (EGF) was first isolated in 1962 (Cohen, 1962). Cohen isolated and characterised a small peptide which he termed 'tooth-lid factor' due to the observation that the heat stable peptide was able to cause the premature eruption of incisors and opening of eyes when injected into newborn mice. Whereas at first Cohen could only guess at a likely molecular weight and empirical amino acid composition, further studies elucidated more precise structural details such as the exact amino acid composition (Taylor et al., 1972), the primary structure (Savage et al., 1972) and the position of the three disulphide bridges (Savage et al., 1973). The latter discovery allowed for the elucidation of the exact structure of the peptide, which by then was known as epidermal growth factor, and is shown in figure 1.3. EGF and other related ligands are characterised by their three intramolecular loops, and are generated by regulated cleavage from a larger transmembrane protein to yield the active growth factor.

Despite proof that EGF had affinity to a cell membrane anchored protein (Carpenter et al., 1975; Hollenberg & Cuatrecasas, 1975), the specific receptor for this protein was not isolated until several years later, when the now familiar 170kD receptor was purified from the membranes of the A431 cell line by affinity chromatography (Cohen et al., 1982). It is now known that the epidermal growth factor receptor, (EGFR) belongs in a larger family of four receptor tyrosine kinase molecules. In addition to EGFR (also known as HER1 and ErbB1, due to its similarity to the previously discovered v-erbB avian oncogene product, which was later characterised as a constitutively active mutant protein derived from EGFR), three other receptor tyrosine kinases have been isolated and characterised. HER2/ErbB2 (Coussens et al., 1985) was identified as a receptor tyrosine kinase with extensive homology to the EGF receptor; of particular interest because it was also shown that this newly discovered protein was the product of the neu oncogene. HER3/ErbB3 was identified as the third

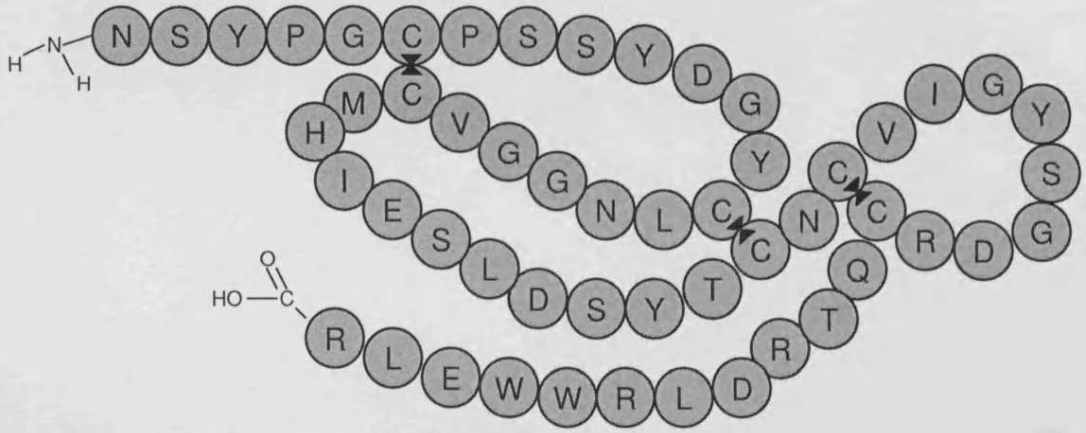


Figure 1.3 Primary structure of epidermal growth factor. As determined by (Taylor et al ., 1972). ►◄ indicates disulphide bond.

member of the EGFR family, and was also shown to be overexpressed in a number of mammary carcinomas (Kraus et al., 1989). The ErbB3 receptor is characterised by its greatly impaired kinase activity compared to other family members. However, the importance of heterodimerisation between members of the EGFR family (discussed in detail later) means that despite lacking significant kinase activity, its role as a heterodimerisation partner for other receptors implicates ErbB3 as a key signalling molecule in certain types of cancer. HER4/ErbB4, the most recently discovered member was isolated and characterised in 1993 (Plowman et al., 1993).

The EGFR family members all share a similar structure, with an extracellular ligand-binding domain, a single membrane spanning region and a cytoplasmic tyrosine kinase domain. All members of the ErbB family transmit growth factor signals across the cell membrane by a process of dimerisation (Yarden & Schlessinger, 1987a) and subsequent autophosphorylation (Yarden & Schlessinger, 1987b) wherein the kinase activity contained within the cytoplasmic c-termini of the receptor molecules phosphorylate tyrosine residues on the cytoplasmic domains of the dimerisation partner. Although it was originally thought that ligand binding at the cell surface facilitated dimerisation of the ErbB family members, and that this dimerisation facilitated autophosphorylation of ErbB hetero-, and homodimers, recent research has shown that inactive dimers can form on the cell surface, which are subsequently activated by the presence of ligands (Yu et al., 2002). This is the first suggestion that dimerisation and autophosphorylation may be separable events. All four ErbB receptors are capable of undergoing dimerisation, with all ten theoretical hetero-, and homodimers being possible subject to the presence of the relevant receptors and a suitable ligand. Although no ligand for ErbB2 has been discovered, ErbB2 functions as a ligand independent co-receptor, and is the preferred dimerisation partner for all other EGFR family members (Olayioye et al., 2000). In cases where ErbB2 is overexpressed ligand independent ErbB2 homodimers can also form (Canbay, 2003). Some dimers are susceptible to stimulation by a wide variety of ligands, whereas

some dimers can only elicit an intracellular signal when stimulated with a specific ligand (Yarden, 2001). Full details of all possible dimers and their ligands are given in table 1.1.

The wealth of potential ligand/dimer combinations and the subtle differences between them allow for a diverse range of signals to be both received and transmitted. This sophisticated multiform signalling system is unavailable to lower animals such as *C. elegans* and *Drosophilla*, who only possess a single EGFR-like receptor tyrosine kinase. Heterodimerisation also allows ErbB family members who do not possess certain fully functional domains to modulate signal transduction. For instance, ErbB2 has no known extracellular ligand (although recent reports have shown that ErbB2 can be activated by an intracellular complex (Carraway et al., 2002)) and ErbB3 has no active intracellular tyrosine kinase domain. Despite this, the ErbB2/ErbB3 heterodimer is an extremely active growth regulator in a number of tumours (Holbro et al., 2003). The ErbB2 receptor, despite having no known ligand, is able to form an exceptionally active heterodimer with EGFR, where ErbB2 acts to decrease ligand dissociation and decreases the rate at which the receptors are internalised (Karunagaran et al., 1996), leading to a more potent and prolonged signal.

It is also possible to activate receptor tyrosine kinase proteins via a number of ligand-independent mechanisms. Signals from a wide variety of growth factors and other signalling events such as Ca^{2+} influx can also activate EGFR. Growth factors frequently use the G-protein coupled receptor to indirectly activate the MAP Kinase pathway via EGFR phosphorylation, but other methods, such as the activation of Rho or the phosphorylation of EGFR by Jak2 enable a wide variety of events to elicit an EGFR-mediated response (Hackel et al., 1999). Ligand independent activation of EGFR can also occur under conditions of oxidative stress, in a mechanism mediated by H_2O_2 (Meves et al., 2001).

Table 1.1. Ligands causing dimerisation of EGFR family members.

Dimer / Ligand		EGF	TGF-α	EG	ER	B-C	HB-EGF	AR	NR
EGFR	EGFR	♦	♦	♦	♦	♦	♦	♦	
EGFR	HER2	♦	♦	♦	♦	♦	♦	♦	
EGFR	HER3	♦	♦	♦	♦	♦	♦	♦	♦
EGFR	HER4	♦	♦	♦	♦	♦	♦	♦	♦
HER2	HER2								
HER2	HER3	♦				♦			♦
HER2	HER4				♦		♦		♦
HER3	HER3								♦
HER3	HER4								♦
HER4	HER4				♦		♦		♦

EGF – Epidermal growth factor receptor, TGF-α – Transforming growth factor alpha, EG – Epigen, ER – Epiregulin, B-C – B-Cellulin, HB-EGF – Heparin-binding EGF, AR – Amphiregulin, NR – Newuregulins 1-4.

Dimerisation and autophosphorylation cause activation of a number of docking sites for intracellular proteins. Phosphorylated EGFR is able to facilitate the binding of molecules which contain SH2 (Src-homology 2) or PTB (phosphotyrosine-binding) domains. There are also a number of other domains which are able to interact directly with activated EGFR, such as the zinc finger protein ZPR1 domain (Galcheva-Gargova et al., 1996).The binding of these proteins either directly activate enzymatic signalling molecules or activate adaptor molecules which allow the transmission of extracellular signals to cytoplasmic signalling molecules and ultimately molecules which modulate gene expression and thus influence cell behaviour.

EGFR signals are primarily transmitted via the Ras-Raf-MAP kinase pathway and the phosphatidylinositol 3-kinase (PI3K) pathway. These signalling pathways, and the relevant adaptors and intermediates are summarised in figure 1.4.

The MAP kinase pathway which contributes to the regulation of proliferation, differentiation, apoptosis, angiogenesis and metastasis can be activated by EGFR via the Ras/Raf/MEK/ERK cascade. Activation of Ras and thus the initialisation of the cascade is reliant upon the exchange of GDP with GTP, mediated by a guanine exchange factor (In this case, Son of Sevenless (SOS)). SOS is recruited to the cell membrane by contact with either of two adaptor proteins which can bind to EGFR only in its phosphorylated state. Once Ras has undergone GDP/GTP exchange it is able to activate Raf and allow the transport of the signal through the cytosolic signalling components of the pathway (Raf, MEK, ERK) (Schlessinger, 2000). The final cytoplasmic event is the phosphorylation of the p90 ribosomal S6 kinase ($p90^{rsk}$), which is able to cross the nuclear envelope and activate a number of transcription factors including CREB, C/EBPβ and members of the Ets family (Roux et al., 2003). Once activated these transcription factors can promote the transcription of genes involved

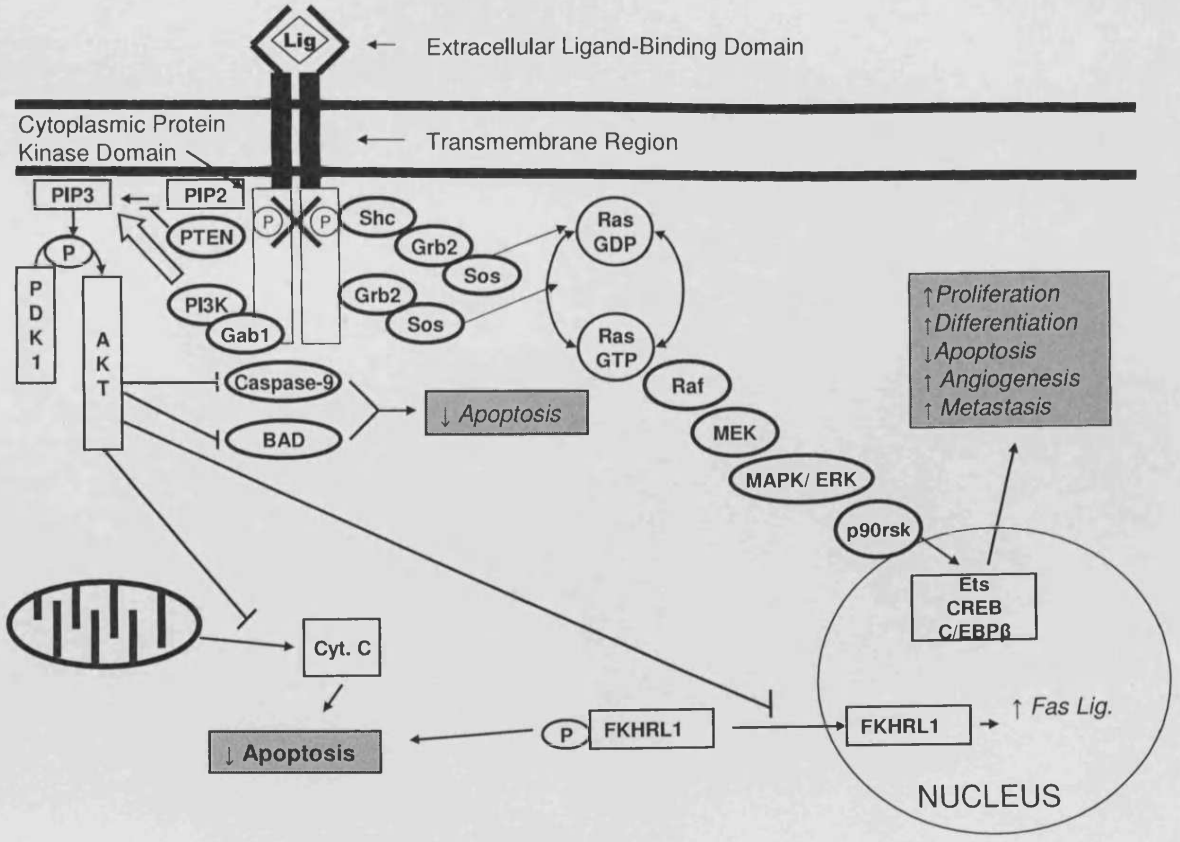


Figure 1.4 - Signalling pathways modulated by EGFR

with proliferation, differentiation, angiogenesis, metastasis and the inhibition of apoptosis.The phosphatidylinositol-3 Kinase (PI3K) pathway is also known to be activated by EGFR. PI3K can directly interact with SH2 domains on some erbB family members (most notably ErbB3 – (Soltoff et al., 1994)), but activation of PI3K through EGFR requires an intermediate, Gab1 (Rodrigues et al., 2000). Activation of PI3K allows for the phosphorylation of PIP2 (phosphatidyl inositol-4, 5-bis-phosphate) in the plasma membrane, forming PIP3 (phosphatidyl inositol-3,4,5-triphosphate). PIP3 provides a docking site in the plasma membrane for protein kinase C (PKC) and AKT (also known as protein kinase B) (Fresno Vara et al., 2004). On the membrane AKT becomes phosphorylated and is free to move between the cytoplasm and the nucleus, allowing it to phosphorylate a number of proteins and transcription factors which can directly influence cell behaviour (Meier & Hemmings, 1999). AKT is known to inhibit the activation of caspase-9 and Bad (She et al., 2005) (a bcl-2 family member), both of which are concerned with the promotion of apoptosis (Cardone et al., 1998; Datta et al., 1997). AKT can also phosphorylate FKHRL1, a member of the forkhead transcription factor family. Phosphorylated FKHRL1 is sequestered in the cytoplasm and is thus unable to function as a transcription factor in the nucleus, where it can promote the transcription of death genes, such as Fas ligand (Brunet et al., 1999). AKT is also able to further protect cells from apoptosis by inhibiting the release of cytochrome C from mitochondria, a key initial stage in the induction of apoptosis via the caspase pathway (Kennedy et al., 1999). EGFR has also been shown to regulate levels of survivin, a member of the inhibitor of apoptosis protein (IAP) family in a PI3K dependent manner (Wang & Greene, 2005), (Nagane et al., 2001).

In common with most other hormone and growth factor receptors, following ligand binding, EGFR is internalised by endocytosis (Haigler et al., 1979). Nullifying the ligand and receptor allows for 'old' signals to be distinguished from later signals, allowing the cell to respond to new influxes of ligand. It is known that activated EGFR maintains its functionality as a

signal transducer during endocytosis, where it is still able to activate downstream signalling pathways. EGFR signalling from within endosomes is slightly different to cell-surface signalling, with the activation of certain pathways only possible at one location. For instance the PLC-γ pathway is only activated at the cell surface, due to a lack of suitable substrate within the endosome (Haugh et al., 1999). Following endocytosis, EGFR-containing endosomes are either re-cycled to the cell membrane or targeted for destruction in lysosomes. This sorting process, which dictates the amount of receptor that is destroyed and the amount that is recycled, is a critical stage in directing the levels of EGFR that are displayed on the cell surface, and therefore the extent to which a cell may respond to stimulation. Endocytosis, sorting, recycling and destruction of EGFR is regulated by a large number of proteins. Of particular importance to these processes are the Cbl family of ubiquitin ligases, which contain TKB (tyrosine-kinase binding) domains that can directly bind EGFR and attach ubiquitin molecules to free lysine residues (Dikic, 2003). In addition to the degree of ubiquitination, di-leucine motifs and conformational changes in the receptor molecule also have a role in determining the fate of endocytosed EGFR (Wiley & Burke, 2001). It should be noted that EGFR (erbB1) undergoes ligand induced endocytosis, while other family members experience only a baseline rate of endocytosis which is unaffected by ligand binding (Baulida et al., 1996). This profound difference in ligand-mediated endocytosis within the erbB family members contributes to the observation that EGFR/erbB2 heterodimers constitute a particularly potent signal. EGFR/erbB2 heterodimers are endocytosed at the same rate at EGFR homodimers, but a far greater proportion of EGFR/erbB2 dimers are recycled to the cell surface than EGFR homodimers (Worthylake et al., 1999).

1.2.2. Aberrant EGFR signalling.

Defective EGFR signalling has been implicated in a number of cancers, with abnormal EGFR signalling being observed in a large number

Table 1.2. Occurrence of EGFR overexpression in tumours.

Carcinoma	Cases in which EGFR is overexpressed
NSCLC*	40-80%
Head & Neck	80-100%
Colorectal	25-77%
Ovarian	35-70%
Breast	15-91%
Glioma	40-63%
Bladder	31-48%
Prostate	40%

* Non-small cell lung cancer

Adapted from (Delbaldo et al., 2003).

of human malignancies (reviewed in (Salomon et al., 1995)). The extent of EGFR overexpression in a variety of tumours is given in table 1.2. As well as simply increasing growth rate, increased EGFR signalling in a tumour is associated with malignant transformation, inhibition of apoptosis, promotion of angiogenesis and metastasis (El-Rayes & LoRusso, 2004). Often this abnormal signalling is caused by a greatly increased number of receptors, along with increased production of EGFR ligands, leading to the establishment of an autocrine loop causing EGFR to become constantly activated. In some cancers, a constantly active EGFR mutant (EGFRvIII, ΔEGFR, de2-7EGFR) is formed following the deletion of exons 2-7 from the EGFR gene (corresponding to the loss of amino acids 6-274) (Wong et al., 1992). The resulting protein lacks a ligand binding domain, but remains active at all times, able to autophosphorylate other ΔEGFR molecules and all other native ErbB family members and causing increased signalling through the more aggressive dimers such as EGFR/Erb2 and EGFR/ErbB3. ΔEGFR also appears to be more resistant to receptor endocytosis than wild-type EGFR (Huang et al., 1997a), essentially facilitating unattenuated EGFR signalling even in the absence of suitable ligands. ΔEGFR contains the same intercellular domains at wild-type EGFR, so therapeutic agents directed towards this part of EGFR are suitable for treating ΔEGFR driven cancers (Han et al., 1996b). Therapies directed at the extracellular domain of EGFR however, do not serve to reduce the activity of ΔEGFR (Nagane et al., 2001). Increased EGFR signalling can also be achieved by a number of other methods, including via increased production of ligands, or via independent activation mediated through alternative receptors (Liu et al., 2002).

The reliance of a large number of tumours on ErbB family signalling molecules (particularly EGFR and ErbB2) for growth and drug resistance means that the inhibition of EGFR signalling is a widely studied therapeutic strategy. This is discussed in detail in section 1.2.3 below.

1.2.3. EGFR-directed cancer therapy.

The frequent observation of increased EGFR activation in carcinomas has highlighted EGFR signalling as a difference between normal and tumour cells which may be exploited for therapeutic gain. The aim of EGFR –directed cancer therapy is simply to prevent the EGFR signal reaching any of the internal signalling molecules which mediate the cellular end-points of EGFR activation. The signal can be blocked at a number of points, with a wide variety of agents being available which inhibit the translation of the EGFR mRNA, ligand binding, dimerisation, autophosphorylation and the docking of cytoplasmic second messengers. While blocking EGFR phosphorylation can have a marked effect on the growth and development of an EGFR-expressing tumour, any systemic treatment directed against EGFR will also interfere with EGFR signalling in non-cancerous tissue, and therefore a number of side effects are observed following EGFR inhibition, the most common of these being acneform skin rashes (Sipples, 2006).

1.2.3.1 Monoclonal antibodies.

There are a number of monoclonal antibodies which have successfully blocked EGFR activation, usually blocking the ligand binding domain of the EGFR molecule and preventing successful dimerisation and subsequent activation. These antibodies are used both as useful experimental tools (Modjtahedi et al., 1998) to study EGFR signalling, or more recently, as approved drugs for the treatment of EGFR overexpressing cancers. Cetuximab (C225, Erbitux®) is a chimeric monoclonal antibody which binds to the extracellular domain of EGFR with greater affinity than EGF or TGF-α (Goldstein et al., 1995). The antibody is murine in origin, with human Fc regions, which significantly reduces the likelihood of an immune response against the antibody itself. Cetuximab is usually given in combination with cytotoxic drugs such as 5-flourouracil. Trastuzumab (Herceptin®) is also frequently used in treatment of certain erbB2-overexpressing cancers. Trastuzumab is a humanised monoclonal antibody targeting the ligand binding domain of erbB2 and preventing

heterodimerisation (Klapper et al., 1997). Trastuzumab can also increase the rate of receptor degradation (Klapper et al., 2000). Other monoclonal antibodies active against EGFR include ABX-EGF, a fully humanised antibody with an IC_{50} of approximately 3nM for EGFR and ICR-62 and EMD-72000, derived from rat and mouse respectively.

1.2.3.2 EGF/TGF molecules attached to toxins (conjugates).

In 1991, it was reported that a conjugate of a cytotoxic drug (peplomycin) and an EGFR receptor-recognising antibody (B4G7) was considerably more effective as an inhibitor of A431 growth than either agent alone (Osaku et al., 1991). This approach allows for the preferential delivery of a cytotoxic agent to EGFR overexpressing cells. More recent studies have utilised the well characterised C225 monoclonal antibody chemically conjugated to paclitaxel, and have demonstrated increased ability to inhibit growth in a number of cell lines compared to either agent alone or a simple mixture of the two (Safavy et al., 2003). In addition to chemical conjugates, DNA recombination has been used to create immunotoxins which combine antibody fragments with peptide toxins. An erbB2 targeting conjugate consisting of a fragment of erbB2 targeting antibody with *Pseudomonas* endotoxin A (OLX-209) has been used to treat non small-cell lung cancer (NSCLC) in clinical trials with some success (King et al., 1996).

1.2.3.3 Small molecule inhibitors of tyrosine kinase activity.

There are a number of small molecule inhibitors which can inhibit the tyrosine kinase activity of EGFR and thus prevent the ligand-induced signal from eliciting an intracellular response. Effective, specific EGFR inhibitors have been synthesised and used to treat patients with EGFR overexpressing cancers, for example gefitinib (ZD1839, Iressa®), an orally-active anilinoquinazoline derivative with a high specificity for EGFR (Ward et al., 1994). Gefitinib competes with adenosine triphosphate for a binding site on the EGFR molecule, thus making the inhibition competitive and reversible. Gefitinib has been licensed in some countries such as Japan

and the USA as a second or third line treatment against non-small cell lung cancers (NSCLC) where promising reports of substantially released tumour volume in 10% of patients have been accompanied by reports of adverse side effects and deaths (FDA website – www.fda.gov). Other Inhibitors of EGFR are available, including OSI-774 (erlotinib, Tarceva®), which has been shown in clinical trials to significantly increase the median survival of patients with advanced NSCLC (Peck 2004) and AG1478, which is frequently used to inhibit EGFR signalling in experimental situations (Partik et al., 1999; Zhu et al., 2001), and is under consideration for trial as a therapeutic agent (Ellis et al., 2006), whilst ZD1839, OSI-774 and AG1478 are receptor tyrosine kinase inhibitors with very specific affinity for EGFR (Herbst, 2004), there are many small molecules which inhibit EGFR along with a wide range of other tyrosine kinases. CL-1033 is a pan-erbB inhibitor capable of inhibiting all members of the erbB family, and the naturally derived RTK inhibitor genistein is capable of inhibiting EGFR along with all other receptor tyrosine kinases. For a comprehensive review of small molecule EGFR small molecule inhibitors see (Ranson, 2004). There is current interest in increasing the potency of monoclonal antibodies and small molecule tyrosine kinase inhibitors by co-administration, such that both the extracellular ligand binding domain and the cytoplasmic tyrosine kinase domain are targeted simultaneously. This is of particular interest following the observation that cells can acquire resistance to monoclonal antibodies such as cetuximab, but still retain the ability to respond to ZD1839 or OSI-774 (Huang et al., 2004).

1.2.3.4 Antisense/siRNA.

In experimental models of cancer such as cultured cells or mouse xenografts, antisense (Akhtar et al., 2002) and more latterly siRNA (Nagy et al., 2003) have been successfully used to reduce EGFR expression, producing a resultant decrease in cell growth. While these approaches show promising results in experimental models, the instability of oligonucleotides in biological milieu means that the delivery of nucleic-acid based therapeutics in a form in which their activity is preserved presents a

serious problem which will need to be overcome before these approaches are of therapeutic value. Despite this, siRNA and antisense are still widely used as experimental tools in cell culture systems. Experiments have also been conducted assessing the feasibility of co-delivering EGFR antisense with the cytotoxic drug 5-fluorouracil with a view to developing a combination therapy and exploiting the apparent ability of EGFR blockade to sensitise cells to the action of cytotoxic drugs (Hussain et al., 2002) which is discussed in detail in section 1.2.4, below.

1.2.4 EGFR Blockade and drug sensitivity.

Following the widely reported observation that a large percentage of tumours which overexpress EGFR also acquire resistance to a variety of cytotoxic drugs (Wosikowski et al., 1997), a number of studies have been conducted investigating the effect of modulating the EGFR signal on response to certain chemotherapeutic agents and ionising radiation (IR). Studies have reported increased effectiveness of a wide variety of cytotoxic agents and ionising radiation when combined with EGFR inhibition therapy in cell culture, xenograft and animal models and in clinical situations. These studies are summarised in table 1.3. Combined regimens of EGFR inhibitors and cytotoxic drugs are becoming increasing well established as a treatment for colorectal cancers (Harari, 2004), and are the subject of a number of current clinical trials for a variety of cancers. These clinical trials are summarised in table 1.4.

1.2.5 EGFR is a mediator of drug resistance and radioresistance.

The studies described in table 1.4 demonstrate that EGFR inhibition is often linked to increased sensitivity of cancer cells to cytotoxic drugs and ionising radiation. Many studies have also demonstrated that increased EGFR signalling can promote resistance to ionising radiation and cytotoxic drugs. EGFR overexpression has been shown to correlate directly with radiocurability in a murine model of tumorogenesis (Akimoto et al., 1999). Studies have also confirmed that increased levels of activated EGF can confer radioresistance to cells in culture (Liang et al., 2003).

Table 1.3. Studies reporting increased sensitivity to cytotoxic drugs following treatment with an EGFR inhibitor.

Ref	Inhibitor	System	Effect on Drug Response
(Shintani et al., 2003)	ZD1839	HSC2 cells	(+) IR
(Stea et al., 2003)	ZD1839	U251 Cells	(+) IR
(Sirotnak et al., 2000)	ZD1839	Mouse Xenograft (A431, A459, SK-LC-16, PC-3, TSU-PR1)	(+) Cisplatin, Carboplatinum, Paclitaxel, Docetaxel, Doxorubicin, Edatrexate,
(Huang & Harari, 2000)	C225	Mouse Xenograft (Primary SCC cells)	(+) IR
(Koizumi et al., 2004)	ZD1839	7 colorectal cancer cell lines	(+) Irinotecal
(Pegram et al., 1998)	rhuMAb HER2 *	Human patients	(+) Cisplatin
(Ciardiello et al., 2000)}	ZD1839	Mouse Xenograft (GEO cells)	(+) Paclitaxel, Topotecan, Raltirexib
(Ciardiello et al., 2000)	ZD1839	GEO, OVCAR-3, MCF-10*ras* and ZR-75-1	(+) Taxol, Topotecan, Doxorubicin, Oxaliplatin, Tomudex, Etoposide, Cisplatin, Carboplatin, Taxotere
(Folprecht et al., 2006)	C225	Human patients	(+) FOLFIRI (irinotecan/5-fluorouracil/folinic acid)
(Pietras et al., 1999)	rhuMAb HER2 *	Mouse Xenografts (MCF-7/HER2)	(+) IR
(Shin et al., 2001)	C225 (Mab)	Human Patients	(+) Cisplatin
(Baselga et al., 1993)	C225/528 (Mabs)	Mouse Xenografts (A431/MDA-468)	(+) Doxorubicin
(Dixit et al., 1997)	Antisense	Cell Culture and Mouse Xenografts (MDA-468)	(+) Cisplatin
(Contessa et al., 1999)	Inducible Dominant Negative	Human Mammary Carcinoma Cells	(+) IR
(Pu et al., 2006)}	PD168 393	DU145 cells in culture (Androgen-independent prostate cancer)	(+) Paclitaxel
(Zhou et al., 2004)	GW572016	Primary Breast Cancer Cell Lines	(+) IR
(Kim et al., 2006)	C225	Mouse Xenografts ARO cells (anaplastic thyroid carcinoma)	(+) Irinotecan
(Taira et al., 2006)	ZD1839	8x TE Human oesophageal cancer cell lines in culture	(+) IR

(Premkumar et al., 2006)	ZD1839	T98G cells in culture (Glioma)	(+) 17-AAG
(Teraishi et al., 2005)	ZD1839	TE8 Cells in culture (Human oesophageal squamous cell carcinoma)	(+) TRAIL (TNF-related apoptosis inducing ligand)
(Al-Hazzaa et al., 2005)	ZD1839	SCC-15 cells in culture (Human squamous cell carcinoma of the tongue)	(+) Cisplatin
(Nakata et al., 2004)	C225	Mouse Xenografts (A431 and MDA468)	(+) IR (+) Docetaxel

IR – Ionising radiation

17-AAG - 17-Allylamino-17-demethoxygeldanamycin

Table 1.4 Clinical trials examining the therapeutic potential of EGFR inhibitors combined with cytotoxic drugs.

clinicaltrials.gov Trial ID	Cancer Type	EGFR Inhibitor	Secondary Therapy
NCT00294762	Advanced NSCLC with EGFR overexpressed/ amplified/ mutated	Erlotinib	Carboplatin
NCT00140556	Advanced head and neck	Erlotinib	Radiotherapy Cisplatin
NCT00080249	Head and neck	Gefitinib	Radiotherapy Paclitaxel
NCT00185835	Squamous cell carcinoma of the head and neck	Gefitinib	Radiotherapy Cisplatin
NCT00232505	ER/PR-Negative, HER-2 nonoverexpressing metastatic breast cancer	Cetuximab	Carboplatin
NCT00260364	Advanced pancreatic cancer	Erlotinib	Gemcitabine Capecitabine Bevacizumab
NCT00251433	Previously untreated ErbB2 overexpressing metastatic breast cancer	Trastuzumab Lapatinib	Docetaxel

Other research has demonstrated elevated levels of EGF can mediate resistance to peplomycin, a radiomimetic drug that mimics the action of ionising radiation (Osaku et al., 2001) and that human breast cancer cells transfected with an EGFR expressing plasmid display an increased resistance to doxorubicin, vinblastine, cisplatin and 5-fluorouracil (Dickstein et al., 1995). Similar results have also been reported in germline tumours (Park et al., 2005). It is thought that EGFR modulates sensitivity to ionising radiation and cytotoxic agents through a variety of different pathways, which are outlined below;

1.2.5.1 Modulation of proliferation and apoptosis.

The ability of EGFR signalling to modulate cell proliferation and survival is well documented and has been discussed previously. This presents a problem when attempting to kill, or inhibit the growth of EGFR overexpressing tumour cells. Cells with increased levels of EGFR signalling are likely to experience an increased growth rate, and therefore attempts to inhibit growth are likely to be less successful, and cells lost to apoptosis following a drug treatment are more readily replaced. The modulation of cell survival pathways by EGFR, mainly through AKT, means that EGFR overexpressing cells are likely to display a more resilient, less apoptotic phenotype when challenged with a cytotoxic agent (Nagane et al., 1998).

1.2.5.2 Increased EGFR activation by IR/cytotoxics.

Following initial observations that ionising radiation causes activation of EGFR (Warmuth et al., 1994; Zheng et al., 1993), it has since been shown that the activation of EGFR, and the subsequent activation of downstream MAPK pathway can be blocked using neutralising antibodies to the known EGFR ligand TGFα (Dent et al., 1999). Thus it appears that in EGFR rich cells, any of the desired detrimental effects on cell growth that might be obtained from radiotherapy are accompanied by (and obscured by) an increase in EGFR stimulated cell growth thus imparting radioresistance onto the cell. Oxidative stress, which can be caused by both ionising radiation and certain cytotoxics has been shown to activate cell proliferation

and survival cascades through activation of EGFR, both via a ligand independent pathway (Meves et al., 2001) and a ligand dependant pathway, dependent on the ADAM mediated cleavage of heparin-binding EGF (HG-EGF), a ligand of EGFR (Fischer et al., 2004).

Several cytotoxic agents have been shown to activate EGFR through a variety of means. Cisplatin, doxorubicin and camptothecin have all been shown to activate EGFR (Benhar et al., 2002), and are therefore also able activate the signalling elements downstream of EGFR that can increase the likelihood of cell survival through the modulation of proliferation and apoptosis mediated through EGFR as previously discussed. Methods of stress induced EGFR phosphorylation are summarised figure 1.5

1.2.6 EGFR and DNA repair.

In addition to the increased rate of proliferation, and cell survival, largely mediated by the MAP Kinase and PI3K/AKT pathways, there is evidence to suggest that EGFR can interact with several proteins involved with the repair of DNA damage caused by ionising radiation and cytotoxic drugs.

The CREB transcription factor, known to be activated by EGFR via the MAPK pathway has been shown to regulate the transcription of the proliferating cell nuclear antigen (PCNA), involved in the repair of DNA damage caused by ionising radiation (Amorino et al., 2003). The DNA repair genes *XRCC1* and *ERCC1* have also shown to be upregulated by ionising radiation in an EGFR and MAPK dependent manner (Yacoub et al., 2003).

Relationships have also been established between EGFR and the DNA dependent protein kinase (DNA-PK), a critical component of the Non-homologous End-Joining (NHEJ) pathway of DNA repair. The exact role of DNA-PK in the repair of DNA damage is discussed in detail in section 1.5.2.2, however, it should be noted that in mammalian cells, the NHEJ

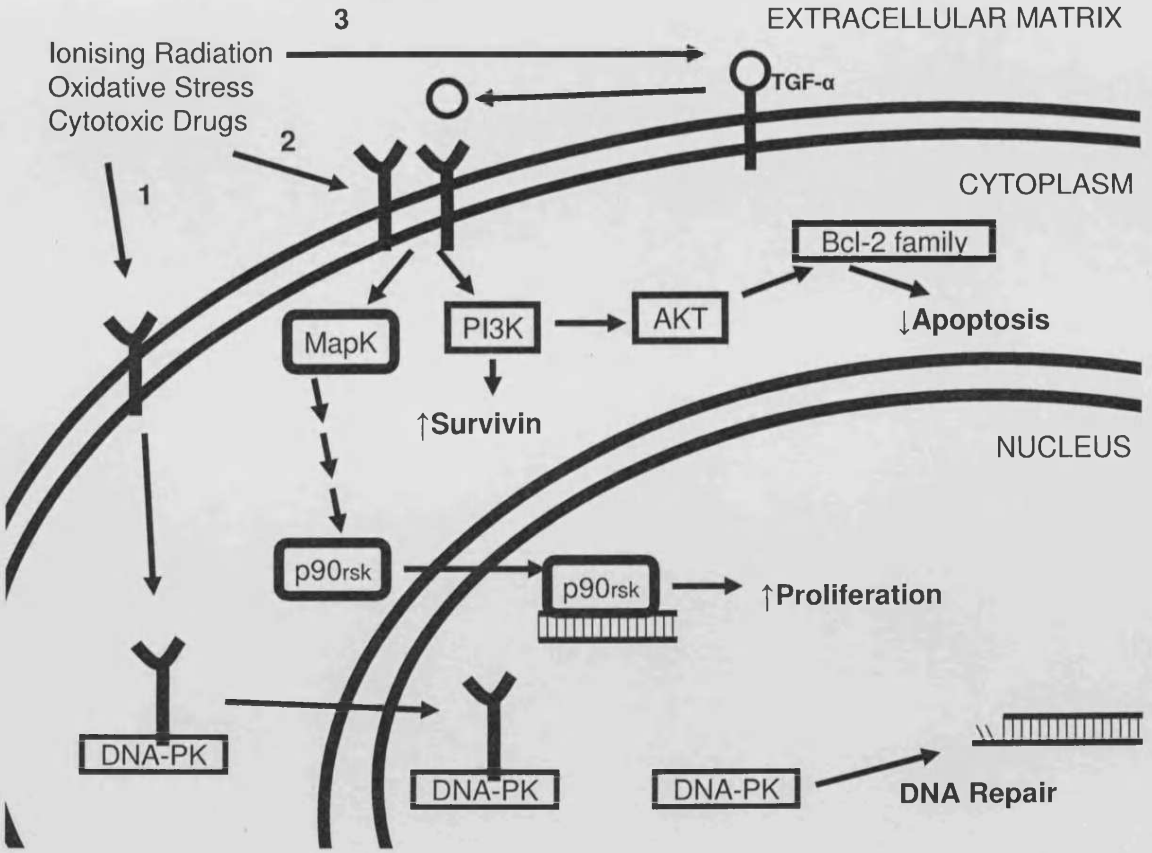


Figure 1.5 EGFR mediated cellular responses to stress from cytotoxic agents, ionising radiation and oxidative stress.

1) Nuclear co-translocation of EGFR and DNA-PK. 2) Stress activated EGFR activation, 3) Ionising radiation/cytotoxic mediated TGF-α cleavage and subsequent activation of EGFR. Both pathways 2 and 3 result in increased activity of proliferative and anti-apoptotic signals within the cell.

pathway is responsible for the repair of the majority of double-stranded breaks (DSBs) in DNA (Rothkamm et al., 2001). DSBs are also the main type of DNA damage caused by ionising radiation and several radiomimetic drugs (described in section 1.3). Initial studies established a physical interaction between EGFR and DNA-PK (Bandyopadhyay et al., 1998), and it is now thought that in certain stressful situations, such as following exposure to ionising radiation, oxidative stress or cytotoxic drugs, EGFR is able to translocate to the nucleus (outlined in fig 1.5) and that monoclonal antibodies can prevent this translocation (Dittmann et al., 2005a). This translocation allows EGFR to facilitate the nuclear import of DNA-PK, where it can repair damaged DNA (Dittmann et al., 2005b). Similar observations have also been made using the small molecule inhibitor ZD1839, which significantly slows the rate of DNA repair some cell culture models (Friedmann et al., 2004).

1.3 Chemotherapeutics.

Not all tumours are suitable for treatment with drugs which work by disrupting hormone signalling. Cytotoxic chemotherapeutics are toxic to cells, and typically work by damaging DNA or by disrupting cell division. Cancerous cells are typically faster growing and therefore have more cells undergoing cell division at any given time, meaning that cytotoxic agents have a greater toxic affect on the tumour cells than other tissues. Other fast growing cells are also adversely affected, such as those involved in hair growth and the maintenance of the gastrointestinal lining, resulting in the frequently observed side effects of hair loss and diarrhoea. There are a wide variety of cytotoxic drugs available, all with distinct modes of action and with distinct side effects.

1.3.1. Bleomycin.

Bleomycin is an antineoplastic mixture of cytotoxic glycopeptides which are produced by *Streptomyces verticillus* (Umezawa et al., 1966). Commercially available bleomycin preparations usually consist of bleomycin A_2, bleomycin B_2 and a small quantity of other bleomycins. The structure of bleomycins A_2 and B_2 are shown in figure 1.6. Bleomycin is currently used to treat well differentiated squamous cell carcinomas of the head and neck, Hodgkin's disease and other malignant lymphomas, testicular teratoma and on occasion metastatic malignant melanoma and carcinomas of the lung, bladder, penis, cervix, vulva and thyroid (Mir et al., 1996). Bleomycin is also used in combination with other cytotoxic drugs and/or radiotherapy in the treatment of some squamous cell carcinomas. Patients receiving bleomycin treatment are closely monitored for chest abnormalities as pneumonia, and occasionally pulmonary fibrosis is observed as a side effect (Ginsberg & Comis, 1982).

Bleomycin elicits a cytotoxic effect by binding iron, activating oxygen and thus forming an activated complex capable of cleaving DNA. It is has been observed that bleomycin shows a preference for damaging DNA at

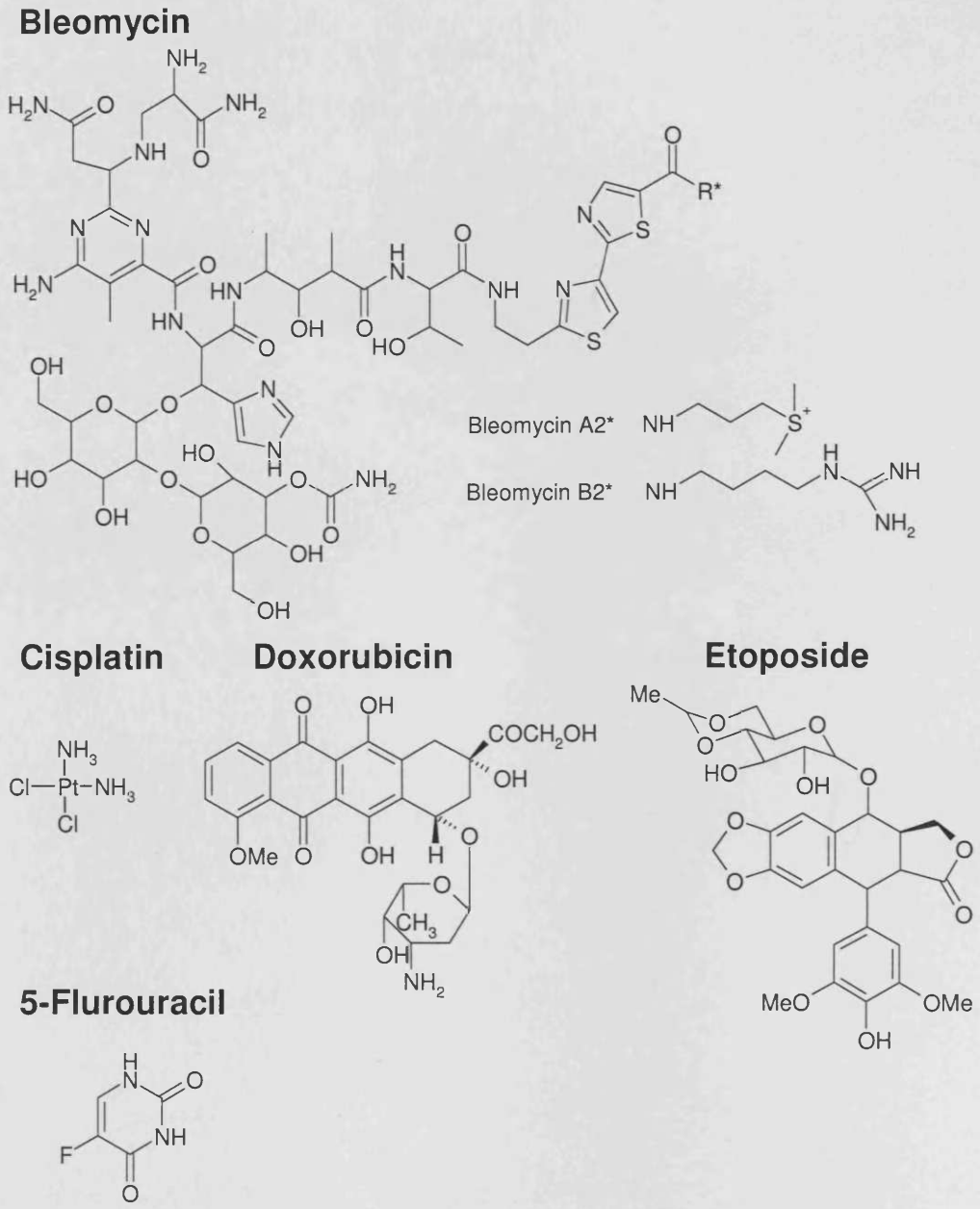


Figure 1.6 Structure of the cytotoxic drugs bleomycin, cisplatin, doxorubicin, etoposide and 5-fluorouracil.

sites containing a particular moiety (5'-GpPy-3'), where it can cause DNA damage, typically double-stranded breaks (Sam et al., 1998) and having a far greater damaging effect on genes in active chromatin than inactive genes (Kuo, 1981). In addition to the DNA damaging effect of bleomycin, it is believed that bleomycin inhibits the synthesis of new DNA by blocking the incorporation of thiamine into the newly synthesised strand. Bleomycin is sometimes referred to as a 'radiomimetic' drug, because the DNA damage it causes (i.e. predominantly double strand breaks) mimics that caused by ionising raditation.

1.3.2 Cisplatin.

Cisplatin (*cis*diamminedichloroplatinum (II), *cis*DDP) is a cytotoxic therapeutic drug which elicits a cytotoxic effect by damaging DNA and preventing its replication. The structure of cisplatin is shown in figure 1.6. The chlorine ligands on the platinum atom can be replaced by nitrogen from the N7 atoms of purines. The spatial arrangement of the ligands on the platinum atom means that cisplatin can cause a number of different types of lesions.

Cisplatin can coordinate nitrogen atoms from adjacent purine bases, causing 1,2 intrastrand adducts, which introduce kinks into the DNA. These adducts are nearly always formed at GpG sites in DNA, but occasionally will form between guanine and an adjacent adenine (Fichtinger-Schepman et al., 1985). When nitrogen atoms from purine bases on opposite strands of a DNA molecule are coordinated by cisplatin, interstrand adducts are formed. Resistance to the cytotoxic effects of cisplatin is observed, and this has been shown to be attributable, at least in part to an ability to repair these adducts (Johnson et al., 1994). Repair of cisplatin adducts by cell extracts has been reported on a number of occasions (Jones et al., 1994; Sibghatullah et al., 1989). It is thought that intrastrand adducts can be repaired via the base excision repair pathway (Jamieson & Lippard, 1999). Interstrand adducts appear to be able to attract a number of other proteins, most notably high motility group proteins (HMG-1) (Hughes et al., 1992)

and the Ku component of DNA-PK (Turchi & Henkels, 1996) suggesting that they might be repaired via the same mechanisms as double stranded breaks, or that they may elicit their apoptotic affect through similar means to double stranded breaks in DNA. Pathways responsible for the detection and repair of double stranded breaks in DNA are discussed in detail in section 1.5.

1.3.3 Doxorubicin.

Doxorubicin (Dox, Adria, Adrimycin®) is the 14-hydroxy derivative of daunorubicin, produced by the fungus *streptomyces peucetius (Arcamone et al., 1969)* and is classed as an anthracycline antibiotic. Its structure is shown in figure 1.6 Doxorubicin can cause DNA damage in a number of ways, including intercalation of the anthracycline moiety into DNA, or by methods similar to bleomycin involving the chelation of metal ions and the activation of oxygen (Gewirtz, 1999). In addition to this, doxorubicin is known to be an inhibitor of topoisomerase II, which further contributes to its status as a major damager and disrupter of normal DNA function (Wassermann et al., 1990). Topoisomerase II makes temporary double stranded breaks in DNA, which it later re-ligates, as part of its function as an untangler of knots in the genetic material. Doxorubicin inhibits only the repair function, turning topoisomerase II into a DNA damaging protein, often termed *topoisomerase II poison* to distinguish it from native topoisomerase II. Doxorubicin is used in the treatment of a wide variety of tumours (Arcamone et al., 1997).

1.3.4 Etoposide.

Etoposide (Eopsin®) is a cytotoxic drug used in the treatment of a number of carcinomas including small cell lung cancer, testicular cancer and monoblastic leukaemia. Like doxorubicin, etoposide is an inhibitor of topoisomerase II (Liu, 1989). Treatment with etoposide results in the introduction of a large number of double strand reaks into DNA (Long et al., 1985), caused by topoisomerase II poison molecules which can break DNA, but lack the ability to rejoin it. Etoposide can be considered a purer

radiomimetic than doxorubicin, due to the fact that etoposide has no other effect on DNA other than that mediated by topoisomerase II poison. It has been shown that causing a double stranded break in DNA requires two molecules of etoposide, affecting two molecules of topoisomerase II at sites close enough together to cause a chromosomal break (Bromberg et al., 2003). The structure of etoposide is shown in figure 1.6.

1.3.5 5-Fluorouracil.

5-Fluorouracil (5-FU, Adrucil, Efudix®) is cytotoxic drug which interferes with DNA replication by inhibiting the enzyme thymidylate synthetase (Cohen et al., 1958) and is used to treat colon, breast, ovarian and prostate cancer. 5-fluorouracil is also occasionally applied topically to the skin in the form of a cream. 5-fluorouracil is mistakenly incorporated into DNA during DNA synthesis, causing a bulky adduct which is eventually removed by the base excision repair enzyme uracil DNA glycosylase (UDG) (Ingraham et al., 1980). There is evidence to suggest that free 5-fluorouracil is able to inhibit UDG, thus causing an additional decrease in DNA repair capacity (Wurzer et al., 1994). The structure of 5-fluorouracil is shown in figure 1.6.

1.3.6 Cellular response to cytotoxic drugs.

Cells typically deal with damaged DNA, whether caused by environmental factors or treatment with cytotoxic drugs in one of three ways. Where low levels of damage are sustained, the damage may be repaired through one of a number of specialised mechanisms that exist in eukaryotes for the repair of DNA. Above this threshold, the cell may enter a state of senescence, where the cell cycle stalls to allow repair of the DNA, and to prevent damaged DNA being copied. Where the damage is too great to be repaired satisfactorily, cells typically undergo apoptosis in order to prevent the spread of the damaged genome. It is the aim of a cytotoxic regimen to induce apoptosis or senescence in the tumour cells, and thus reduce the size or growth rate of the tumour. The fate of a cell is typically dealt with by a complex and overlapping network of signalling molecules

which can recognise DNA damage, and regulate DNA repair, cell cycling and apoptosis (Bernstein et al., 2002). The exact signalling molecules responsible for the range of responses are discussed in detail later

.

Tumours can eventually become resistant to treatment with cytotoxic drugs, and this resistance can be achieved by a variety of mechanisms. The different mode of action of cytotoxics compared with hormonal drugs such as tamoxifen means that the mechanisms of resistance are usually distinct from the manner in which resistance to hormonal drugs is achieved. Cells can acquire an increased ability to actively pump drugs out of the cytoplasm across the cell membrane utilising a variety of drug resistance proteins which are capable of transporting a variety of cytotoxic drugs out of cells in an ATP dependent process. The first of these to be discovered was P-glycoprotein (Pgp) (Juliano & Ling, 1976), which has since been shown to be able to actively transport scores of drug compounds out of cells (Seelig, 1998). Since the discovery of Pgp, further drug resistance proteins have been discovered and characterised, and these are discussed in detail in section 1.4.

Changes in activity of DNA repair proteins can also impart cytotoxic resistance to cells, and allow cells to grow unhindered in the presence of DNA damaging agents. Increased DNA repair has been shown to be important in the development of resistance to cisplatin (Parker et al., 1991), and to ionising radiation (Abbott et al., 1999). The cellular machinery used to sense and repair DNA damage is also discussed in section 1.5.

Resistance to drugs can also be achieved by changes in apoptotic signalling. Changes in levels of apoptosis-related proteins in tumours can result in cells which are reluctant to undergo apoptosis and can therefore survive and proliferate in the presence of high concentrations of cytotoxic agents (Eliopoulos et al., 1995). Apoptotic signalling is discussed in detail in section 1.6.

1.4 Drug Resistance Proteins.

Drug resistance proteins, which can actively pump cytotoxic agents out of cells, are frequently found to be overexpressed in drug resistant tumours and are at least partly responsible for facilitating cytotoxic resistance in tumours. Drug resistance proteins are members of the ATP-binding cassette (ABC) protein superfamily, and in common with other members of this family, they derive the energy required to pump cytotoxics (and other substrates) out of cells against strong concentration gradients from the hydrolysis of ATP. ABC superfamily members typically comprise one, two or three transmembrane domains, each consisting of five or six membrane spanning regions (Schinkel & Jonker, 2003). The extracelluar loops are typically N-glycosylated on at least one site. Studies with P-glycoprotien (Pgp), a very well characterised member of the ABC superfamily have revealed that this glycosylation is not necessary for drug transport, but may have a role in routing and stability of the proteins (Schinkel et al., 1993). The structure of Pgp, which displays two such domains each consisting of six membrane spanning regions is shown in figure 1.7. There are numerous subfamilies of the ABC superfamily, and subfamilies ABCB, ABCC and ABCG all contain drug resistance proteins.

1.4.1 ABCB subfamily.

1.4.1.1 P-glycoprotein.

The multidrug resistance protein P-glycoprotein (Pgp, MDR1, ABCB1), discovered over 30 years ago (Juliano & Ling, 1976) is a member of the ABCB subfamily and an important mediator of drug resistance in a wide variety of cells. Pgp displays a very broad substrate specificity, and as a result cells which overexpress Pgp display resistance to many cytotoxic agents and are said to be multidrug resistant (MDR). Cells from epithelial tissue from organs such as the kidneys, liver, colon and brain express Pgp intrinsically and as a result tumours which arise in these tissues are more likely to exhibit MDR. Pgp expression in other tumour types is typically heterogeneous, and as a result, over the course of a cytotoxic treatment

there will be a selective advantage on high Pgp containing cells (Ambudkar et al., 2005). It has now been shown that functional Pgp can be passed between cells, allowing high Pgp expressing cells to impart drug resistance to neighbouring cells (Levchenko et al., 2005).

1.4.2 ABCC Subfamily.

The ABCC subfamily includes a family of nine multidrug resistance proteins (MRP1-9) in addition to several other transporters which are not involved in the transport of cytotoxic drugs, such as the cystic fibrosis transmembrane conductance regulator (CFTR), the protein which is mutated in cystic fibrosis.

1.4.2.1 The MRP family.

The discovery of a multidrug-resistant cell line that did not overexpress Pgp (McGrath & Center, 1987) led to the isolation and further characterisation of MRP1 (Cole et al., 1992; Krishnamachary & Center, 1993), a membrane bound 190kd glycoprotein with a low level of homology to Pgp (around 15%). It is now known that MRP1 is part of a family of related drug transporters, nine of which have been characterised to date. Like Pgp, MDR family members contain two intracellular ligand binding domains and two membrane spanning domains but most MRP family members (MRPs 1,2,3,6,7) also contain a third membrane spanning domain. The individual structure and function of all family members is reviewed in (Kruh & Belinsky, 2003; Schinkel & Jonker, 2003) The structure of MRP1 is represented in figure 1.8.

All 9 MRP family members are able to pump a variety of cytotoxic substrates across cell membranes. The MRP family members and substrates pertinent to this study that they are able to confer resistance to are summarised in table 1.5.

1.4.3 ABCG Subfamily

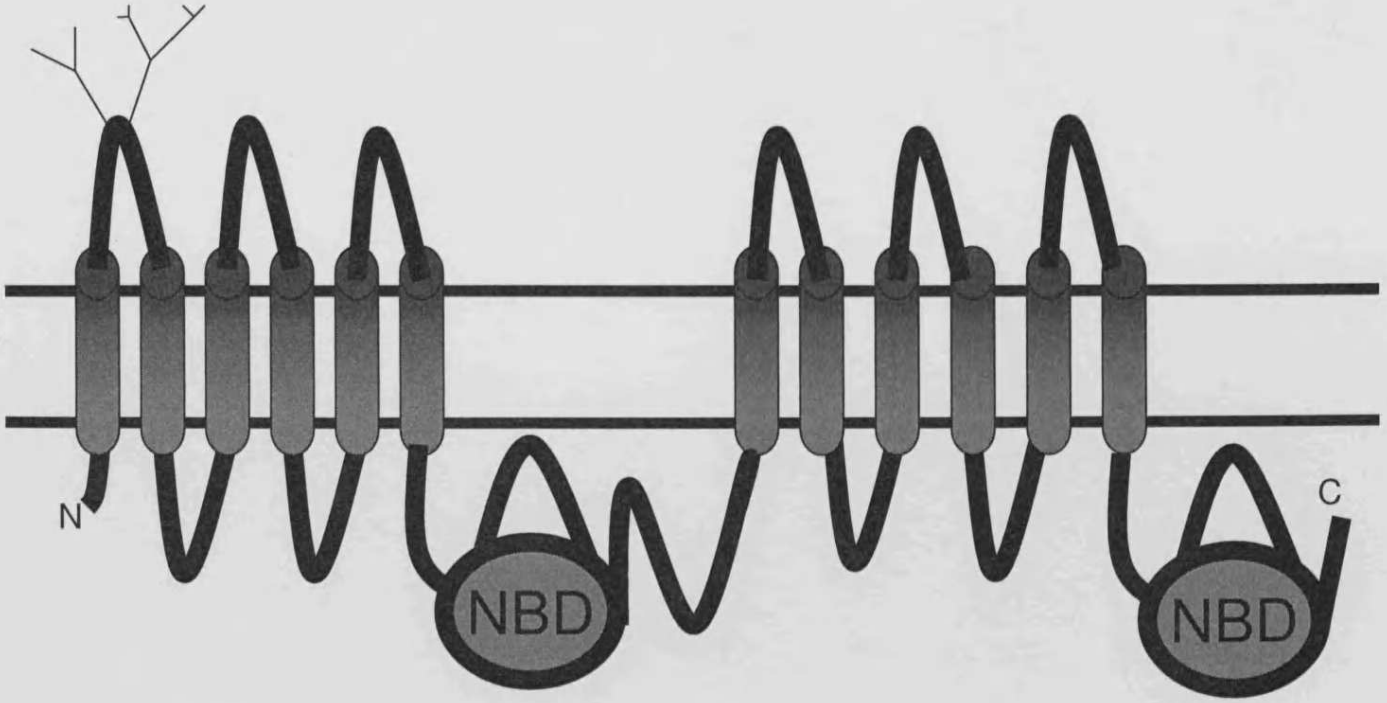


Figure 1.7 Structure of p-glycoprotein (pgp). Pgp consists of two domains each made up of six membrane-spanning regions. Both the N and C termini are located on the cytoplasmic side, as are the two nucleotide binding domains (NBDs). The first extracellular loop is N-glycosylated).

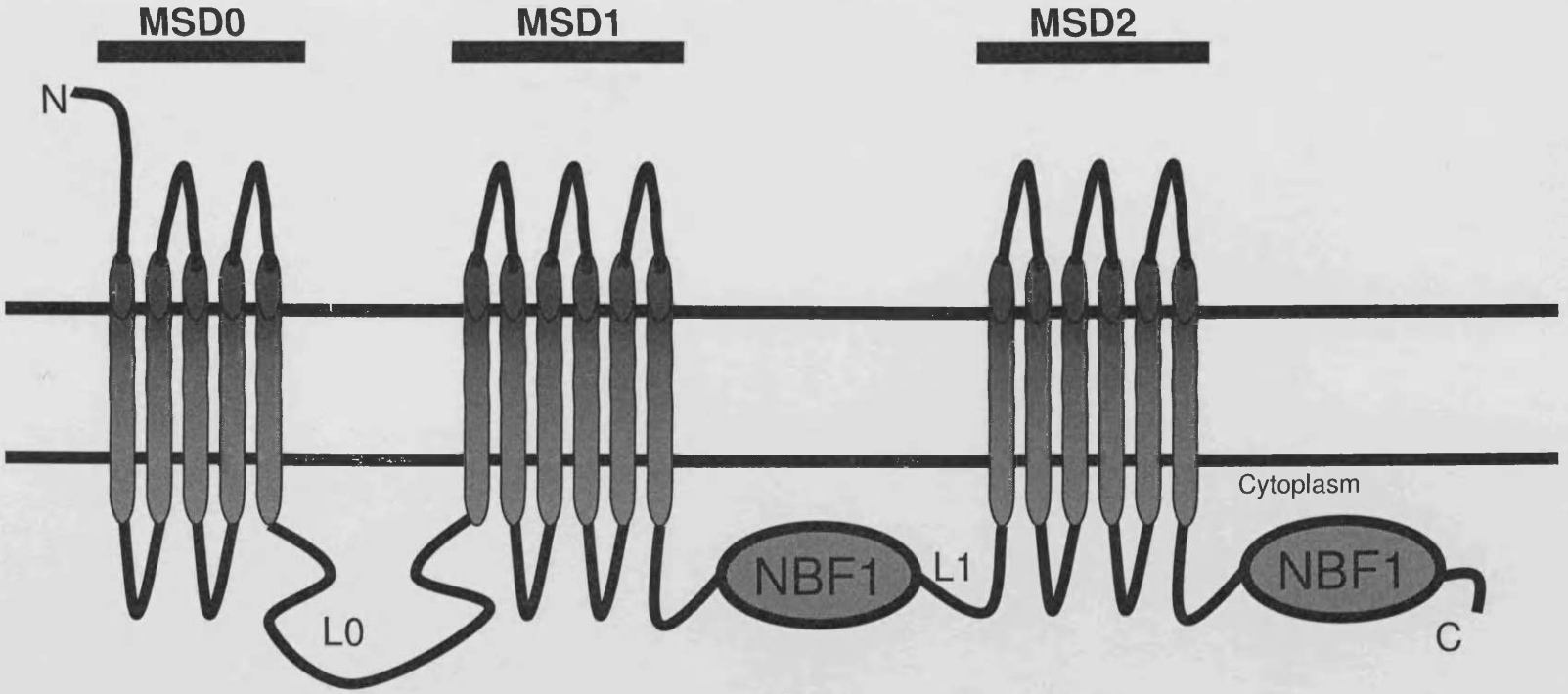


Figure 1.8 – Structure of MRP1. MRP1 contains two six-pass membrane spanning domains (MSD1 and MSD2) and two intracellular nucleotide binding domains. In addition to this, MRP1 also contains an additional five-pass membrane spanning domain (MSD0) joined to MSD1 via a cytoplasmic loop (L0). The additional five pass MSD0 domain means that the N terminal domain of MRP1 lies on the extracelluar side of MRP1. Not all MRP1 family members contain a MSD0 domain, MRP4, MRP5, MRP8 and MRP9 all lack an MSD0 domain, although they do all have a L0 intracellular link. Adapted from (Kruh & Belinsky, 2003)

Many of the members of the ABCG subfamily of transporters are concerned in normal physiological processes, in particular the transport of steroids in the brain, gut and liver (Lorkowski & Cullen, 2002). Some members of this family are known to be able to transport cytotoxics across cell membranes and are therefore able to confer drug resistance upon cells. Breast cancer resistance protein (BCRP) was first isolated from a multidrug resistant MCF-7 cell line (Doyle et al., 1998). Despite its name, BCRP is overexpressed in a wide variety of tumour types, and is present in a number of healthy tissues, particularly in the gut, where it serves to reduce the uptake of xenobiotics, for a review see (Allen & Schinkel, 2002). The relevant substrates of ABCG subfamily members are summarised in table 1.5.

Table 1.5 Drug efflux pumps, accession numbers and relevant substrates.

PROTEIN	SYNONYMS	Accession no	U133-a No*	Relevant Substrates
MDR1	ABCB1, PGY1, P-gp, GP170	NM_000927	209994_s_at 209993_at	Doxorubicin (Turton et al., 2001). Etoposide (Sohn et al., 2006).
MRP1	ABCC1	NM_004996 (sv1) NM_019862 (sv2) NM_019898 (sv3) NM_019899 (sv4) NM_019900 (sv5) NM_019901 (sv6) NM_019902 (sv7)	215559_at 202804_at 202805_s_at	Doxorubicin (Grant et al., 1994). Etoposide (Cole et al., 1994).
MRP2	ABCC2, cMOAT, cMRP	NM_000392	206155_at	Cisplatin (Hinoshita et al., 2000). Doxorubicin (Tada et al., 2002). Etoposide (Guo et al., 2002).
MRP3	ABCC3, MOAT-D, cMOAT-2	NM_003786 (MRP3) NM_020037 (MRP3a) NM_020038 (MRP3b)	N/A	Cisplatin (Haga et al., 2001). Doxorubicin (Tada et al., 2002). Etoposide (Zelcer et al., 2001).
MRP4	ABCC4, MOAT-B	NM_005845	203196_at	None.
MRP5	ABCC5, MOAT-C, pABC11	NM_005688 (tv1) NM_001023587 (tv2)	209380_s_at	None.
MRP6	ABCC6, MOAT-E, MLP1	NM_001171	215559_at 214033_at 208480_s_at	Cisplatin (Belinsky et al., 2002). Doxorubicin (Belinsky et al., 2002). Etoposide (Belinsky et al., 2002).
MRP7	ABCC10	NM_033450	213485_s_at	None

			215873_x_at	
MRP8	ABCC11	NM_032583. (tv1) NM_033151 (tv2) NM_145186 (tv3)	N/A	5-Fluorouracil (Guo et al., 2003).
WHITE1	ABCG1, ABC8	NM_207630 (tv1) NM_016818.(tv2) NM_207174 (tv3) NM_004915 (tv4) NM_207627 (tv5) NM_207628 (tv6) NM_207629.(tv7)	204567_s_at 211113_s_at	None
BCRP	ABCG2, MXR ABCP	NM_004827	209735_at	Doxorubicin (Robey et al., 2003)

* Refers to reference number for gene on Affymetrix U133A Genechip.

1.5 Double Stranded breaks in DNA.

Double stranded breaks most frequently occur as a result of exposure to ionising radiation or radiomimetic drugs such as bleomycin, but can also occur occasionally as a result of mechanical stress or by the collapse of replication machinery caused by a more minor DNA lesion. A DSB requires breaks in both strands sufficiently close together to overpower the Watson-Crick hydrogen bonding which holds the strands together around the break. As a result, double stranded breaks are rarely clean, and will often display overhangs containing several unpaired bases, or fragments of sugars which have to be processed prior to repair. Left unrepaired, DSBs can cause aneuploidy, as the broken fragments of chromosomes are unable to segregate correctly. A single DSB is thought to be enough to cause cell death (Huang et al., 1996), either through inactivation of a vital gene of more likely, through the induction of apoptosis. Sustaining and surviving a double stranded break can have severe consequences, due to the fact that the two broken half-chromosomes have no physical attachment. This situation can lead to chromosomal exchanges, with the resulting daughter cells containing incorrect numbers of particular genes, a possible step toward carcinogenesis.

Eukaryotic cells have two principal pathways for dealing with DSBs. Non-homologous end joining (NHEJ), where two ends are simply processed and ligated back together with an occasional loss of several bases of genetic information and the more energy-intensive non-homologous recombinational repair (HRR) pathway, which is capable of replacing any genetic information which may have been lost upon the acquisition of DNA damage. Both pathways are well conserved throughout the eukaryotes from yeast to humans (Shinohara et al., 1993). However, it should be noted that higher eukaryotes (including humans) repair the majority (50-90%) of DSBs using NHEJ, whereas lower eukaryotes such as yeast are more dependent on the HRR pathway (Jackson & Jeggo, 1995). The reasons that higher eukaryotes are more dependent on NHEJ are not fully understood, but the presence of large repetitive regions of DNA in

higher eukaryotes, along with relatively large distance between regions of homology (except during the G2 and M phases) might well result in the formation of multiple chromosomal translocations during HRR, as DSBs in repetitive regions are mis-matched to incorrect templates (Lieber et al., 2003). The likelihood and severity of this has to be weighed up against the chance of losing several bases of genetic information during NHEJ if damage is sustained in an exon of an active gene. Again the low percentage of DNA which codes for critical protein seems to bias higher eukaryotic cells towards NHEJ, especially considering the implications of progression through the cell cycle with an unrepaired DSB.

The mechanism by which one DSB repair pathway is selected above another is not particularly well understood. It is known that more HRR occurs in cells which have defects in NHEJ proteins, suggesting a passive competitive relationship. A more recent school of thought is that there is a large amount of cross talk between the DNA-PK holoenzyme and components of the HRR pathway such as p53, ATM and RPA (Allen et al., 2003).

1.5.1 Homologous Recombinational Repair (HRR).

Homologous recombination repair involves the use of sequence information from an homologous double stranded DNA molecule to repair DNA damage and replace any genetic information which may have been lost as a result of this damage. Considering its reliance on either a sister chromatid or homologous chromosome to act as template, it is not surprising that most HRR activity is detected in the D/G2 phase of the cell cycle, when additional copies of the genome are present.

The first stage of HRR involves formation of a complex around the broken DNA ends. The Histone H2AX is phosphorylated by ATM, and BRCA1, along with the MRN complex are recruited to the damage site, along with nucleases which digest in a 5'-3' direction from the break site, creating large 3' single-stranded DNA tails. These 'tails' are then coated with many copies of RPA which in turn facilitates the binding of Rad51 and

BRCA2, which in turn recruits the Rad52 and Rad54 proteins (reviewed in (Valerie & Povirk, 2003)). These proteins facilitate the location and invasion of an homologous region and a Holiday junction is established. The homologous strand is then used as a template to synthesise the missing bases. The HRR pathway is not nearly as well understood as the NHEJ pathway, and the polymerase responsible for this synthesis is not known. It is however thought that DNA-ligase I is responsible for the ligation of the final nick.

1.5.2 Non-Homologous End Joining (NHEJ).

Non-Homologous end joining is a method of repairing DSBs without the use of template. It can occasionally result in a loss of genetic information, varying from a point mutation at the site of breakage to the translocation of large amounts of genetic information. The NHEJ pathway has the unique ability to ligate otherwise unligateable DNA ends. There is large degree of overlap between the NHEJ pathway and V(D)J recombination, as both pathways are involved with the ligation of double stranded breaks.

Arguably the most important complex in NHEJ, the DNA-dependent protein kinase (DNA-PK) has been known for a long time to be critical in NHEJ (Finnie et al., 1995), and is made of two subunits. A catalytic subunit (DNA-PK_{cs}) A member of the phosphoinositol-3-kinase related protein kinase (PIKK) family, harbouring the serine/threonine kinase activity, and the Ku heterodimer, comprising an 86kDa subunit (Ku80) and a 73kDa subunit (Ku70). This complex rapidly forms at the broken ends of DNA, with the Ku heterodimer binding first, and then recruiting DNA-PK_{cs} to form the DNA-PK holoenzyme.

1.5.2.1 The Ku Heterodimer.

The binding of Ku to a DSB is known to be vitally important to the repair of DNA via the NHEJ pathway, with Ku activity directly correlating to the activity of the DNA-PK holoenzyme (Zhao et al., 2000). Cells treated with Ku86 antisense have been shown to be more sensitive to ionizing

radiation, bleomycin and etoposide, all agents which cause DSBs (Belenkov et al., 2002). Consistent with a role in both DNA repair and V(D)J recombination, a lack of Ku is associated with hypersensitivity to DNA damage and immunodeficiency, and it was from patients with immune system defects that the Ku protein was first discovered as an autoantigen (Koike, 2002). Ku has a very high affinity for DNA ends, and is able to translocate along DNA in an ATP independent fashion. The two components of Ku share a similar central domain which is concerned with binding DNA, but have unique terminal domains. The carboxy-terminal of Ku80 contains the region which interacts with DNA-PK_{cs} (Gell & Jackson, 1999).

1.5.2.2 DNA-PK_{cs}.

The catalytic subunit of DNA-PK is also a critical mediator of radiosensitivity, with studies in both animals (Okayasu et al., 2000) and breast carcinoma cells (Kim et al., 2002) demonstrating that a lack of DNA-PK_{cs} results in sensitivity to DSBs. With this in mind, DNA-PK has been identified as a possible target for chemotherapeutics which will sensitise tumour cells to other DSB-causing drugs (Kashishian et al., 2003). DNA-PK_{cs} is believed to be an extremely ancient and well conserved component of the cellular machinery, with DNA-PK_{cs} orthologues being found in *Anopheles gambiae* (mosquito) and *Apis mellifera ligustica* (honey bee), (Dore et al., 2004), indicating that NHEJ may have developed very early in the development of eukaryotic cells.

DNA-PK is recruited to the site of DNA damage where it becomes activated, and as well as physically protecting the ends from further degradation is able to activate and recruit other factors (such as DNA Ligase IV and XRCC4) to the damage site. The binding of DNA-PK facilitates the translocation of DNA-PK_{cs} and enables the phosphorylation of XRCC4, but this interaction has been shown not to be critical for NHEJ (Lee et al., 2004) or V(D)J (Yu et al., 2003). DNA-PK_{cs} also phosphorylates p53, an event critical for the induction of DSB mediated apoptosis (Bernstein et al., 2002). DNA-PK is also able to phosphorylate Ku, WRN (The Werners

Syndrome Protein), the Atermis nuclease and is also capable of self-phosphorylation, which results in the dissociation of DNA-PK from the DNA.

DSBs are very rarely clean breaks and often nucleotides are lost or damaged around the break. site. Sugars around the site of damaged can be damaged by free radicals and covalent hairpins can also form at the ends of strands. While these residues to not interfere with the binding of Ku and DNA-PK, any damaged sugar or phosphate residues need to be removed before ligation can take place. The protein responsible for this step is Artemis, a factor known to be critical for NHEJ which also has exonuclease activity. Lack of Artemis results in a reduced ability to deal with DSBs as well as a more pronounced inability to repair bleomycin-induced DSBs which require end processing (Rooney et al., 2003). Other possible contributors to DNA processing prior to ligation are Human terminal deoxytransferase (TdT), The Werners syndrome protein (WRN), polynucleotide kinase (PNK), Human tyosyl-DNA phosphodiesterase (hTdp1) and the MRN complex (Lees-Miller & Meek, 2003).

Once the ends have been processed they are spatially arranged by the DNA-PK complex. There is often no natural homology between the broken ends, as bases very near to the site of damage will have been destroyed or damaged and removed by Artemis. Thus regions of microhomology are used to align the strands, with as little as one nucleotide of homology being required for repair to proceed. DNA polymerase μ (pol- μ) is then recruited to add in the relevant homologous nucleotides. Pol- μ then assists with the recruitment of the ligation machinery, namely XRRC4 and DNA Ligase IV, both of which are essential for NHEJ (Adachi et al., 2001; van Heemst et al., 2004). These proteins are known to form a complex containing twice as many XRCC4 molecules as Ligase-IV molecules, sometimes referred to as the LX complex, This LX complex exists as either a trimer of 2 XRCC4 and one Ligase-IV or a Hexamer of 4 XRCC4 and 2 Ligase-IV molecules and completes the ligation before the complex dissociates. NHEJ is summarised in figure 1.9.

1.5.3 DNA repair and apoptotic signalling.

Both repair pathways contain proteins that are able to generate apoptotic signals in addition to having a function in DNA repair. This allows information on the extent of DNA damage to be collated by the cellular machinery, and protects against genomic instability by promoting apoptosis in cases where irreparable amounts of damage are sustained.

In HRR, BRCA1 regulates the assembly and activity of the BRCA1-associated genome surveillance complex (BASC), a large multi-protein assembly which senses and repairs DNA damage (Wang et al., 2000c). Studies with dominant negative BRCA1 have demonstrated that functional BRCA1 is critical for apoptosis in breast and ovarian cancer cell lines, and that BRCA1 mediates apoptosis through a pathway involving H-Ras, MEKK4, and Fas ligand, which results in the activation of caspase-9 (Harkin et al., 1999; Thangaraju et al., 2000). The protein mutated in ataxia telangiectasia, ATM, also forms part of BASC, and following the induction of DSBs by ionising radiation is able to stimulate apoptosis through the phosphorylation of p53 (Canman et al., 1998). ATM can also influence apoptosis through its ability to phosphorylate and stabilise the transcription factor E2F1 in response to DNA damage (Lin et al., 2001) E2F1is able to induce apoptosis by promoting the transcription of the p53 homolog p73, and by stabilising p53 through the p14ARF-mediated neutralisation of HDM2. In the NHEJ pathway, DNA-PK, which serves to detect DSBs and align the ends ready for ligation by DNA ligase IV (Karran, 2000) also has the capacity to activate apoptosis through the phosphorylation of p53 (Shieh et al., 1997), and the inactivation of HDM2, the endogenous inhibitor of p53 (Mayo et al., 1997). Experiments with DNA-PK null mice have demonstrated that DNA-PK is necessary for p53 activation and accumulation following radiation damage, and that without DNA-PK, irradiation does not produce the reduction in Bax levels normally effected

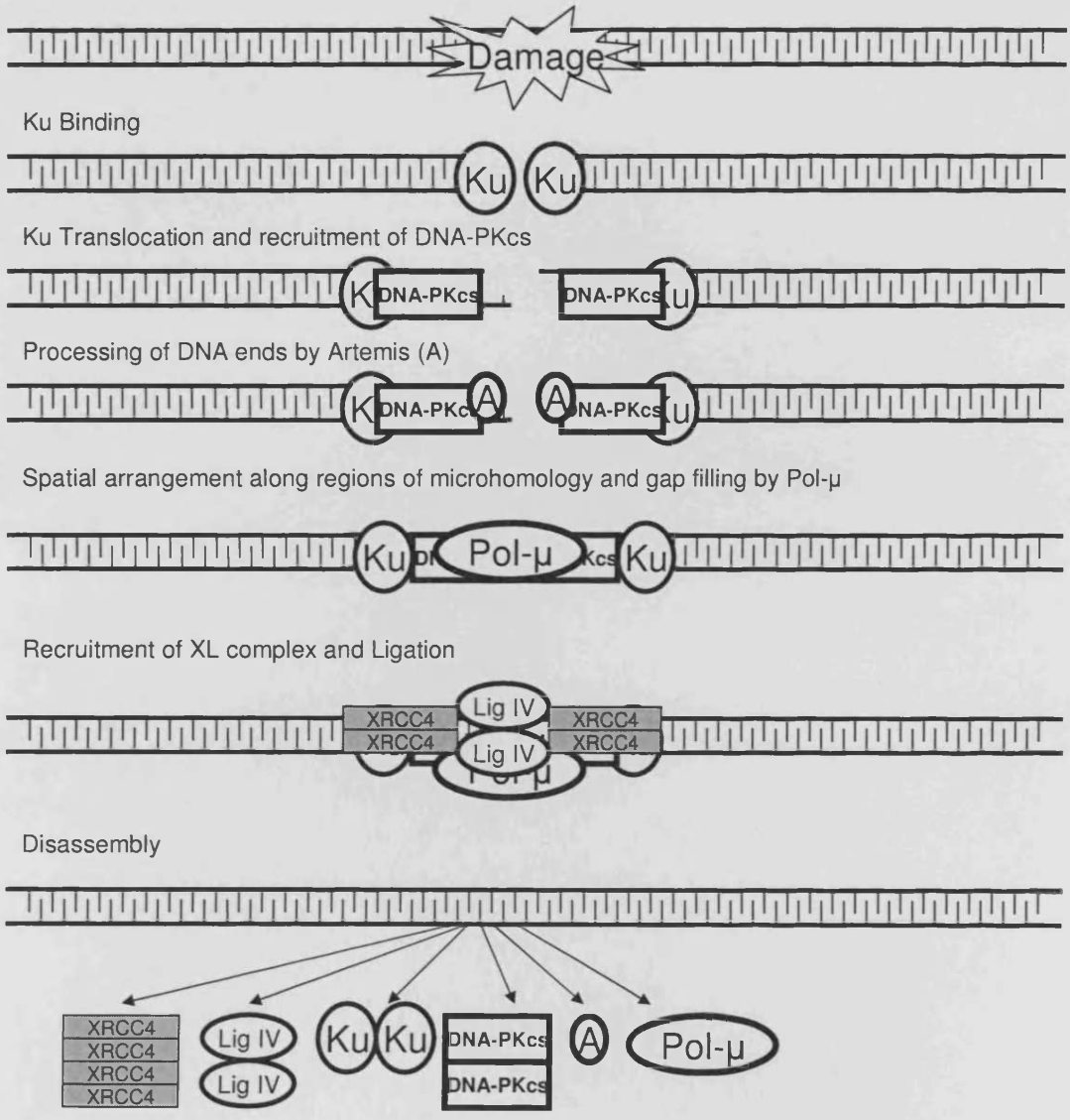


Figure 1.9 The non-homologous end joining pathway. Responsible for the repair of the majority of DSBs in higher eukaryotes.

by p53 (Wang et al., 2000b). It was initially thought that p53 was necessary for apoptosis following the induction of double stranded breaks by radiation (Lowe et al., 1993), but more recently, experiments in fibroblasts from p53 null mice have demonstrated apoptosis being caused by both ionising radiation, and 'clean' DSBs generated through electroporation of the *Pvu*II restriction enzyme (Lips & Kaina, 2001). This apoptotic pathway involved a decrease in bcl-2 transcription, indicating that pathways exist that link DSBs to apoptosis that do not involve p53.

1.6 Apoptosis.

Apoptosis is the programmed death of cells. In many self-renewing tissues, the constant turnover of cells is maintained via regulation of the balance between proliferation and apoptosis. The programmed death of cells is also vital during embryonic development, where many cells which make up the foetus are destined to die before the end of gestation (Runic et al., 1998). Apoptosis is of particular importance in the maintenance of genomic integrity. As cells accumulate damage in their DNA, as result of exposure to cytotoxic agents such a radiation or chemotherapeutic drugs, cells with badly damaged DNA are able to activate apoptotic pathways and remove themselves from the organism to avoid the propagation of damaged genetic information which may lead to loss of genomic stability and the establishment, or further development of cancer (Zhivotovsky & Kroemer, 2004).

1.6.1 Apoptotic signals.

There are a wide variety of cellular signals which contribute to the development of an apoptotic signalling cascade. It should be noted that apoptosis can be regulated by a large number of signalling molecules and that the levels of many proteins throughout the whole apoptotic pathway can effect the decision of a cell to commit to apoptosis. A cell can be 'told' to undergo apoptosis by the receipt of a death signal via a receptor on the cell surface, for example the Fas receptor. This mechanism is relatively well understood (Nagata & Golstein, 1995) and is the mechanism by which cytotoxic t-cells are able to initiate cell death in other cells through surface to surface contact. Death signals can also be received in a similar manner by other well understood death receptors such as the tumour necrosis factor receptor-1 (TNFR-1) and the binding of the TNF-related apoptosis inducing ligand (TRAIL) to its receptors DR4 and DR5 (Pan et al., 1997b). Unlike other death factors, TRAIL appears to be constituently produced by a large number of cell types. It is thought that most cells are protected from the apoptotic action of TRAIL by the action of 'decoy receptors' which

sequester TRAIL but lack the intracellular activity to initiate apoptosis (Pan et al., 1997a).

1.6.2 The FAS ligand and CD95 receptor.

While the FasL / CD95 will be examined in detail, it should be noted that all three of these pathways operate in a similar manner, namely through the facilitation of receptor trimerisation by the presence of the correct ligand which in turn assembles death domains on the cytoplasmic side of the plasma membrane which initiate apoptosis through the cleavage and activation of caspase 8.

The FasL ligand (FasL, CD95L, Apo-1L) is itself activated by trimerisation, and the trimeric ligand, which can be displayed on the surface of cytotoxic t-cells, or solublised by shedding through the action of metalloprotienases (Tanaka et al., 1998) in turn promotes trimerisation of the receptor. Trimerisation of CD95 causes the cytosolic death domains (DDs) of CD95 to cluster, and recruit other proteins also in possession of a DD, most importantly Fas-associated protein with death domain (FADD) which is vital for the activation of caspase 8 (Juo et al., 1999). Caspase 8 is able to interact and be cleaved by FADD due to the death effector domains (DEDs) possessed by FADD. The complex comprising CD95, FADD and caspase 8 is sometimes termed the death-inducing signalling complex (DISC) and is summarised in figure 1.10. Apoptosis can be regulated at this stage through the action of the FLICE-like inhibitory protein (FLIP or cFLIP), a protein which shares a large degree of homology with caspase 8 and is thus able to enter DISC, but which lacks any of the proteolytic activity necessary to initiate apoptosis (Wang et al., 2000a).

1.6.3 Downstream of Caspase 8.

Caspase 8 is able to bring about apoptosis by two mechanisms. It can either directly cleave and therefore activate downstream 'effector' caspases (caspases 3 and 7) seemingly independent of other apoptotic regulation (Salvesen & Dixit, 1997), or through the direct cleavage of Bid (Li et al., 1998). Bid is a member of the bcl-2 family which is able to initiate a

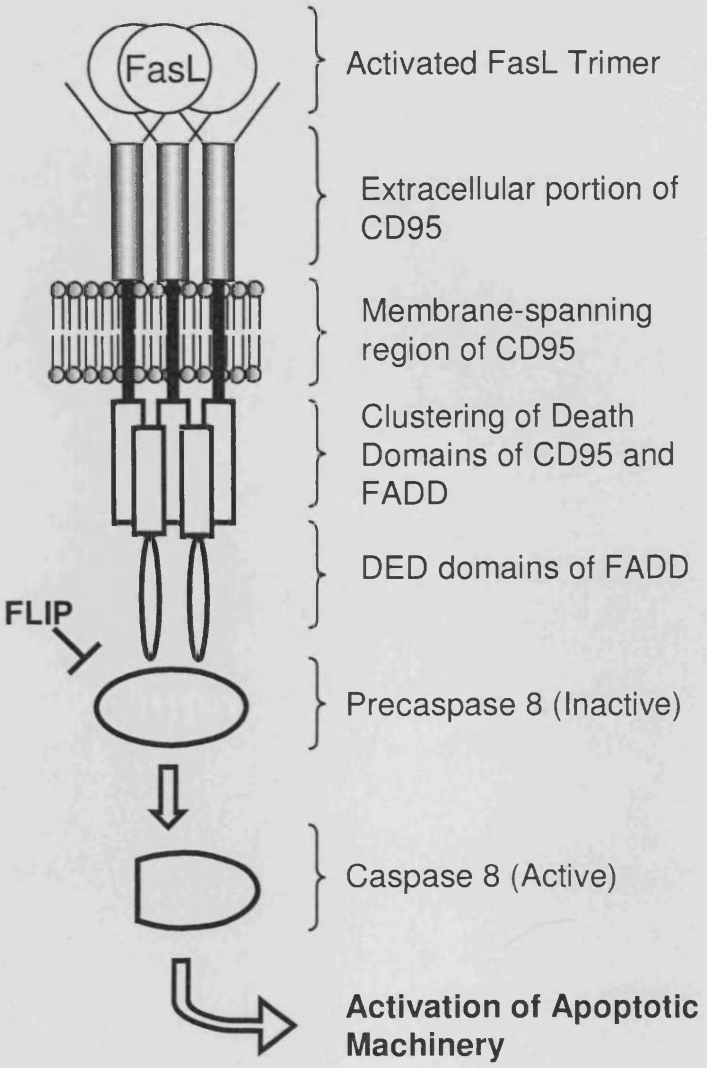


Figure 1.10 Activation of the apoptotic initiator caspase 8 following stimulation of a cell death receptor. This multiprotien assembly is sometimes termed the death-inducing signalling complex (DISC).

more readily regulated apoptotic pathway involving more bcl-2 family members and the loss of cytochrome c from mitochondria, also ultimately resulting in the cleavage and therefore activation of the effector caspases. Experimentation has confirmed the ability of caspase 8 to initiate two separate apoptotic pathways, one in which apoptosis can be blocked by bcl-2 overexpression, and where a loss in mitochondrial membrane potential is observed and a second mitochondria independent pathway (Scaffidi et al., 1998). It is thought that the amount of active caspase 8 is critical in deciding which of these apoptotic pathways is used.

1.6.4 Mitochondria dependent apoptosis.

This pathway is used to relay the apoptotic signal in the majority of cell types and apoptotic scenarios where there is insufficient activated caspase 8 to bring about apoptosis by direct cleavage of the effector caspases (Kuwana et al., 1998). This pathway effectively amplifies the apoptotic signal, allowing apoptosis to arise from a relatively small signal and can also be regulated at many stages and thus can serve as a point at which pro- and anti-apoptotic signals can be collated, and apoptosis can be prevented or promoted depending on the circumstances of the cell (Green & Reed, 1998). In addition to enabling apoptosis as a response to extrinsic signals, apoptosis can also occur as a result of signals contained within a single cell, so called 'intrinsic apoptosis'. bcl-2, and other members of the bcl-2 family are responsible for the majority of the regulation which occurs during mitochondria-dependent apoptosis (Kluck et al., 1997). It is a bcl-2 family member which receives the initial apoptotic signal from caspase 8, and the interplay of other bcl-2 family members are responsible for the maintenance of mitochondrial membrane integrity.

1.6.5 The Bcl-2 family.

Twenty years ago it was known that certain genes in *C. elegans* were vital for programmed cell death to occur. Studies showed that mutations in the *ced-3* and *ced-4* genes resulted in the survival of cells which were normally fated to die during *C. elegans* development (Ellis &

Horvitz, 1986) and it was later shown that there were also genes in *C. elegans* (*ced-9*) which protected cells from cell death (Hengartner et al., 1992).

The first mammalian gene with homology to these *ced* genes displayed homology to *ced-3* and was termed bcl-2, as the gene was first observed as the site of a common translocation in B-cell lymphoma. Introduction of the gene cDNA into lymphoid and myloid cell lines promoted cell survival following the removal of interleukin-3 (IL-3) (Vaux et al., 1988).

Since the discovery of bcl-2, many other proteins have been discovered which share homology with bcl-2, and contain at least one of the four identified bcl-2 homology (BH) domains, numbered 1-4. These proteins comprise the bcl-2 family. The family is broadly divided into three sub-families. The pro-survival bcl-2 family containing bcl-2, bcl-X_L and bcl-w, The pro-apoptotic or bax subfamily containing bax, bak and bok, and a third family which only contain a region homologous to the BH3 domain of bcl-2 and are unrelated to any other known protein (Kirkin et al., 2004). these comprise the BH3 subfamily which contains the pro-apoptotic proteins bad, bid, bik, blk hrk, BNIP3 and bimL (Shibue & Taniguchi, 2006). Certain viral proteins such as the Epstein-Barr virus protein E1B-19K are also homologous to bcl-2 (Han et al., 1996a).

The structure of bcl-2 consists of 6 α-helices with a hydrophobic groove known to bind the BH3 domains of other family members and is thus important in dimerisation (Petros et al., 2001). The structure of the other bcl-2 family members show remarkable structural similarity to bcl-2, despite marked differences in amino acid sequence and regardless of whether they serve to promote apoptosis or to prevent it (Petros et al., 2004).

1.6.6 Cleavage of bid by caspase 8.

The first stage in mitochondria dependent apoptosis is the cleavage of the bcl-2 family member Bid by caspase 8. Bid is a cytoplasmic 29kd protein which is readily cleaved into a 15kd C-terminal and 14kd N-terminal fragments. The C-terminal fragment is termed truncated Bid (tBid) and is a potent apoptosis-inducing agent, and retains the ability to interact with other bcl-2 family members via a bcl-2 homology 3 (BH3) domain and to form pores in the mitochondrial membrane (Li et al., 1998). The structural integrity of Bid is unharmed by this cleavage, and it is able to promote apoptosis both by binding Bcl-X_L (an antiapoptotic protein closely related to bcl-2) via its BH3 domain and by independently promoting mitochondrial damage (Chou et al., 1999). It has been shown that the proposed cytochrome c releasing factor (CCRF) responsible for the apoptotic activity contained in caspase-8 treated HeLa cytosol is in fact tBid (Luo et al., 1998). Granzyme B (GrB), an aspartyl serine protease released in the granules of cytotoxic T-lymphocytes is also able to initiate apoptosis via cleavage of Bid into tBid (Alimonti et al., 2001), although cleavage occurs at a slightly different site to caspase 8 mediated cleavage.

1.6.7 Mitochondrial integrity and cytochrome c release.

The bcl-2 family serves to regulate the formation of the apoptosome, a multimeric complex which can activate caspase 9 and thus begin the cascade that leads to the activation of the effector caspases. The apoptosome is a heptameric assembly of Apaf-1 and cytochrome c, arranged in a circular pattern with sevenfold symmetry (Acehan et al., 2002). Cytochrome c, which is normally localised in the mitochondrial matrix assists in the hydrolysis of dATP which is bound as a cofactor to Apaf-1. This hydrolysis provides the energy for Apaf-1 and cytochrome c to form an active apoptosome, and while cytochrome c is critical for successful apoptosome formation, the presence of dATP is also critical for Apaf-1 and cytochrome C to be able to form a complex capable of caspase activation (Kim et al., 2005). This complex then goes on to recruit caspase 9, which it is able to activate by cleavage. Activated caspase 9 is then free to activate the effector caspases (caspases 3 and 7), which are able to

bring about chromatin condensation, DNA fragmentation and other apoptotic processes.

1.6.8 The pro-apoptotic bcl-2 family members.

It is bax, and other related members of the pro-apoptotic bcl-2 family that are responsible for the formation of pores in the mitochondrial membrane which allow cytochrome C to escape into the cytosol and promote the formation of the apoptosome. bax is ordinarily located in the cytosol of healthy cells, but following an apoptotic stimulus it becomes localised to membranes, in particular the mitochondrial membrane (Wolter et al., 1997). bax also heterodimerises following an apoptotic stimulus (Gross et al., 1998) and then goes on to form oligomers of molecular weight between 96,000 and 260,000 kDa which form pores in the mitochondrial membrane (Antonsson et al., 2001). This oligomerisation is promoted by the presence of activated Bid (Eskes et al., 2000), providing an opportunity for ligand-mediated apoptotic signals to bring about cytochrome C translocation. The pore-forming ability of bax is greatly reduced by the presence of anti-apoptotic bcl-2 family members such as bcl-2 (Antonsson et al., 1997) or bcl-xL (Finucane et al., 1999). It was initially thought that these pores physically assisted in the lysis of the apoptotic cell, but it has been subsequently demonstrated that pores formed by bax and other pro-apoptotic family members permeablise the mitochondrial membrane and allow cytochrome C to be released from the mitochondrial matrix into the cytosol (Jurgensmeier et al., 1998). Anti-apoptotic bcl-2 family members, which are normally found around the mitochondria regardless of the presence of an apoptotic stimulus (Monaghan et al., 1992) disrupt this translocation (Kluck et al., 1997).

In addition to indirectly facilitating apoptosome formation, bcl-2 is also able to regulate apoptosis in apaf-1 deficient mice (Marsden et al., 2002), indicating that bcl-2 contributes to apoptosis through an additional pathway not related to apoptosome formation.

1.6.9 BH3 subfamily.

Members of the BH3 subfamily, which share homology only with the BH3 domain of bcl-2, and include bad, bid, bim and blk also contribute to the regulation of apoptosis. All the BH3 proteins have a pro-apoptotic effect (Huang & Strasser, 2000), and can illicit this effect either through interactions with both pro-, and anti-apoptotic bcl-2 family members. The BH3 protein Bid, which is activated by cleavage by caspase 8 (see above) is known to be essential for cytochrome c release, where it induces a conformational change in the pro-apoptotic protein BAK (similar to BAX), which facilitates oligomerisation and allows the formation of channels in the mitochondrial membrane (Wei et al., 2000). Other BH3 family members interact with anti-apoptotic family members such as bcl-2, and prevent them from eliciting their antiapoptotic effects (Cheng et al., 2001; Huang & Strasser, 2000). The contribution of bcl-2 family members to the regulation of apoptosis is shown in figure 1.11.

1.6.10 Regulation.

The activity of bcl-2 family members can be regulated by a number of mechanisms, all of which serve to collate information on the status of the cell through regulation of the balance between pro-, and anti-apoptotic bcl-2 family members. Signals can be integrated into the bcl-2 regulated apoptotic pathway through the altering the proteins at the transcriptional level, for example the levels of pro-apoptotic bim are increased with cytokine withdrawal in lymphocytes through increased transcription mediated through the forkhead transcription factor (Dijkers et al., 2000), and the bcl-2 gene is known to contain two oestrogen response elements and can be upregulated by 17β-oestradiol (Perillo et al., 2000) and downregulated by treatment with antioestrogens (Diel et al., 1999). The activity of bcl-2 family proteins can also be modulated through direct interaction with signalling molecules. In addition to upregulating bcl-2 expression through the cAMP response element binding protein (Pugazhenthi et al., 2000), the survival factor Akt can also directly

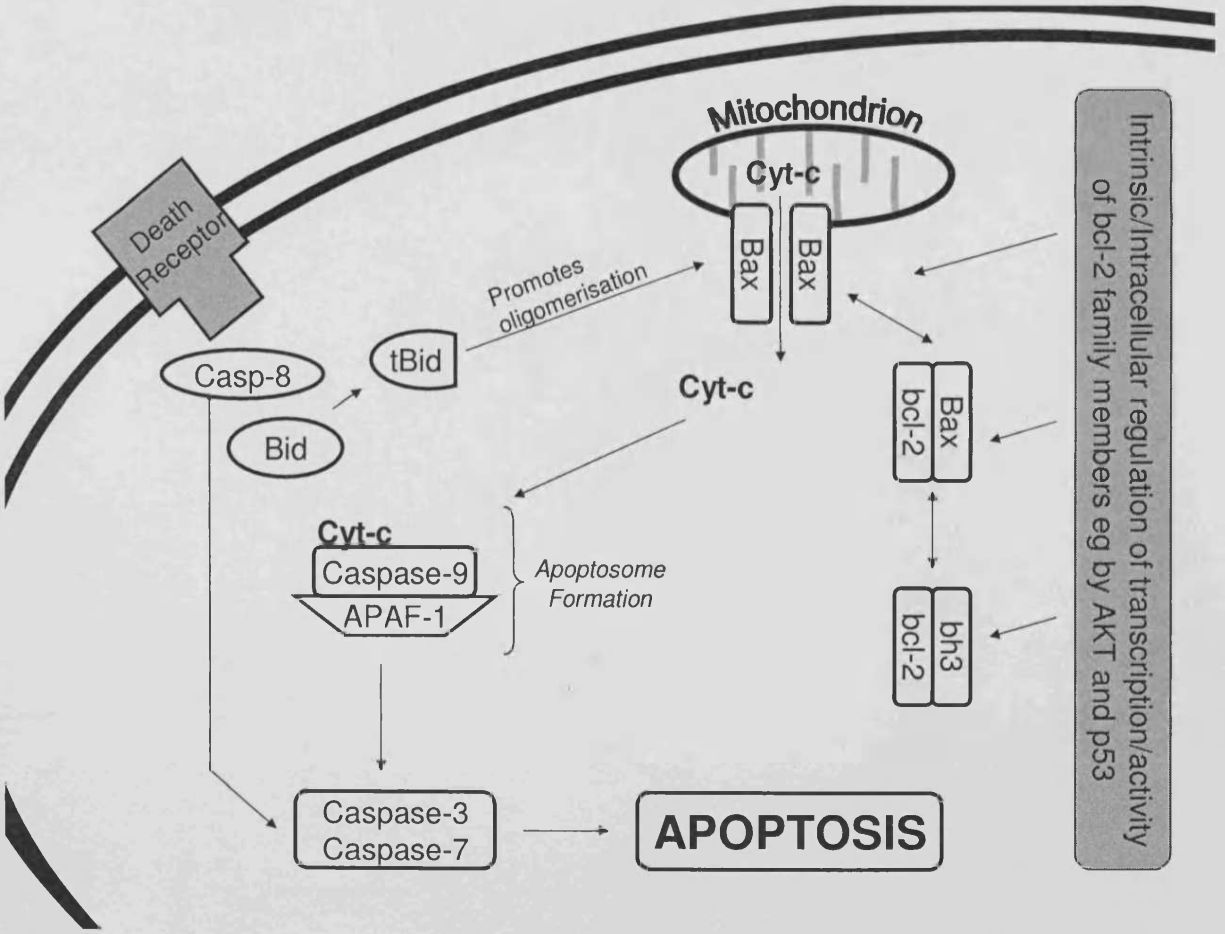


Figure 1.11 The bcl-2 family and regulation of apoptosis. bcl-2 family members primarily regulate the permeability of the mitochondrial membrane to cytochrome c, which is essential for apoptosome formation in the cytoplasm

phosphorylate and inactivate bad (Datta et al., 1997), preventing it from fulfilling its pro-apoptotic role. Consistent with its role as a tumour suppressor and cell cycle regulator, p53 interacts with a wealth of bcl-2 family members, having the ability to regulate many at the transcriptional level, for example bcl-2 and bax (Miyashita et al., 1994b) and Noxa (a pro-apoptotic BH3 protein) (Oda et al., 2000). p53 can also directly activate bax (Chipuk et al., 2004).

1.6.11 Effector Caspases.

Once activated by the formation of the apoptosome, caspase 9 is able to cleave and activate the effector caspases, such as caspase 3 and caspase 7, the activity of which carry out apoptosis through the cleavage of proteins such as gelosin (Kothakota et al., 1997), PAK2 (Rudel & Bokoch, 1997), focal adhesion kinase (Wen et al., 1997) and rabaptin -5 (Cosulich et al., 1997), bringing about the typical apoptotic phenomena of rounding up of cells, membrane blebbing, formation of apoptotic bodies and the cessation of membrane transport. Caspase-3 is also known to be responsible for DNA fragmentation (Janicke et al., 1998), through the cleavage and inactivation of ICAD, the inhibitor of caspase-activated DNase (CAD), which becomes available to degrade the DNA at the linker regions between nucleosomes (Sakahira et al., 1998).

As well as carrying out the cleavage of structural and functional molecules that bring about apoptosis, caspases are now known to be able to further sustain the apoptotic signal through the cleavage and inactivation of survival factors. For instance, EGFR is known to be cleaved and inactivated by caspase-3 (Bae et al., 2001), and caspase-3 is able to further increase the potency of the apoptotic signal by cleaving bcl-2 into a smaller, proapoptotic molecule (Cheng et al., 1997), creating a positive feedback loop which continues to generate a self propagating apoptotic signal.

1.7 Experimental aims of this study

It is the aim of this study to examine what effect the development of tamoxifen resistance has on the sensitivity of breast cancer cells to cytotoxic agents. A close correlation between EGFR expression and cytotoxic resistance has been demonstrated in a number of experimental and clinical systems and it will be of interest to discover whether the increased expression of EGFR that is known to accompany the development of tamoxifen resistance also results in a phenotype more resistant to treatment with cytotoxic drugs.

The extent to which cytotoxic sensitivity is altered will be examined, as will changes in the expression of proteins that may serve to regulate the response of tamoxifen resistant cells to cytotoxic insult, including proteins concerned with the detection and repair of DNA damage, proteints responisible for the efflux of drug molecules, and the proteins which regulate apoptosis.

Chapter 2

Materials and methods.

2.1 Cell Culture.

2.1.1 Cell lines used in this study.

Wild-type MCF-7 – MCF-7.

MCF-7 cells are an epithelial human metastatic mammary carcinoma cell line, originally isolated from a pleural effusion of a woman with metastatic breast cancer in 1973. These cells were quickly identified as expressing the oestrogen receptor (Brooks et al., 1973) and were latterly identified as being responsive to the growth stimulatory and inhibitory action of oestrogens and antioestrogens respectively (Lippman et al., 1976). MCF-7 cells used in this study were obtained from the Tenovus Centre for Cancer Research (Welsh School of Pharmacy, Cardiff, UK).

Tamoxifen resistant MCF-7 MCF-7(TamR).

A tamoxifen resistant cell line was derived by the continuous culture of MCF-7 cells in media deprived of phenol red, which has an weak oestrogenic effect (Welshons et al., 1988). Steroid hormones were also removed by charcoal stripping of serum, and media was supplemented with 10^{-7}M 4-hydroxytamoxifen (Sigma, UK). Initially tamoxifen inhibits the growth rate; however following six months in culture the rate of growth increases indicating the development of tamoxifen resistance (Knowlden et al., 2003). Following establishment of tamoxifen resistance cells, are routinely cultured for a further four months before experimental use. In this study MCF-7(TamR) cells were obtained from the Tenovus Centre for Cancer Research (Welsh School of Pharmacy, Cardiff, UK).

Tamoxifen & gefitinib resistant MCF-7.- MCF-7(DR).

The selective EGFR inhibitor gefitinib (Iressa™, ZD1839) initially causes a drastic reduction in growth rate of MCF-7(TamR) cells. When MCF-7(TamR) cells are cultured in media containing 10^{-6}M gefitinib for four months, the growth rate improves, and a cell line able to proliferate in the presence of tamoxifen and gefitinib is derived. MCF-7(DR) cells were obtained from the Tenovus Centre for Cancer Research (Welsh School of Pharmacy, Cardiff, UK).

Growth factor deprived MCF-7 cells - -MCF-7 (gfd).

MCF-7 cells were grown in phenol red free RPMI medium in which all oestrogens and exogenous growth factors had been removed by charcoal stripping, and subsequent heat inactivation (65ºC, 40mins) of the foetal calf serum (5%) prior to adding to the medium (van der Burg et al., 1988). Growth factor deprivation initially causes a 80% decrease in growth rate (Staka et al., 2005), but following four months in culture the rate of growth increases until it is equivalent to that of the parental MCF-7 line. MCF-7(gfd) cells were obtained from the Tenovus Centre for Cancer Research (Welsh School of Pharmacy, Cardiff, UK).

T47D.

T47D are an epithelial human mammary carcinoma cell line, originally isolated from pleural effusion of a woman with breast cancer. T47D cells used in this study were obtained from the Tenovus Centre for Cancer Research (Welsh School of Pharmacy, Cardiff, UK).

Tamoxifen resistant T47D – T47D(TamR).

In manner identical to that used to generate the tamoxifen-resistant MCF-7 cell line, MCF-7(TamR), T47D cells were cultured for six months in the presence of 4-hydroxytamoxifen (10^{-7} M) in order to generate a cell line able to proliferate in the presence of tamoxifen. T47D(TamR) cells used in this study were obtained from the Tenovus Centre for Cancer Research (Welsh School of Pharmacy, Cardiff, UK).

2.1.2 General Cell Culture Methods.

Cell Culture Media.

Cell lines were routinely cultured in the following media. All reagents were purchased from Invitogen, UK unless otherwise stated.

MCF-7 and T47D Cells.

Full RMPI medium supplemented with;

5% (v/v) foetal calf serum.

10µg/ml penicillin streptomycin.

2.5µg/ml Fungizone.

.Hereafter referred to as RPMI+5%.

To minimise the unwanted oestrogenic effects of phenol red and foetal calf serum, cells were transferred to alternative media prior to experimentation, containing

RMPI medium (w/o phenol red and L-glutamine) supplemented with;

5% (v/v) charcoal stripped foetal calf serum.

4mM L-glutamine.

10µg/ml penicillin Streptomycin.

2.5µg/ml Fungizone.

Hereafter referred to as W+5%.

MCF-7(TamR) and T47D(TamR) Cells.

W+5% media supplemented with 10^{-7}M 4-hydroxytamoxifen (Sigma, UK). Hereafter referred to as W+5%+Tam.

MCF-7(DR) cells..

W+5%+Tam media supplemented with 10^{-6}M gefitinib (AstraZenica, UK). Hereafter referred to as W+5%+Tam+Gefitinib.

MCF-7(gfd) cells.

RMPI medium (w/o phenol red and L-glutamine) supplemented with;

5% (v/v) heat-inactivated charcoal stripped foetal calf serum.

4mM L-glutamine.

10µg/ml penicillin Streptomycin.

2.5µg/ml Fungizone.

Hereafter referred to as W+5%HIS.

Foetal calf serum was stripped by adjusting its pH to 4.2 with 5M HCl (Fisher, UK) and leaving to equilibrate at 4˚C for 30mins. 5mls of activated

charcoal solution was added (2g activated charcoal (Sigma, UK), 0.01g Dextran T-70 (Fisher, UK), 18ml water) per 100ml foetal calf serum. The resultant mixture was left overnight at 4˚C with stirring. The charcoal was removed by centrifugation at 12,000g for 40 mins in high-speed refrigerated centrifuge, then filtered 3 times through Whatmans No.4 filter paper (Whatman, UK). The pH was then readjusted to 7.2 with NaOH (Fisher, UK). Finally the serum was filtered through a 0.2µm filter (Millipore, UK) under sterile conditions to remove microorganisims.
Where necessary serum was heat inactivated by heating to 65ºC for 40 minutes.

Passaging.

Upon reaching around 90% confluency, cells were passaged by trypsinisation in Trypsin/EDTA for 5 minutes at 37ºC. Cells were resuspended in an appropriate medium and washed by centrifugation at 1000rpm in a Heraeus Multifuge 3_{S-R} fitted with 6441 rotor and resuspended in the same medium. Cells were counted as described below then seeded out in an appropriate vessel (Nunclon, UK) at an approximate density of 40,000 cells/cm^2.

Freezing and reviving cells.

As required, cells were centrifuged as above and resuspended at a concentration of 1million cells/ml in a freezing medium, consisting of their usual medium additionally supplemented with 10% DMSO (Sigma, UK). This cell suspension was frozen in 1ml aliquots overnight at -80ºC before being stored at -130ºC until required.

When required, cells were revived by thawing to room temperature and washing twice by centrifugation and resuspension in normal culture medium. Finally cells were resuspended in 5mls of culture medium, then left to grow to 90% confluency in a 25cm^2 flask (Nunclon, UK).

Counting.

Following culture in 24-well format tissue culture plates (Nunclon, UK), cells were counted by either dual-chamber Neubauer haemocytometer (Fisher Scientific, UK) or by a Coulter Multisizer II counter (Beckman Coulter, UK). For counting by haemocytometer, cells were trypsinised as previously described in 200µl Trypsin/EDTA for 5 minutes. Cells were then removed to 1.5ml centrifuge tubes (Elkay, UK), the action of trypsin was reduced with the addition of 100µl culture media and the resulting 300µl was stored briefly at 4ºC until counting. Immediately prior to counting, 10µl 0.03% Trypan blue (Sigma, UK) was added, and the resulting mixture was mixed several times with a needle and syringe before being pipetted into the chambers of the haemocytometer. The average of four measurements was taken for each well. For counting by Multisizer II, each well was trypsinised with 1ml of Trypsin/EDTA for 10 minutes. The 1ml cell suspension was then added to a cup (Sarstedt AG and Co, Germany) containing 6ml of Isoton (Beckman Coulter, UK) using a syringe and needle. The empty well was then washed three times with 1ml of Isoton again using a syringe and needle. The product of each wash was added to the cup giving a total volume of 10ml. Two separate 500µl portions of this suspension was counted per well, and the average of these two measurements was taken.

Light Microscopy / Photography.

Cells were observed microscopically using a Nikon Eclipse TE200 light microscope, and when required, photographic images were taken using an attached Nikon F70 camera.

2.2 Drug treatments.

The following drugs were used throughout this study. All drug treatments were made up using sterile reagents in a class II laminar flow cabinet. Where several concentrations of the same drug were required, serial dilutions of the drug were made prior to addition to cell culture media. Where solvents other than water were used, control cells were cultured in the presence of the appropriate solvent. Where toxic solvents, such as DMSO and ethanol were used, the total concentration of solvent in the media never exceeded 0.1%.

Cells were plated in 24-well plates (Nunclon, UK) at a concentration of 40,000 cells/well and left overnight. Drugs were then administered at concentrations described below dissolved in fresh W+5% media, with 4-hydroxytamoxifen being included at a concentration of 10^{-7}M for tamoxifen resistant cell lines. For 7 day cytotoxic treatments media was refreshed on the fourth day, and where EGFR inhibitors were used alongside cytotoxics the EGFR inhibitor was present in the media for the full 7 day cytotoxic treatment. Where 24 cytotoxic treatments were used, the initial 24 hour cytotoxic treatment (including EGFR inhibitors where used) was followed by a four day recovery period in normal media (also including EGFR inhibitors where used).

4-hydroxytamoxifen.

4-hydroxytamoxifen was obtained from Sigma, UK and was dissolved in ethanol (Fisher Scientific, UK). A 10^{-2}M stock solution was stored at -20°C until required, whereupon a working solution of 10^{-3}M was made up and stored at -20°C for short periods of time during use. 4-hydroxytamoxifen was included in the routine growth media for all tamoxifen resistant cell lines at a concentration of 10^{-7}M.

AG1478.

AG1478 (4-(3-Chloroanillino)-6,7-dimethoxyquinazoline) was obtained from Tocris Bioscience (USA) and was dissolved in DMSO to a concentration of 1mM. AG1478 was stored at -20°C until required.

Bleomycin.

Bleomycin (Bleo-kyowa™) was obtained from Velindre hospital (Cardiff, UK) and was supplied in vials of freeze dried powder containing 10mg of bleomycin sulphate with an activity of 15,000IU. Bleomycin was dissolved in sterile water to a concentration of 10mg/ml and stored at -20°C until required.

Cisplatin.

Cisplatin (cis-Diammineplatinum(II) dichloride) was obtained from Sigma, UK and stored as a powder. As required, cisplatin was dissolved in cell culture grade DMSO (also from Sigma, UK), diluted to the required concentration and stored at -20°C for short periods of time.

Camptothecin.

Camptothecin was obtained from Sigma, UK, dissolved in DMSO (10mg/ml) and stored at -20°C until required.

Doxorubicin.

Doxorubicin was obtained from Sigma, UK and was stored in aqueous solution (10mg/ml) at -20°C until required.

Etoposide.

Etoposide (Etoside™) was obtained from Velindre hospital (Cardiff, UK) as a liquid and was stored at -4°C until required.

Faslodex.

Faslodex was obtained from AstraZeneca (Macclesfield, UK) and was stored dissolved in ethanol to 10^{-3}M at -20°C until required.

5-Fluorouracil.

5-fluorouracil was obtained from Sigma, UK. It was stored in aqueous solution at -20°C until required.

Gefitinib.

Gefitinib (Iressa™) was obtained from AstraZeneca (Macclesfield, UK) and was diluted in DMSO to 10^{-2}M until required.

Oestradiol.

Oestradiol (17-β-oestradiol) was obtained from Sigma, UK, and was diluted in ethanol to 10^{-5}M and stored at -20°C until required.

2.3 Immunocytochemistry.

The expression levels of several proteins were assayed using immunocytochemistry. MCF-7 and MCF-7(TamR) cells growing in log phase were examined for expression and activation of EGFR, c-erbB2, AKT and ERK1/2., and immunocytochemistry was also used to examine levels of bcl-2 expression in MCF-7, MCF-7(TamR), MCF-7(DR) and MCF-7(gfd) cells, either during normal log phase growth, or following treatment with oestradiol, faslodex or tamoxifen. Cells were cultured on 6cm^2 dishes (Nunclon, UK) containing autoclaved coverslips (Fisher Scientific, UK) and the appropriate medium and drug treatment. Following treatment, cells on the coverslips were fixed by one of the following methods.

Acetone fixation.

Coverslips were fixed by immersion in chilled (-10°C) acetone (Fisher Scientific, UK) for 10 minutes. Coverslips were then removed and left to dry in air for 20 minutes before being stored until required at -80°C.

ER-ICA fixation.

Coverslips were initially immersed in 3.7% formaldehyde solution at room temperature for 15 minutes before being immersed in PBS at room temperature for a further five minutes. This was followed by a five minute immersion in cold (-10°C) methanol (Fisher Scientific, UK) and a five minute immersion in cold (-10°C) acetone before being immersed in PBS at room temperature for a further five minutes. Coverslips were stored at -20°C in sucrose storage medium until required (see below).

Methanol/Vanadate/Acetone fixation.

Following the removal of media from coverslip dishes, 1ml of 2mM sodium orthovanadate (Sigma, UK) in methanol (Fisher Scientific, UK) chilled to -10°C was added to coverslips for 5 minutes. Coverslips were then rinsed in 1ml cold methanol (-10°C). Coverslips were placed in a rack situated in a bath of cold methanol (-10°C) before being transferred to a

bath of cold acetone (-10℃) for five minutes. Coverslips were then air dried for 20 minutes and stored at -80℃ until required.

Formal saline fixation.

3.7% Formal saline solution was prepared as follows;

4.5g Sodium Chloride (Fisher Scientific, UK)

50ml 37% Formaldehyde solution (Fisher Scientific, UK)

450ml Tap water.

Coverslips were immersed in 3.7% formal saline solution for 10 minutes, then 100% ethanol (Fisher Scientific, UK) for five minutes, followed by PBS for five minutes. Coverslips were stored at -20℃ in sucrose storage medium until required (see below).

Phenol formal saline fixation.

3.7% Formal saline solution was prepared as above, with the addition of phenol (Fisher Scientific, UK) to 2.5%. Fixation was then carried out exactly as for formal saline fixation.

Sucrose storage medium.

Sucrose storage medium was prepared as follows; all chemicals were purchased from Sigma. UK.

42.8g sucrose

0.33g magnesium chloride

250ml PBS

250ml glycerol

Stored at -20℃ prior to use.

Blocking, developing and mounting.

After fixation, coverslips were washed in PBS, followed by 0.02% PBS/TWEEN prior to blocking. Coverslips were then incubated first with primary antibody, then with a secondary antibody, with one wash in PBS and two washes in 0.02% PBS/TWEEN after each incubation. The specific blocking method used in the detection of each protein of interest is given in

table 2.1 along with details of fixation method and the primary and secondary antibody incubation conditions. After the relevant antibody incubations, all coverslips were incubated with 70µl DAB chromogen (DAKO, USA). Following this treatment coverslips were washed twice with distilled water before being counterstained with 0.5% methyl green (Fisher Scientific, UK) for thirty seconds. After further washing in distilled water coverslips were dried and mounted on slides using DPX mounting medium (Fisher Scientific, UK).

Imaging.

Images were captured on an Olympus BH2 microscope fitted with an attached Olympus DP12 digital camera system.

Table 2.1. Fixation, blocking and antibody incubation conditions for detection of proteins by immunocytochemistry

Protein of interest	Fixation	Blocking Conditions	Primary antibody incubation	Secondary antibody incubation
Total EGFR	Phenol formal saline.	0.02% PBS/TWEEN for 10 mins.	Neomarker EGFR (Cat no. Ab-10 111.6) 1/50 in PBS overnight.	DAKO mouse EnVision (Cat no. k4007) for 2 hours.
p-EGFR	Phenol formal saline.	0.02% PBS/TWEEN for 10 mins	Biosource pEGFR (tyr1068) (Cat no. 44-788g) 1/40 in1% BSA[1]/PBS overnight.	DAKO rabbit EnVision (Cat no. k4011) for 2 hours.
Total c-erbB2	ERICA.	5% Goat[2]+ 5% human[3] serum in PBS for 10 mins.	DAKO c-erbB2 (Cat no. A0485) 1/100 in 5% goat / 5% human serum / PBS for 2 hours.	Sigma goat anti-rabbit peroxidase conjugate (Cat no. A4914) 1/50 in 5% goat / 5% human serum / PBS for 1 hour.
p-c-erbB2	Methanol vanadate acetone.	0.02% PBS/TWEEN for 10 mins.	Upstate p-erbB2 (tyr1248) (Cat no. 66-229) 1/20 in PBS overnight.	Sigma goat anti-rabbit peroxidase conjugate (Cat no. A4914) 1/50 in 0.1% BSA
pAKT	ERICA	0.02% PBS/TWEEN for 10 mins	Cell signalling technologies pAKT (ser473) (Cat no. 9277) 1/70 in PBS overnight.	DAKO rabbit EnVision (Cat no. k4011) for 2 hours.
pERK1/2	Formal saline	0.02% PBS/TWEEN for 10 minutes	Cell signalling technologies ERK1/2 (thr202/thr204) (Cat no. 9101) 1/20 in PBS for 1 hour.	DAKO rabbit EnVision (Cat no. k4011) for 1 hour.
bcl-2	Acetone	5% Goat+ 5% human serum in PBS for 10 mins.	DAKO bcl-2 (Cat no. M0887) pre-incubated with two volumes of human serum for 90 minutes. 1/30 antibody concentration in 0.1% BSA/PBS overnight.	DAKO mouse EnVision (Cat no. k4007) for 2 hours.

[1]Bovine serum albumin obtained from Sigma, UK
[2]Goat serum obtained from DAKO, USA
[3]Human serum obtained from Golden West Biologicals, USA

2.4 Protein analysis by western blotting.

Expression and activation levels of several proteins were assayed by western blot analysis, carried out according to the following method (all reagents from Sigma, UK unless otherwise indicated).

Cells were lysed on ice with a lysis solution (50mM TRIS base (Fisher Scientific, UK), 5mM EGTA, 150mM NaCl (Fisher Scientific, UK), 1% Triton) containg a cocktail of protease inhibitors (2mM NaVO4, 50mM NaF, 1mM PMSF, 20μM phenylarsnine, 10mM sodium molybdate, 10μg/ml leupeptin, 8μg/ml aprotinin), the latter two reagents both being added on day of use. The lysates were then centrifuged at 15,300g for 15mins at 4℃. The protein content of the supernatants was subsequently quantified with a protein estimation kit (Biorad, Hemel Hempstead, UK) according to the manufacturer's instructions. A series of BSA solutions of known concentrations were also quantified in order to construct a calibration curve. The supernatants were then aliquoted and stored at -20℃. Samples were subjected to SDS-PAGE in Protean 3 electrophoresis equipment (Biorad, Hemel Hempstead, UK) at a constant voltage of 150V for at least 90 minutes through a two layered polyacrylamide gel, containing a 5% stacking section (1.67ml 30% acrylamide, 5.83ml water, 2.5ml 0.5M TRIS (pH 6.6), 0.1ml 10% SDS, 50μl 10% ammonium persulphate, 10μl TEMED) and a 7.5% resolving section (2.5ml 30% acrylamide, 4.8ml water, 2.5ml 1.5M TRIS 9 (pH 8.8), 0.1ml 10% SDS, 100μl ammonium persulphate, 20μl TEMED). For each sample, 60mg of protein was loaded per lane, along with the appropriate volume of 2x sample buffer (4% SDS, 20% glycerol, 120mM TRIS, 0.01% bromophenol blue in water). Blotting was carried out in a blotting tank (Biorad, Hemel Hempstead, UK) according to the manufacturers' instructions. Briefly, polyacrylamide gels were placed on top of a piece of Protrans™ nitrocellulose membrane and were held in a cassette along with filter papers and sponges. All gels, membranes, filter papers and sponges were soaked in transfer buffer (0.25M TRIS base, 1.92M glycine, 20% methanol in water) prior to assembly of the cassette. Care was taken to ensure that following the construction of the cassette,

the membrane lay on the cathode side of the gel. The cassette was immersed in transfer buffer and run for 2hrs at a constant voltage of 100V. All blotting apparatus was kept at 4℃ during the blotting process.

The resulting membranes were blocked overnight in a solution (1ml blocking substrate/19mls water) of POD Blocking Substrate (Roche, UK), then probed for 90mins-4hrs with an appropriate primary antibody, diluted in POD Blocking Substrate and 0.05% sodium azide. Details of primary antibody incubations are given in table 2.2. Membranes were then washed three times in TBS/tween (10mM TRIS base, 150mM NaCl, 0.05% Tween) before being incubated with a secondary antibody. Details of secondary incubations are given in table (2.2). Membranes were then washed three times in TBS/tween before being visualised on Kodak MXB Film using a Horseradish Peroxidase Chemiluminescence kit (Supersignal ® West Dura Extended Duration Substrate #34075. Pierce, UK). Even loading was confirmed by reprobing the membranes for β-actin.

Table 2.2 Primary and secondary incubation conditions for protein detection by western blotting.

Protein	Primary Incubation	Secondary Incubations
Total EGFR	Overnight at 4°C in a 1:1000 solution of rabbit-derived EGFR specific antibody, Cell Signalling Technologies, UK (Cat no 2232)	Room temperature incubation for 2hrs with 1:10,000 solution of HRP-conjugated anti-rabbit secondary antibody (Amersham, UK)
p-EGFR	Overnight at 4°C in a 1:1000 solution of rabbit-derived p-EGFR (tyr1068) specific antibody, Cell Signalling Technologies, UK (Cat no. 2234).	Room temperature incubation for 2hrs with 1:10,000 solution of HRP-conjugated anti-rabbit secondary antibody (Amersham, UK).
Total c-erbB2	Overnight at 4°C in a 1:1000 solution of rabbit-derived c-erbB2 specific antibody, Santa Cruz Biotechnology, USA (Cat no. sc-284).	Room temperature incubation for 2hrs with 1:10,000 solution of HRP-conjugated anti-rabbit secondary antibody (Amersham, UK).
p-c-erbB2	Overnight at 4°C in a 1:1000 solution of rabbit-derived p-c-erbB2 (tyr1248) specific antibody (Upstate Biotechnology, UK (Cat no. 06-229)	Room temperature incubation for 2hrs with 1:10,000 solution of HRP-conjugated anti-rabbit secondary antibody (Amersham, UK)
Total AKT	Overnight at 4°C in a 1:1000 solution of rabbit-derived AKT specific antibody. (Cell Signalling Technologies, UK – Cat No #9272).	Room temperature incubation for 2hrs with 1:10,000 solution of HRP-conjugated anti-rabbit secondary antibody (Amersham, UK)
p-AKT	Overnight at 4°C in a 1:1000 solution of mouse-derived p-AKT (ser473) specific antibody (Cell Signalling Technologies, UK – Cat No #9272).	Room temperature incubation for 2hrs with 1:10,000 solution of HRP-conjugated anti-mouse secondary antibody (Amersham, UK)
p-ERK1/2	Overnight at 4°C in a 1:1000 solution of rabbit-derived p-ERK1/.2 (thr202/thr204) specific antibody. Cell Signalling Technologies, UK (Cat no. 9101).	Room temperature incubation for 2hrs with 1:10,000 solution of HRP-conjugated anti-rabbit secondary a antibody (Amersham, UK)
Total ERK1/2	Overnight at 4°C in a 1:1000 solution of rabbit-derived ERK1/2 specific antibody, Cell Signalling Technologies, UK. (Cat no. #9212).	Room temperature incubation for 2hrs with 1:10,000 solution of HRP-conjugated anti-rabbit secondary antibody (Amersham, UK).
β-actin	Room temperature incubation for 90 minutes with a 1:10,000 solution of mouse-derived β-actin specific antibody (Cell Signalling Technologies, UK – Cat No #4967)	Room temperature incubation for 2hrs with 1:10,000 solution of HRP-conjugated anti-mouse secondary antibody (Amersham, UK)

2.5 Measurement of doxorubicin uptake.

Doxorubcin uptake was measured by flow cytometry, exploiting the In order to quantify cellular uptake of doxorubicin, cells were seeded in 24 well plates at 40,000 cells / cm^2 and left for 24 hours at 37℃ in the relevant growth media. The media was then aspirated and replaced with media containing doxorubicin at various concentrations and left on the cells for either 4hrs or 24hrs. Following this treatment, cells were washed three times with PBS before being typsinised for 5 minutes in 200µl trypsin/EDTA (Gibco, UK). Following trypsinisation cells were pelleted by centrifugation (5mins, 1000rpm in a Heraeus Multifuge 3_{S-R} fitted with 6441 rotor) in round bottomed polycarbonate tubes (BD Biosciences, UK) and resuspended in ice cold PBS before the amount of doxorubicin present in cells was measured using the FL-2 channel of a FACScalibur (BD Biosciences, UK) flow cytometer. Data obtained was analysed using WinMDI 2.8 (Freeware from http://facs.scripps.edu/software.html).

2.6 Measurement of fluid phase endocytosis by FITC-dextran uptake.

In this study, the rate of fluid phase endocytosis was measured as a marker of the rate of membrane turnover, as the rate of membrane turnover has been postulated as a key factor in the rate of bleomycin uptake by cells.

The rate of fluid-phase endocytosis was measured by monitoring the uptake of 10k FITC-dextran both by flow cytometry and by microscopic evaluation. I

Flow cytometry.

Cells were seeded at a density of 40,000 cells/cm^2 in a 24 well plate (Nunclon, UK) and left overnight before being treated with medium containing 5mg/ml FITC-dextran (Sigma, UK) for 4hrs. Cells were then washed *in-situ* three times with PBS (37℃) before being trypsinised for 5 mins in 200µl trypsin/EDTA (Gibco, UK). Following trypsinisation cells were transferred to centrifuge tubes (Elkay, UK), pelleted by centrifugation and resuspended in PBS as described in section 2.5. The amount of FITC-dextran present in cells was measured using the FL-1 channel of a FACScalibur (BD Biosciences, UK) flow cytometer. Data obtained was analysed using WinMDI 2.8 (Freeware from http://facs.scripps.edu/software.html).

2.7 Neutral comet assay.

Double strand breaks in DNA caused by bleomycin were examined using the neutral comet assay. The assay was performed in accordance with the standard method (Rojas et al., 1999; Singh et al., 1988), with a number of small modifications. All reagents were from Sigma, UK unless otherwise stated. In brief, a suspension of cells in low melting point agarose (LMPA) (5 x 10^4 cells/ml LMPA) at 37°C was pipetted into a dual-window CometSlide™ (Trevigen, Gaithersburg, USA), such that each window contained approx. 4000 cells suspended in 75µl LMPA. The slide was then chilled to 4°C to allow the agarose to set. Cell membranes were then dissolved by submerging in a lysis solution (2.5M NaCl, 100mM Na2EDTA, 10mM Tris, 10% (v/v) DMSO, 1% Triton X-100 – pH10) overnight. Slides were then neutralised in a TBE solution (0.089M Tris, 0.089M borate, 0.002M EDTA in water) for 30mins, before being subjected to electrophoresis for 20 minutes in the same buffer at 2v/cm in a refrigerated room. This allowed fragments of DNA which had sustained double stranded breaks to move free of the nucleus and migrate towards the anode, forming a 'tail' and giving the impression of a comet.

All solutions were kept at 4°C until immediately prior to use in order to minimise the risk of LMPA melting and lifting from the slide. Following electrophoresis, the slides were neutralised with a commercially available neutralising buffer (Ikzus, Genoa, Italy), before being fixed in ethanol and air dried. Slides were stained with FLUO-Plus DNA stain (Ikzus, Genoa) and visualised on a DRAM2 epifluorescent microscope (Leica. Cambridge, UK) equipped with a Retiga 1300 digital camera (QImagin, Canada). The extent to which broken fragments had been moved by electrophoresis was analysed using CASP software (Konca et al 2003) to calculate Olive tail moments (OTM) of 50 cells from each window of each slide.

2.8 Gene expression analysis by microarray

RNA was isolated from triplicate cultures of MCF-7 and MCF-7(TamR) cells during log phase growth in their normal growth media. Cells were lysed *in situ* with TriReagent (Sigma, UK), scraped into 1.5ml microcentrifuge tubes and stored at -80°C. Total RNA was isolated as per reagent protocol with subsequent DNase1 treatment and RNA clean-up using RNeasy Mini Columns (Qiagen, UK). RNA was quantified by UV-spectrophotometry at 260nm, and initial determination of the purity of the nucleic acid by determination of the 260/280nm ratio which was >1.7. Integrity of RNA was established by visualisation of horizontal gel electrophoresed material using ethidium bromide (Sigma, UK). Integrity and concentration of samples were then further checked using an Agilent Bioanalyser system prior to their reverse transcription, amplification, biotin-tag labelling and hybridisation to the Affymetrix HG-U133A Genechip (Central Biotechnology services, Cardiff University). Following automated washing of chips hybridised material was fluorescently labelled and scanned by confocal laser. Specific normalised signal levels were determined (using mismatch control signal levels for non-specificity) using the Affymetrix MAS5 analysis software and data exported as excel format datasheets. Data was uploaded into the Affymetrix analyis specific programs of GeneSifter.net, a web-based analysis package. Here triplicate data sets for each experimental arm were median-normalised and data was log transformed. Control gene expression and MVA plots to assess comparability between data sets were performed prior to any project analyses. Expression levels for experimental genes were then analysed to examine relative gene expression profiles with appropriate cutoffs for fold changes in expression.

2.9 CaspACE™ apoptosis detection Assay.

Apoptosis was detected using the commercially available CaspACE™ reagent (Promega, UK), a conjugate of the fluorophore FITC and the irreversible caspase inhibitor VAD-FMK. This reagent binds irreversibly to the activated forms of caspases 1 and 3 and allows for fluorometric calculation of the extent of apoptosis by FACS. CaspACE™ is provided as a 5mM solution in DMSO and was used as follows.

Following treatment with a suspected inducer of apoptosis, media was removed into round bottomed polycarbonate tubes (BD Biosciences, UK) while the cell monolayer was trypsinised with a mixture of trypsin/EDTA (Gibco, UK) and PBS in equal volumes for 3 minutes. The resulting cell suspension was returned to the appropriate tube and the resulting cells were pelleted from the suspension by centrifugation as described in section 2.5. The supernatant was removed and the cell pellet was resuspended in 150µl of a 10µM solution of CaspACE™ in PBS and left to incubate in the dark for 20 minutes. Following incubation, cells were pelleted as described above and resuspended in 2ml PBS. This solution was then spun down and resuspended in a smaller volume (500µl) of PBS. The amount of CaspACE™ present in cells was measured using the FL-1 channel of a FACScalibur (BD Biosciences, UK) flow cytometer. Data obtained was analysed using WinMDI 2.8 (Freeware from http://facs.scripps.edu/software.html).

2.9 Transfection with bcl-2 siRNA.

Levels of bcl-2 protein in MCF-7 cells were reduced by transfection with commercially available ON-TARGETplus SMARTpool siRNA mixture specifically directed against bcl-2 mRNA (Dharmacon, UK – Cat #L-003307-00). siRNA was made up to 5nM using the siRNA buffer provided and DNAse/RNAase free water (Ambion, UK). siRNA was mixed with Dharmafect™ transfection reagent (Dharmacon, UK) in a 2:1 ratio (v/v) and left at room temperature for 20 mins. This mixture was then added to the appropriate cell culture media at a concentration of 7.5µl per ml. The media was applied to cells 24 hours post seeding at a density of 40,000 cells/cm^2. Control transfections were carried out for all experiments using an siRNA targeting a non endogenous gene. Control transfections were carried out exactly as described for bcl-2, with the use of anti-luciferase duplex 1(Dharmacon, UK) siRNA in place of anti-bcl-2 siRNA.

2.10 Statistical analysis of data

Statistical significance of data was calculated using the SPSS programme for Windows (SPSS inc, Chicago, US). ANOVA was performed on each set of data, and a Levine statistic calculated to establish whether variances were equal in sets of data that were to be analysed. Where the Levine statistic was equal to or more than 0.05 and therefore variances were assumed to be equal Dunnett's t-test was used to establish whether each treatment had an effect relative to the untreated control group. Where the Levine statistic was larger than 0.05, unequal variances were assumed and Tamahane's *post hoc* test was used to establish whether each treatments had a significant effect compared with the untreated control group.

Where two data sets were directly compared (for example the effect of a fixed dose of drug on the growth of two different cell lines), a paired t-test was performed to establish whether the particular treatment regimen affected the two cell lines to a significantly different extent.

Chapter 3

Results.

It was the initial aim of this study to confirm that during the development of tamoxifen resistance, levels of expression and activation of several key signalling molecules such as EGFR and AKT are increased. Given that increased levels of these proteins, in particularly EGFR are generally associated with tumours that have increased resistance to cytotoxic drugs (see section 1.2.2), the sensitivity of breast cancer cells to several cytotoxic agents was measured before and after the development of tamoxifen resistance.

3.1 Characterisation of cell lines.

Firstly, the tamoxifen resistant cells developed by long term culture in 10^{-7}M tamoxifen were examined to ensure that they were able to proliferate in the presence of tamoxifen, and that they displayed behaviour consistent with previous cell culture models of tamoxifen resistance.

A number of changes were observed in MCF-7 cells following the development of tamoxifen resistance. The morphology of the cells was significantly altered, with the tamoxifen resistant MCF-7(TamR) cells growing in a more scattered fashion compared to the wild-type MCF-7 cells which generally grew in more clearly defined colonies. MCF-7(TamR) cells also had a more angular appearance, and displayed the longer processes previously observed in tamoxifen resistant cells in culture (fig 3.1.1 A) (Knowlden et al., 2003). While tamoxifen initially inhibited the growth of MCF-7 cells in culture, once tamoxifen resistance had developed the rate of growth of the tamoxifen resistant cells increased (fig 3.1.1 B) and this was reflected in the marked decrease from 64hrs to 41hrs in the mean doubling time of MCF-7 and MCF-7(TamR) cells respectively. In addition to their routine growth in 10^{-7}M tamoxifen MCF-7(TamR) cells also demonstrated the ability to proliferate in a range of concentrations of tamoxifen which were shown to inhibit the growth of wild-type MCF-7 cells (fig 3.1.2), with only a 20% reduction in growth of MCF-7(TamR) cells being observed in a

3.1.1 Appearance and growth rate of MCF-7 and MCF-7(TamR).

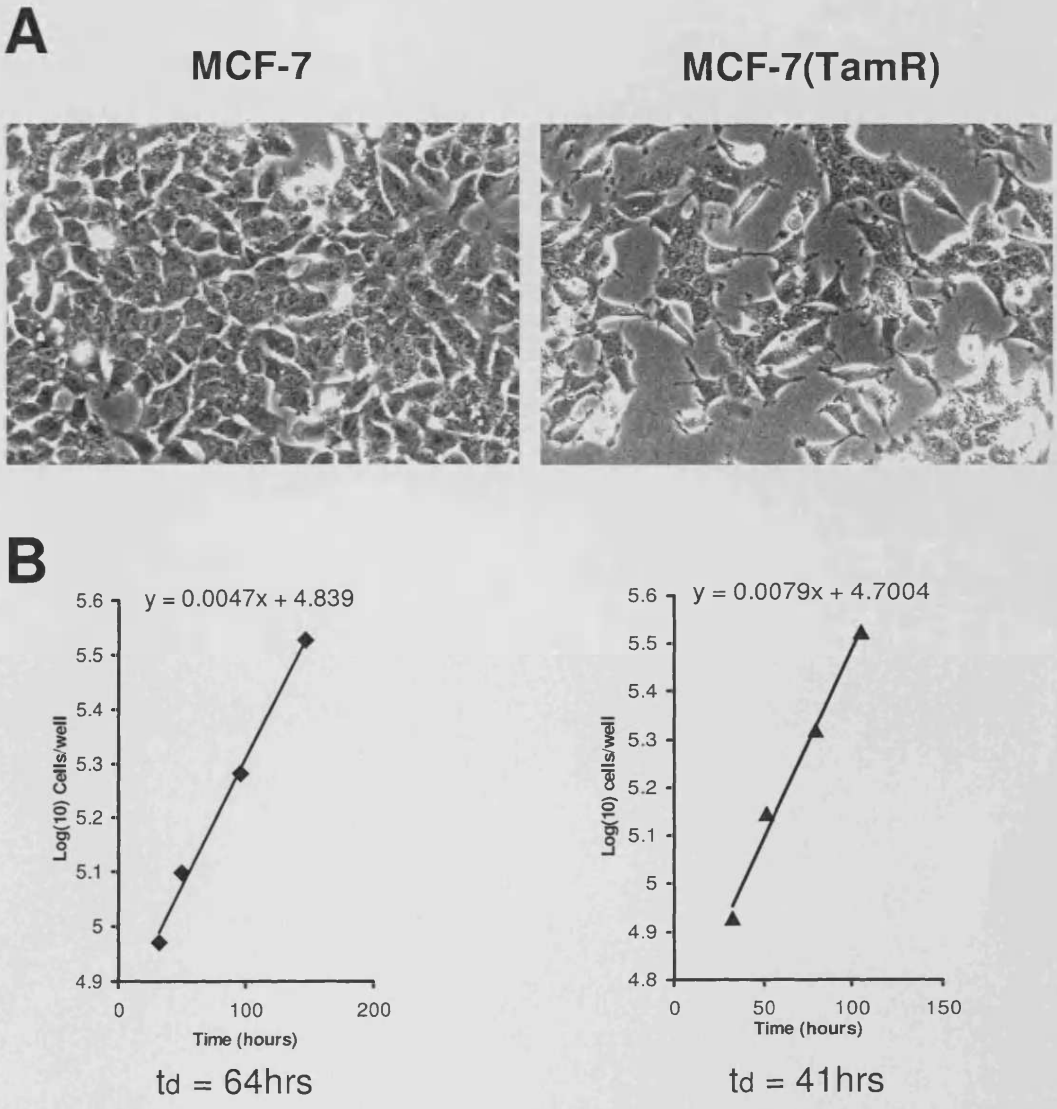


Figure 3.1.1 Appearance and growth rate of wild-type MCF-7 cells and their tamoxifen resistant derivative, MCF-7(TamR). (A). Light microscope images of the MCF-7 and MCF-7(TamR) taken at 10x magnification. (B). Log plots of growth of MCF-7 and MCF-7(TamR) cells over time with calculation of doubling time (td).

3.1.2 Effect of tamoxifen on growth of MCF-7 and MCF-7(TamR) cells.

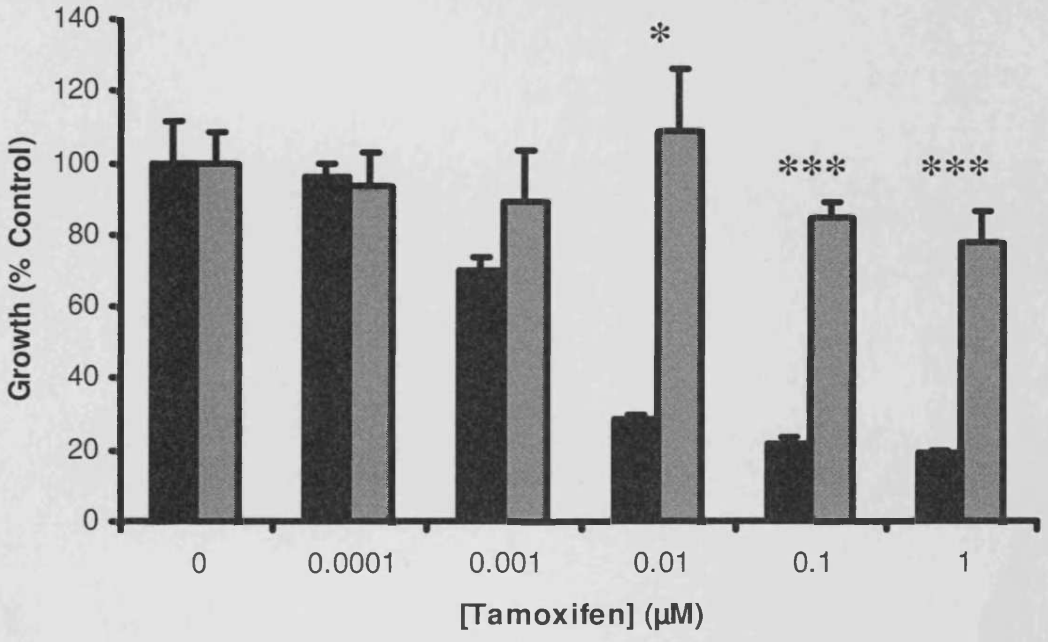


Figure 3.1.2 Effect on growth of MCF-7 (■) and MCF-7(TamR) (▒) cells of a 7 day treatment with tamoxifen, added to culture medium and refreshed on day 4. n=3. p values calculated from a paired t-test comparing growth inhibition between cell lines at a given concentration of tamoxifen, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars indicate standard deviation.

7-day treatment with 10^{-5}M tamoxifen, compared to a 75% reduction in growth of MCF-7 cells.

Once it had been confirmed that the MCF-7(TamR) cells were resistant to tamoxifen, and were displaying behaviour typical of tamoxifen resistant cells, the expression and activation of several signalling molecules was examined. Immunocytochemistry and western blotting confirmed that, consistent with previously observed models of tamoxifen resistance (Hiscox et al., 2006; Knowlden et al., 2003) the tamoxifen resistant cells demonstrated increased expression of the receptor tyrosine kinases EGFR and c-erbB2 as measured by immunocytochemical staining (fig 3.1.3 A). This observation was further validated by the analysis of protein expression by western blot, which clearly showed the increased levels of EGFR and c-erbB2 present in the tamoxifen resistant cells (fig 3.1.3 B). In addition to increased expression of EGFR and c-erbB2, increased activation of these proteins was also observed, with clearly increased levels of phosphorylation of both molecules detected by immunocytochemistry (fig 3.1.4 A) and western blotting (fig 3.1.4 B).

In addition to increased expression and activation of receptor tyrosine kinases, signalling molecules downstream of EGFR and c-erbB2 were also seen to display increased activation following the development of tamoxifen resistance. Immunocytochemical analysis of ERK1/2 activation revealed a clear increase in ERK1/2 phosphorylation, with MCF-7(TamR) cells exhibiting both an increase in staining density, and an increase in the number of densely staining cells (fig 3.1.5 A). Examination of AKT activation also displayed a similar pattern, with a clear increase in staining density following the development of tamoxifen resistance (fig 3.1.5 A). Western blot analysis confirmed the increase in activation of both ERK1/2 and AKT compared to their total expression levels. The total expression of ERK1/2 seems to increase with the development of tamoxifen resistance, while levels of total AKT seem to remain comparable (fig 3.1.5 B).

3.1.3 EGFR and c-erbB2 expression in MCF-7 and MCF-7(TamR) cells.

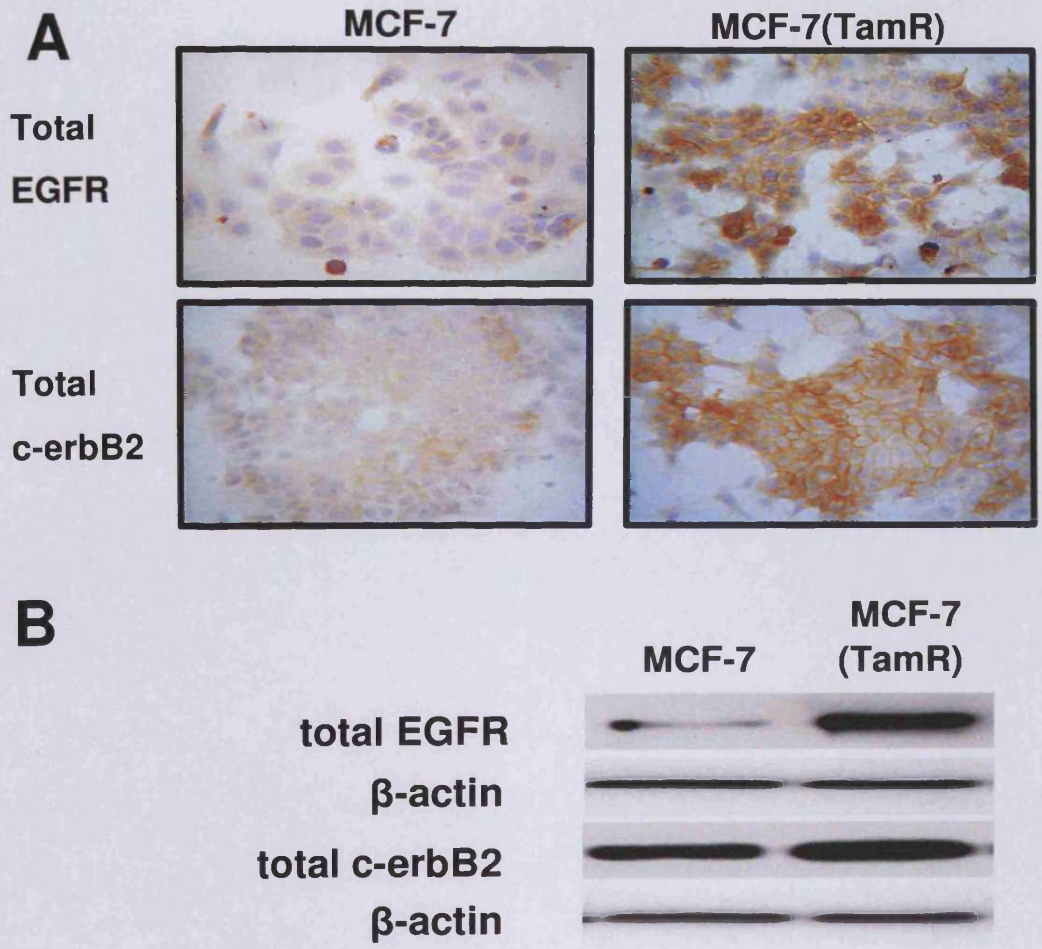


Figure 3.1.3 Changes in expression of EGFR and c-erbB2 following development of tamoxifen resistance in MCF-7 cells as measured by (A) immunocytochemistry (original magnification x10) and (B) western blot.

3.1.4 EGFR and c-erbB2 activation in MCF-7 and MCF-7(TamR) cells.

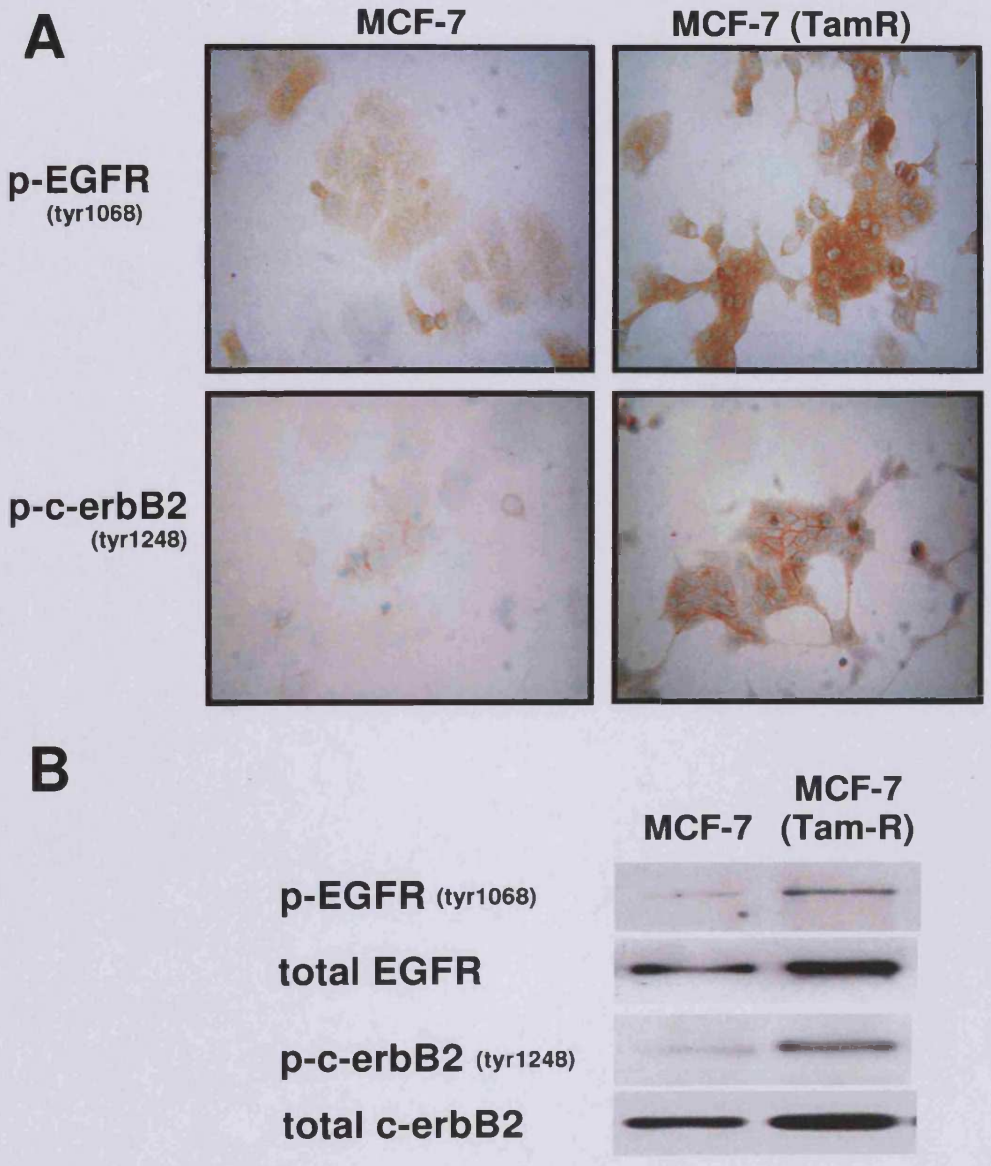


Figure 3.1.4 Changes in levels of activated EGFR and c-erbB2 following development of tamoxifen resistance in MCF-7 cells as measured by (A) immunocytochemistry (original magnification x10) and (B) western blot.

3.1.5 Activation of ERK1/2 and AKT in MCF-7 and MCF-7(TamR) cells.

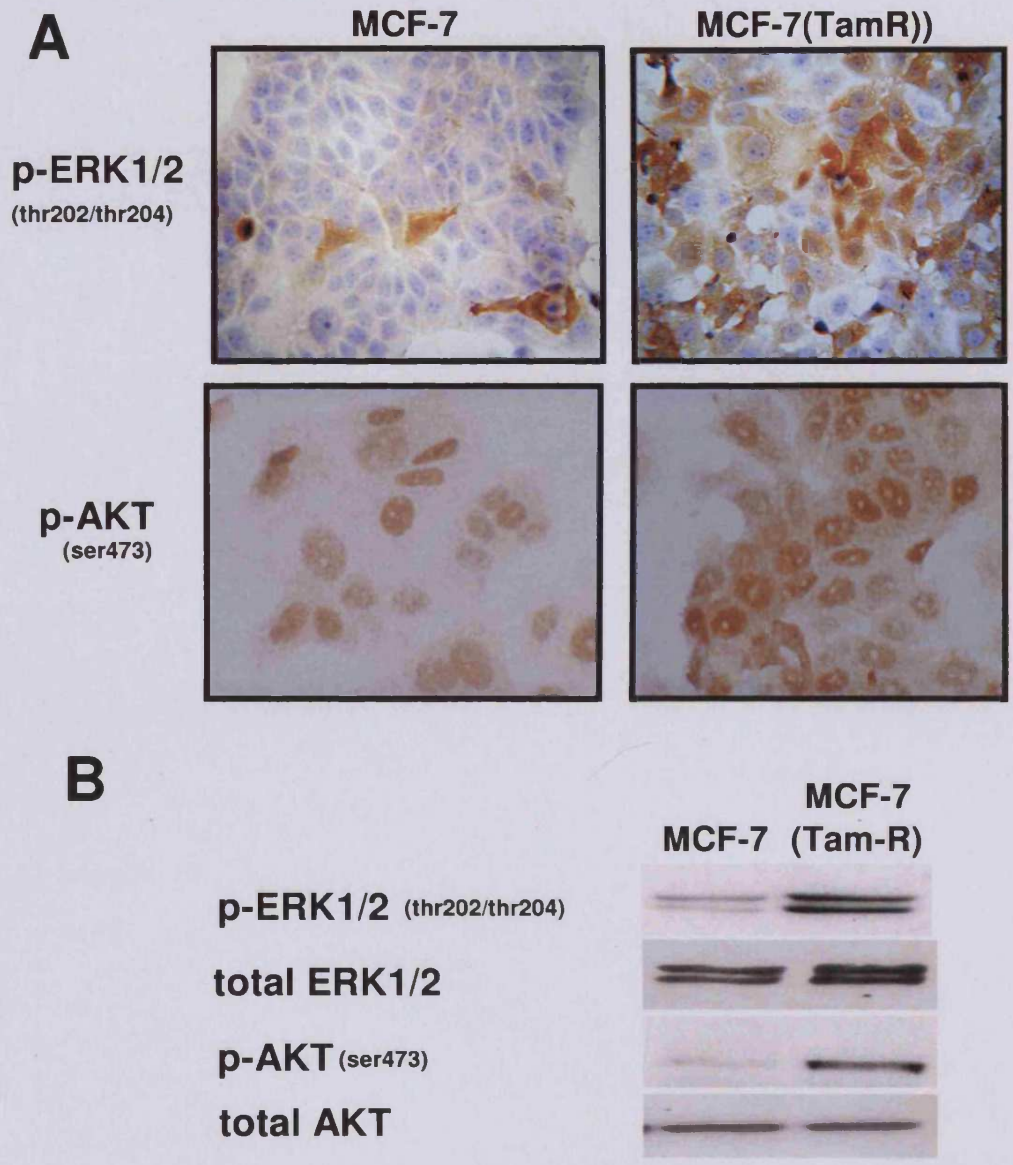


Figure 3.1.5 Changes in levels of expression and activation of ERK1/2 and AKT following development of tamoxifen resistance in MCF-7 cells as measured by (A) immunocytochemistry (original magnification x10) and (B) western blot.

As well as displaying increased activation of receptor tyrosine kinases and several associated downstream signalling molecules, MCF-7(TamR) cells became much more sensitive to the growth inhibitory effects of EGFR inhibitors, highlighting their reliance on EGFR signalling for the transmission of proliferative signals. The small molecule inhibitor AG1478, delivered at a concentration of 1μM over 7 days, inhibited the growth of MCF-7(TamR) cells by around 50%, while having no detectable effect on the growth of MCF-7 cells (fig 3.1.6 A). Another small molecule inhibitor of EGFR, gefitinib, caused a 50% reduction in growth of MCF-7(TamR) cells over 7 days at 0.5μM, while MCF-7 cells treated in an identical manner displayed only a 10% reduction in growth (fig 3.1.6 B). Much higher concentrations of gefitinib (around 10μM) were able to significantly reduce growth of both cell lines, however at all concentrations of gefitinib, the growth inhibitory effect was significantly higher in MCF-7(TamR) cells than MCF-7. This is consistent with observations made in several other studies using various inhibitors of EGFR and EGFR family members, including gefitinib (Gee et al., 2001), and Herceptin (Knowlden et al., 2003).

3.1.6 Effect of the EGFR inhibitors AG1478 and gefitinib on growth of MCF-7 and MCF-7(TamR) cells.

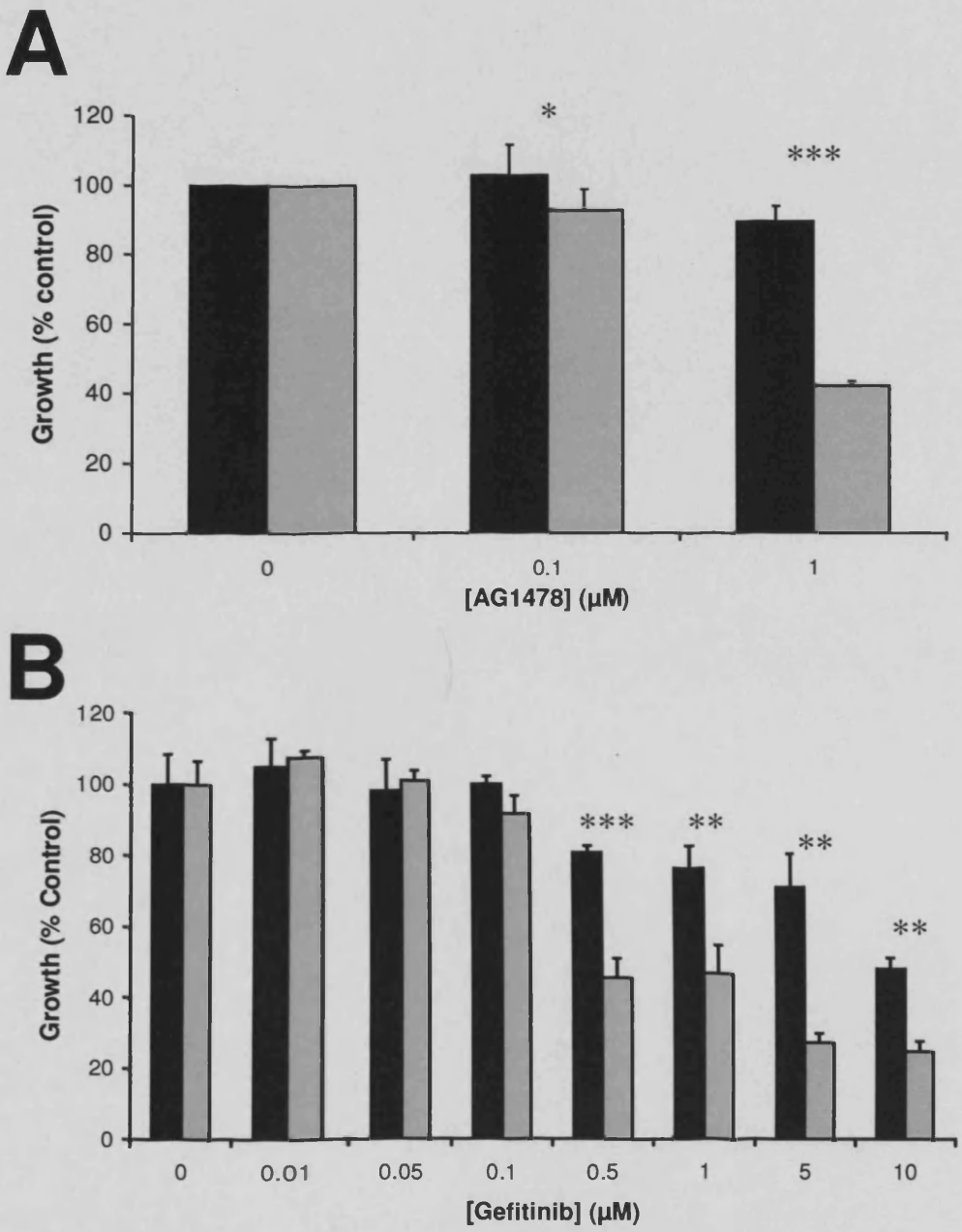


Figure 3.1.6 Effect on growth of MCF-7 (■) and MCF-7(TamR) (■) cells of 7 day treatment of the EGFR inhibitors (A) AG1478 and (B) gefitinib added to the cell culture medium and refreshed on day 4. cells. n=3. p values calculated from a paired t-test comparing growth inhibition between cell lines for a given concentration of EGFR inhibitor, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars indicate standard deviation.

3.2 Sensitivity of tamoxifen resistant breast cancer cells to cytotoxic agents.

Once the expected changes in the activity of signalling molecules in tamoxifen resistant cells had been confirmed, the effect of these changes on cytotoxic sensitivity was examined. The sensitivity of wild-type, low EGFR expressing MCF-7 cells and tamoxifen resistant, high EGFR expressing MCF-7(TamR) cells to a panel of cytotoxic agents was compared. When treated with bleomycin there was a marked difference in sensitivity observed between MCF-7 and MCF-7(TamR) cells. Bleomycin reduced the growth of both cell lines, but had a much larger effect on the growth of MCF-7(TamR) cells. Over a 7 day treatment, 0.05μg/ml bleomycin reduced the growth rate of MCF-7(TamR) by around 50%, while having no significant effect on the growth of MCF-7 cells. Higher concentrations of bleomycin over 7 days reduced the growth rate of both cell lines, but had a much larger effect on MCF-7(TamR) compared to MCF-7 (fig 3.2.1 A). Although a shorter (24 hour) treatment period with bleomycin reduced the growth of both cell lines, a significantly larger inhibitory effect was once again observed on MCF-7(TamR) in comparison to MCF-7cells at all concentrations of bleomycin used (fig 3.2.1 B).

Treatment with cisplatin also caused a reduction in growth rate of both cell lines, but unlike bleomycin, had a comparable effect across a wide concentration range. Growth was reduced to a similar extent in both cell lines over the course of a 7 day (fig 3.2.2 A) or 24 hour (fig 3.2.2 B) treatment period, with MCF-7(TamR) displaying a slightly increased sensitivity at the highest concentrations of cisplatin in both cases.

Doxorubicin reduced the growth rate of both MCF-7 and MCF-7(TamR) over a 7 day treatment, with a slightly increased effect on MCF-7(TamR) at the highest concentrations (fig 3.2.3 A). Higher concentrations of doxorubicin over 24 hours reduced the growth of both cell lines, but resulted in a much larger difference in response between MCF-7 and MCF-7(TamR) (fig 3.2.3 B).

Aside from bleomycin, the second largest difference in response between MCF-7 and MCF-7(TamR) cells was seen following a 7 day treatment with etoposide with the drug having a significantly larger effect on MCF-7(TamR) cells over a range of concentrations (fig 3.2.4 A). MCF-7(TamR) also displayed an increased sensitivity to etoposide following a 24 treatment (fig 3.2.4 B).

MCF-7 and MCF-7(TamR) cells displayed broadly similar sensitivity to 5-fluorouracil over 7 day (fig 3.2.5 A) and 24 hour (fig 3.2.5 B) treatments. The highest concentrations of 5-fluorouracil did however have a greater growth reduction effect on MCF-7(TamR) cells in both treatment regimens.

3.2.1 Effect of tamoxifen resistance on bleomycin sensitivity.

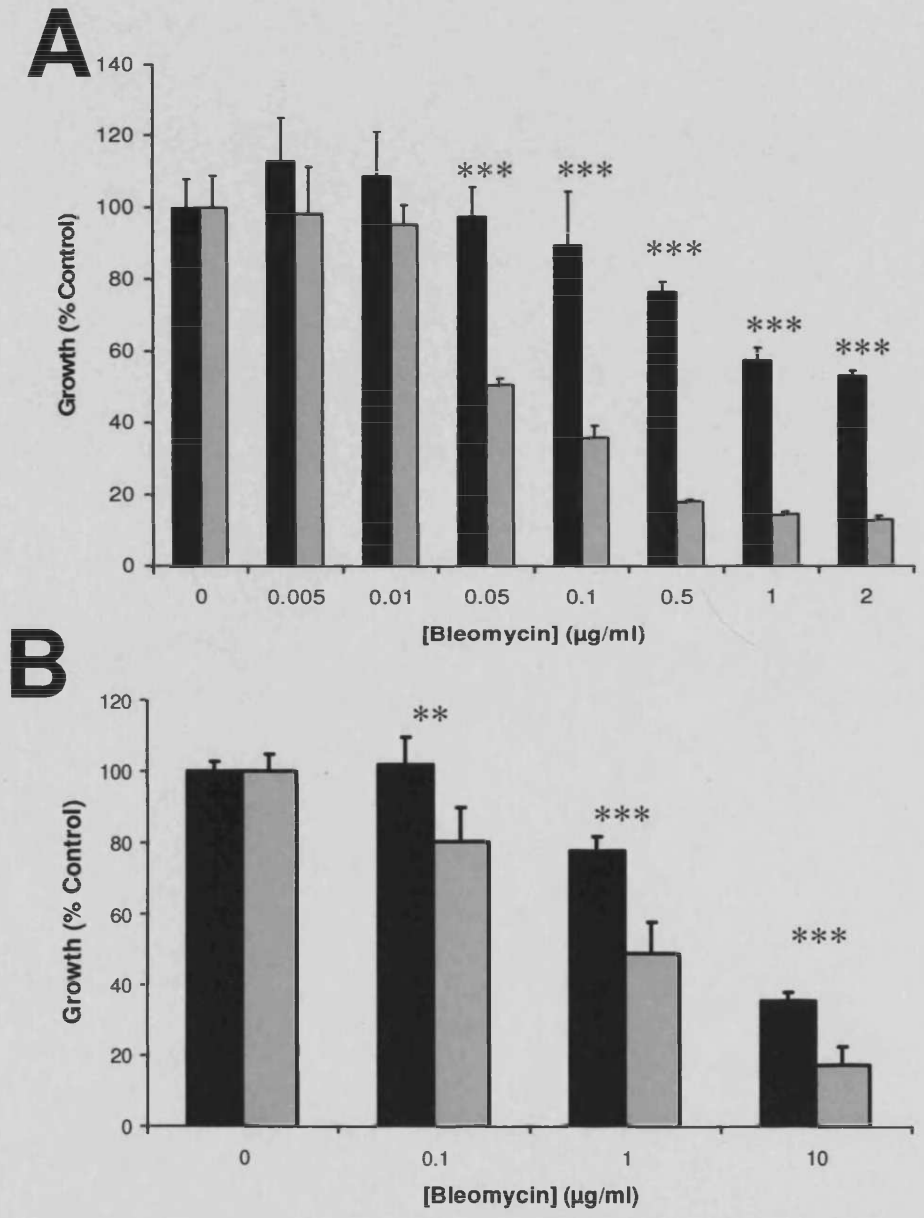


Figure 3.2.1 (A) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing bleomycin for 7 days. (B) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing bleomycin for 24 hours before returning to bleomycin-free media for a further 4 days. n=6. p values calculated from paired t-test comparing growth inhibition between MCF-7 and MCF-7(TamR) cells caused by a given concentration of bleomycin, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars indicate standard deviation.

3.2.2. Effect of tamoxifen resistance on cisplatin sensitivity.

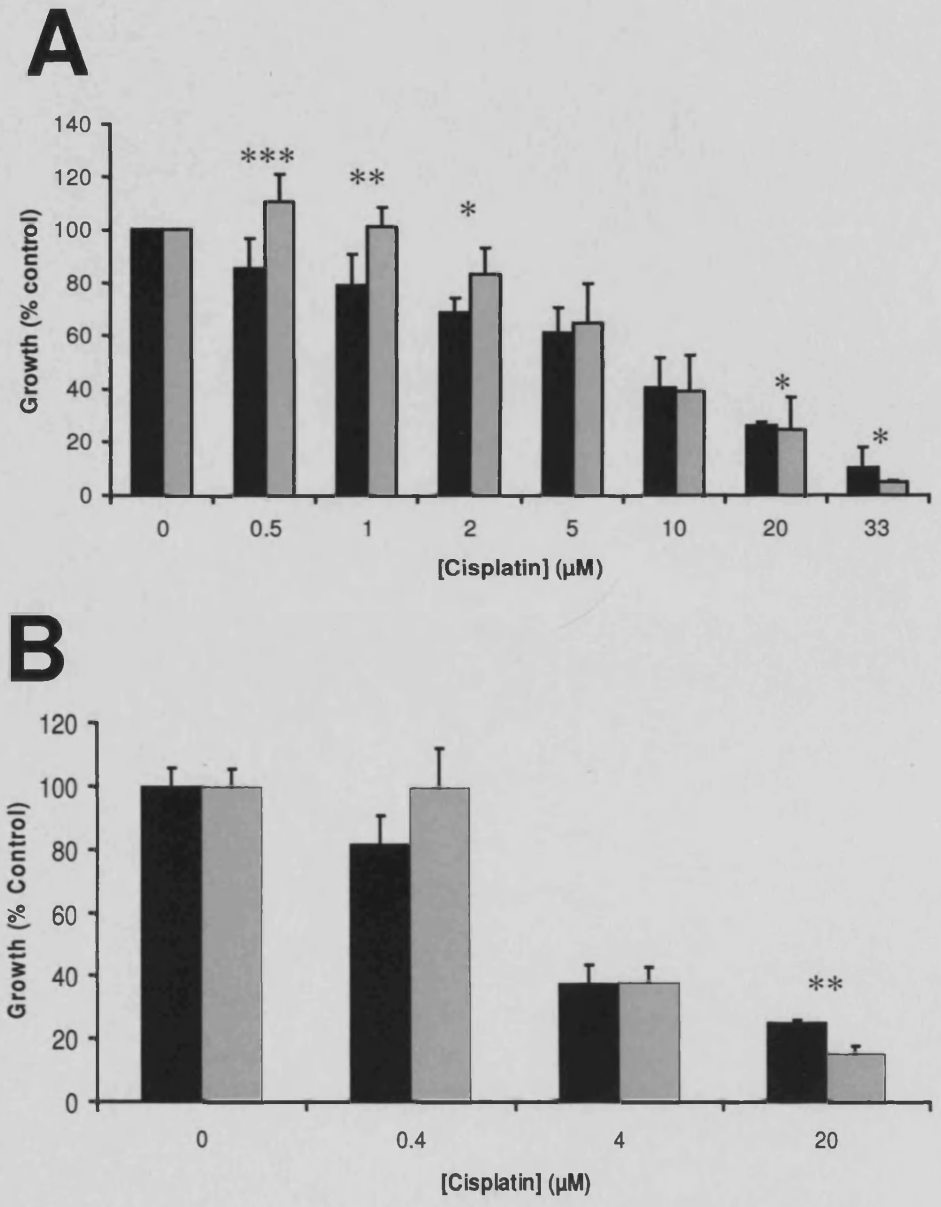


Figure 3.2.2 (A) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing cisplatin for 7 days. (B) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing cisplatin for 24 hours before returning to cisplatin-free media for a further 4 days. n=6. p values calculated from paired t-test comparing growth inhibition between MCF-7 and MCF-7(TamR) cells caused by a given concentration of cisplatin, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars indicate standard deviation.

3.2.3 Effect of tamoxifen resistance on doxorubicin sensitivity.

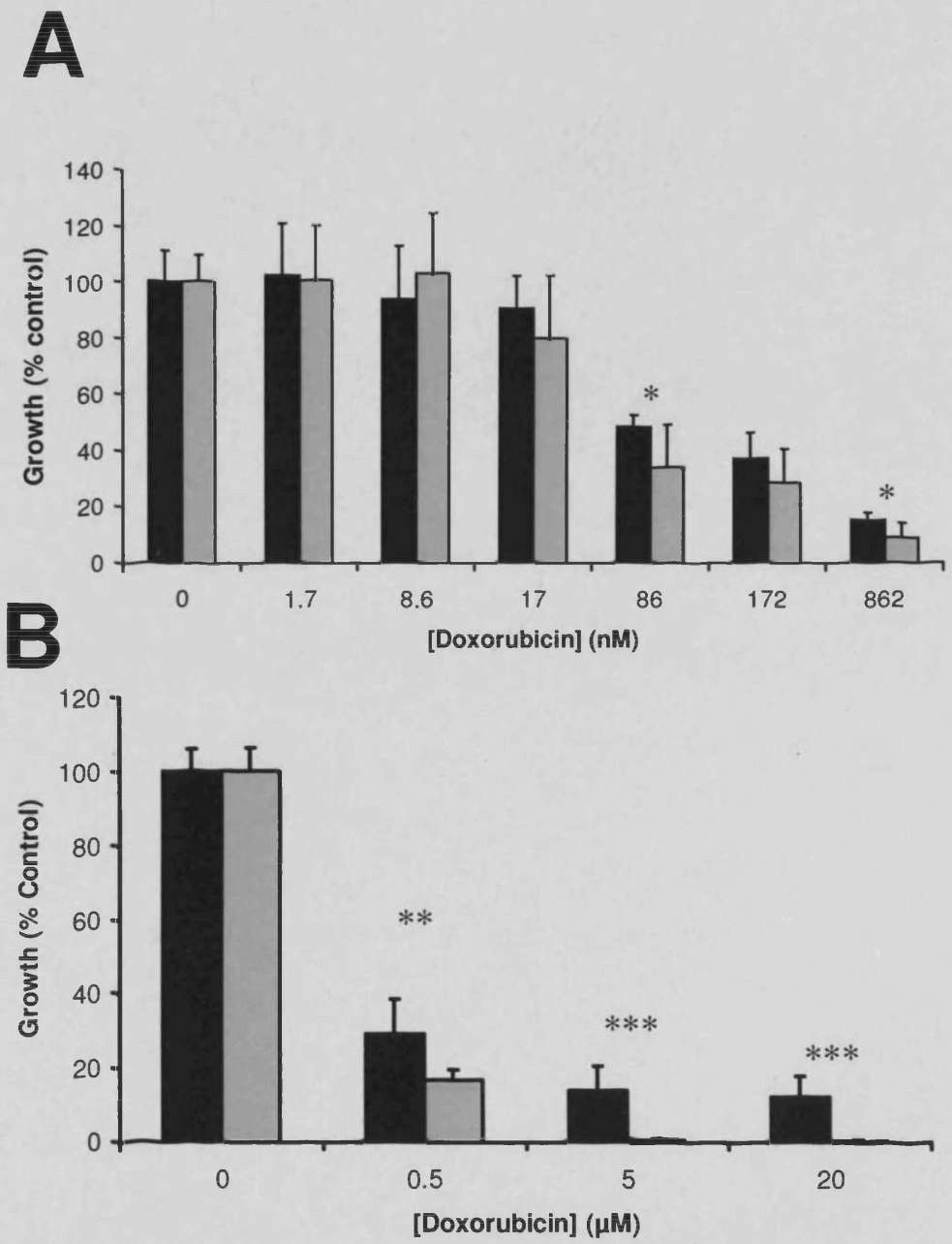


Figure 3.2.3 (A) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing doxorubicin for 7 days. (B) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing doxorubicin for 24 hours before returning to doxorubicin-free media for a further 4 days. n=6. p values calculated from paired t-test comparing growth inhibition between MCF-7 and MCF-7(TamR) cells caused by a given concentration of doxorubicin, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars indicate standard deviation.

3.2.4 Effect of tamoxifen resistance on etoposide sensitivity.

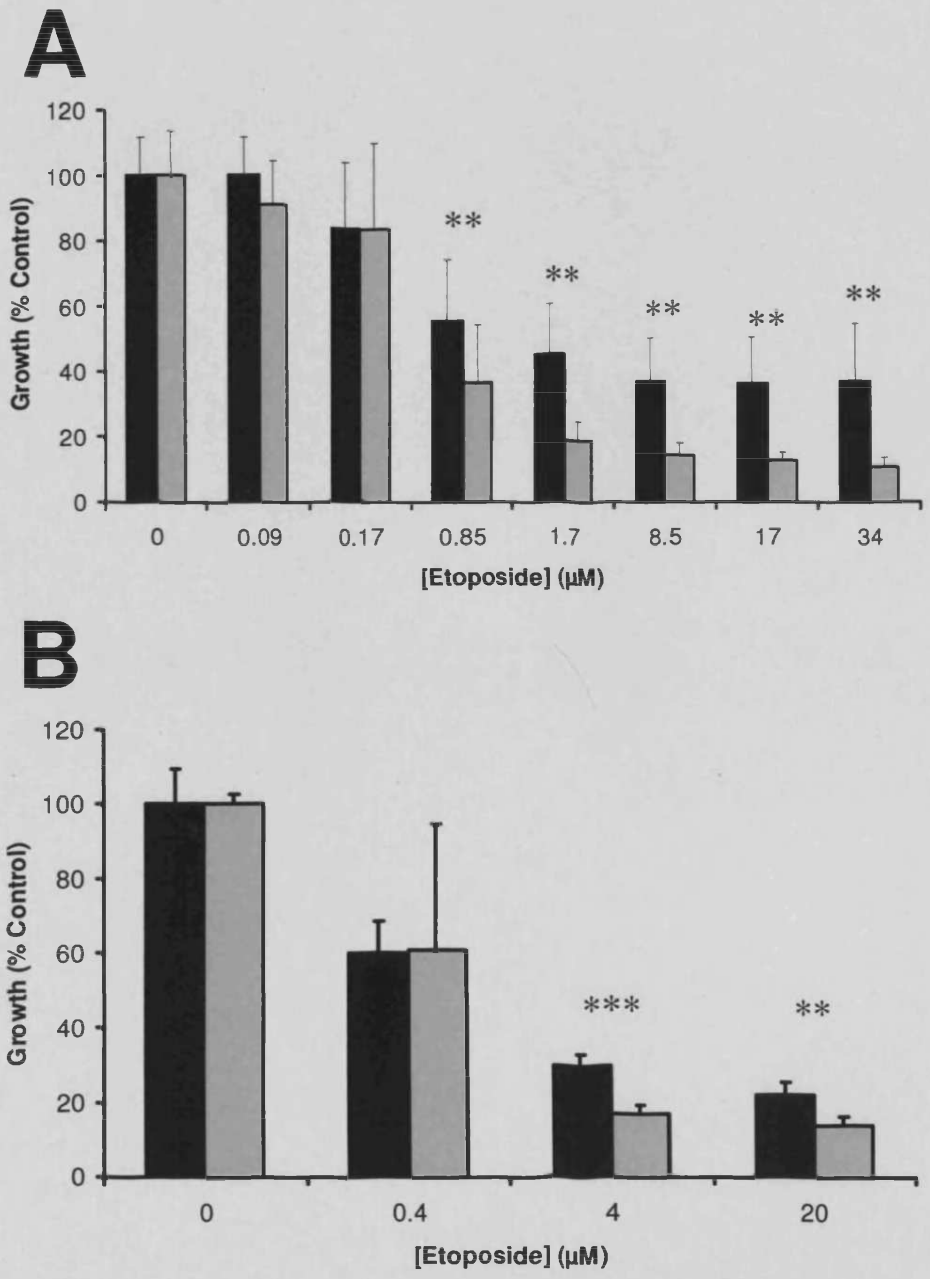


Figure 3.2.4 (A) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing etoposide for 7 days. (B) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing etoposide for 24 hours before returning to etoposide-free media for a further 4 days. n=6. p values calculated from paired t-test comparing growth inhibition between MCF-7 and MCF-7(TamR) cells caused by a given concentration of etoposide * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars indicate standard deviation.

3.2.5 Effect of tamoxifen resistance on 5-flurouracil sensitivity.

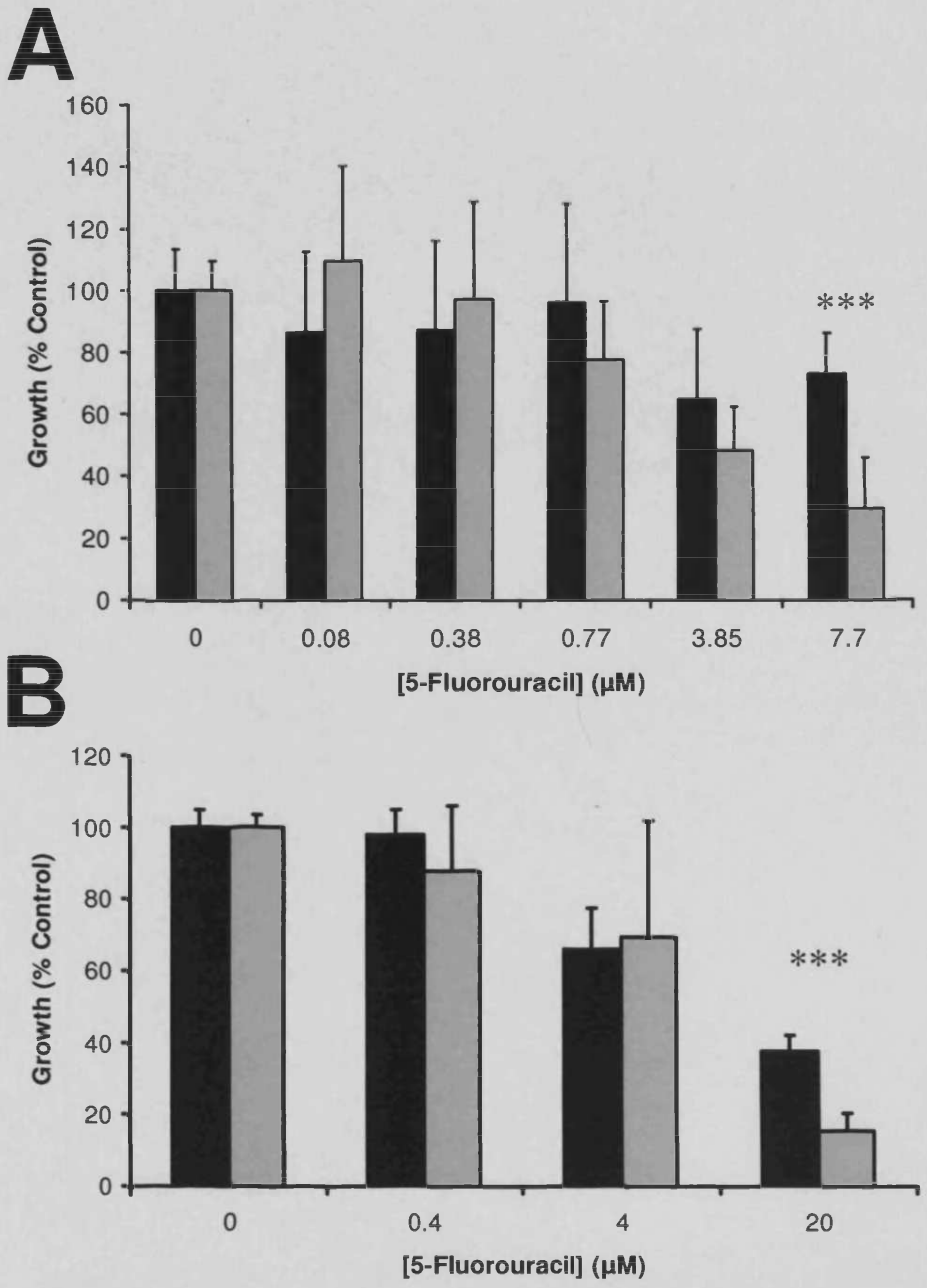


Figure 3.2.5 (A) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing 5-flurouracil for 7 days. (B) Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing 5-fluorouracil for 24 hours before returning to 5-fluorouracil-free media for a further 4 days. n=6. p values calculated from paired t-test comparing growth inhibition between MCF-7 and MCF-7(TamR) cells caused by a given concentration of 5-fluorouracil. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars indicate standard deviation.

3.3. Effect of EGFR signalling on drug sensitivity in breast cancer cells.

The increased EGFR signalling observed in tamoxifen resistant MCF-7 cells appeared to offer no protection at all from the cytotoxic effects of the five drugs studied in 3.2, and in many cases (most strikingly following treatment with bleomycin) the higher-EGFR expressing, tamoxifen resistant MCF-7(TamR) cells displayed increased sensitisation to drug treatments. In order to examine whether cytotoxic sensitivity of MCF-7 cells and their tamoxifen-resistant derivatives is mediated by EGFR signalling, cytotoxic agents were co-delivered with EGFR inhibitors. Inhibition of EGFR signalling with AG1478 had no effect on the sensitivity of either MCF-7 or MCF-7(TamR) cells to the cytotoxic effects of bleomycin (fig 3.3.1 A) or doxorubicin (fig 3.3.1 B) when administered as a 24 hour treatment of EGFR inhibitor and cytotoxic agent, followed by a four-day incubation in the EGFR inhibitor alone. In all cases the response to the cytotoxic was the same regardless of whether AG1478 (at either 1μM or 0.1μM) was present or not. Addition of AG1478 (at either 1μM or 0.1μM) to the culture media for the duration of a 7 day treatment with bleomycin also had no effect on the sensitivity of either MCF-7(fig 3.3.2 A) or MCF-7(TamR) (fig 3.3.2 B) cells to the cytotoxicity of bleomycin.

3.3.1 Effect of EGFR inhibition with AG1478 on sensitivity of MCF-7 and MCF-7(TamR) cells to a 24 hour treatment with bleomycin and doxorubicin.

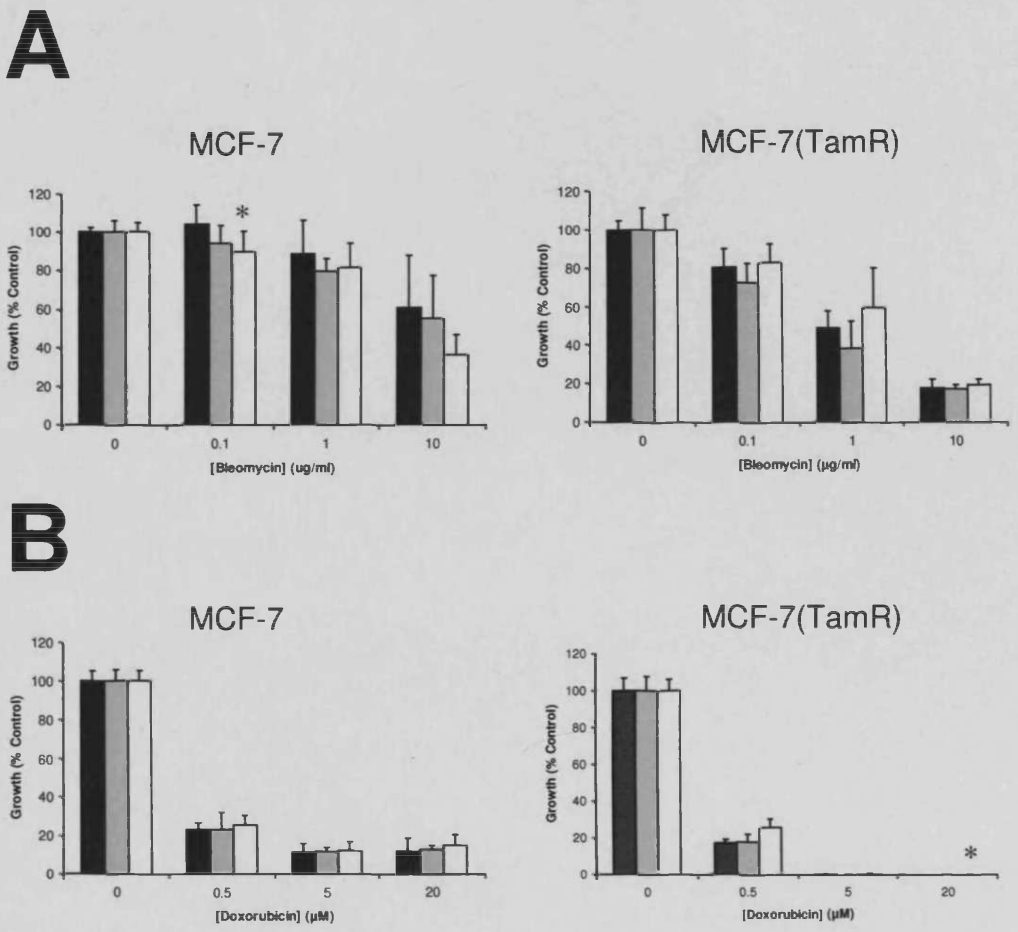


Figure 3.3.1 Effect of AG1478 treatment on sensitivity of MCF-7 and MCF-7(TamR) cells to (A) bleomycin and (B) doxorubicin. Cells were subjected to a 24 hour cytotoxic treatment with the addition of (■) 0µM, (■) 0.1µM or (□) 1µM AG1478 before 4 days recovery in the same medium minus the cytotoxic. p values calculated from paired t-test comparing growth inhibition between cells untreated with AG1478 and cells treated with AG1478, both of which were treated with a given concentration of doxorubicin or bleomycin. * indicates $p<0.05$ *c.f.* 0µm AG1478. Error bars indicate standard deviation.

3.3.2 Effect of EGFR inhibition with AG1478 on sensitivity of MCF-7 and MCF-7(TamR) cells to a 7 day treatment with bleomycin.

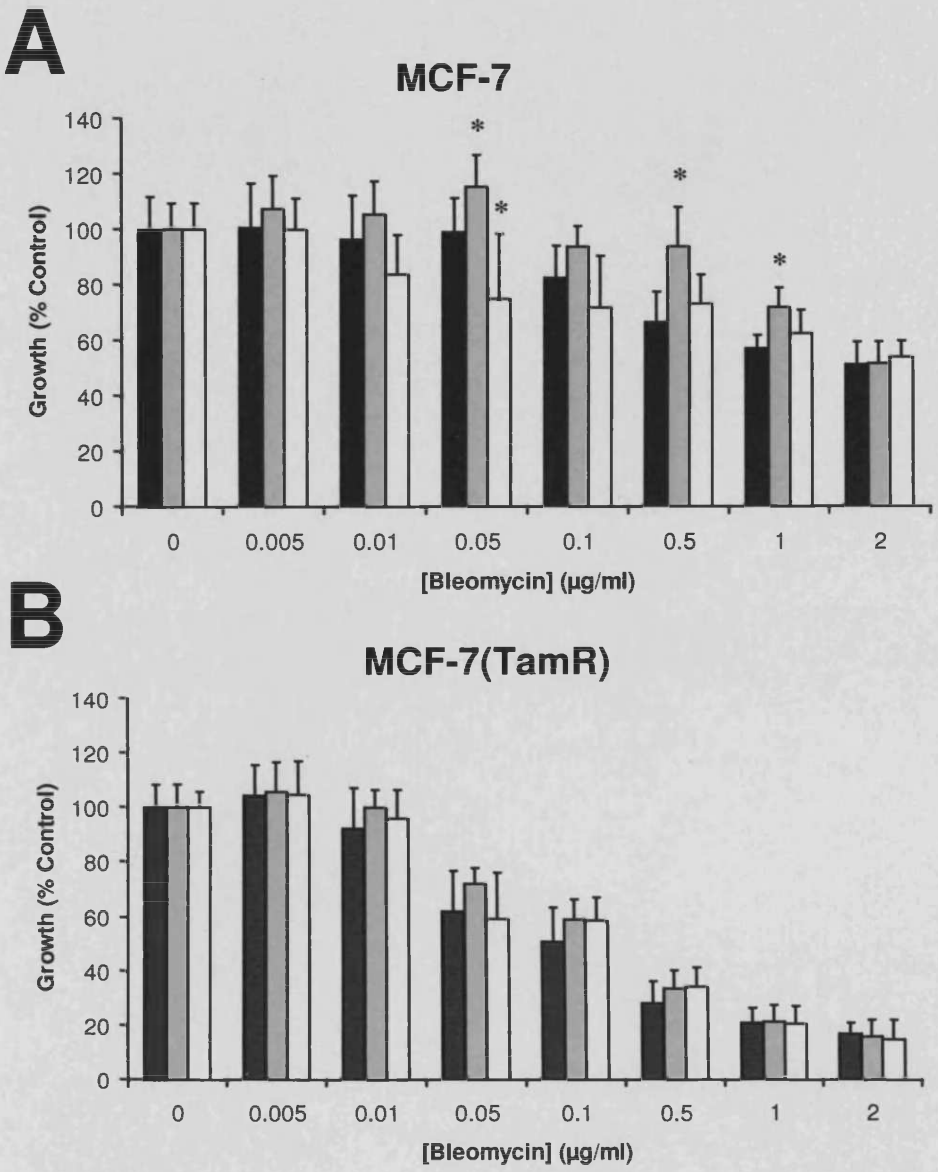


Figure 3.3.2 Effect of AG1478 treatment on sensitivity of (A) MCF-7 and (B) MCF-7(TamR) cells to bleomycin. Cells were subjected to a 7 day treatment with bleomycin with the addition of (■) 0µM, (■) 0.1µM or (□) 1µM AG1478 to the culture medium for the full length of the treatment, refreshed on day 4. p values calculated from paired t-test comparing growth inhibition between cells treated with no AG1478 and cells treated with AG1478 in addition to given concentration of bleomycin. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ *c.f.* 0µm AG1478. Error bars indicate standard deviation.

3.4 Bleomycin sensitivity in alternative cell culture models of tamoxifen resistance.

In order to establish that the marked increase in sensitivity of tamoxifen resistant breast cancers cells to bleomycin was not unique to the MCF-7 model of tamoxifen resistance, the effect of the development of tamoxifen resistance on bleomycin sensitivity in an alternative cell culture model, based on the t47d epithelial breast cancer cell line was assayed. t47d cells can develop tamoxifen resistance in culture in the same way as MCF-7 cells and similarly resistant to the growth inhibitory effects of tamoxifen in the culture media (fig 3.4.1 A), and as in MCF-7 cells, this is accompanied by an increase in EGFR activation, although unlike MCF-7 cells, the level of EGFR expression remains unaffected by tamoxifen resistance (fig 3.4.1 B). Like MCF-7(TamR) cells, the tamoxifen-resistant cells derived from t47d cells displayed a massively increased sensitivity to bleomycin (fig 3.4.2), suggesting that the phenomenon of bleomycin sensitivity in tamoxifen-resistant cells is not a peculiarity of the MCF-7 cell culture model, and may represent a general trend in tamoxifen resistant cells.

3.4.1. Characterisation of the breast cancer cell line t47d and its tamoxifen resistant derivative, t47d(TamR)

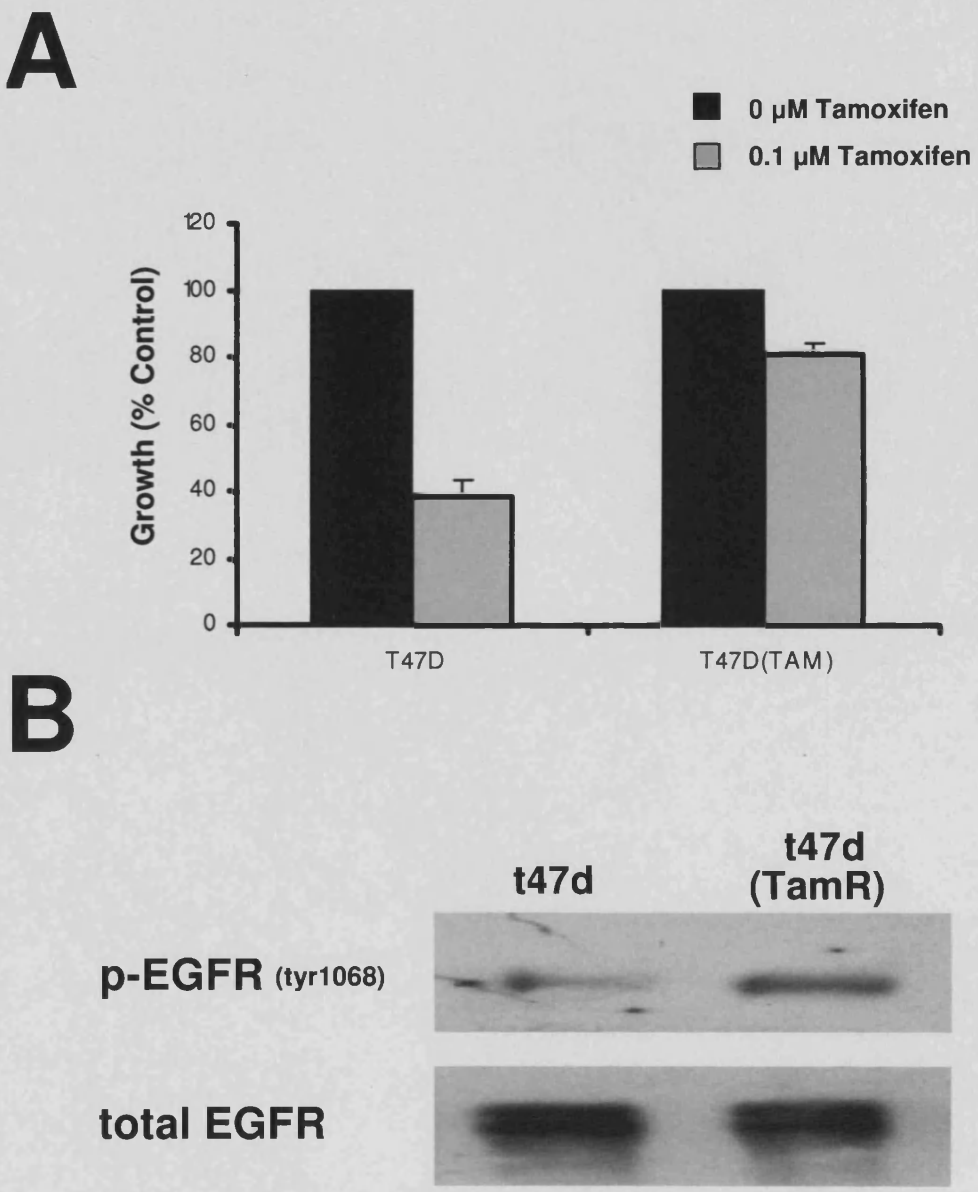


Figure 3.4.1 (A) Effect of a 14-day tamoxifen treatment (0.1µM tamoxifen added to normal growth media) on t47d cells, and t47d(TamR), a tamoxifen-resistant cell line derived from t47D. (B) Levels of EGFR expression and activation in t47d cells before and after the development of tamoxifen resistance.

3.4.2 Bleomycin sensitivity of t47d and t47d(TamR)

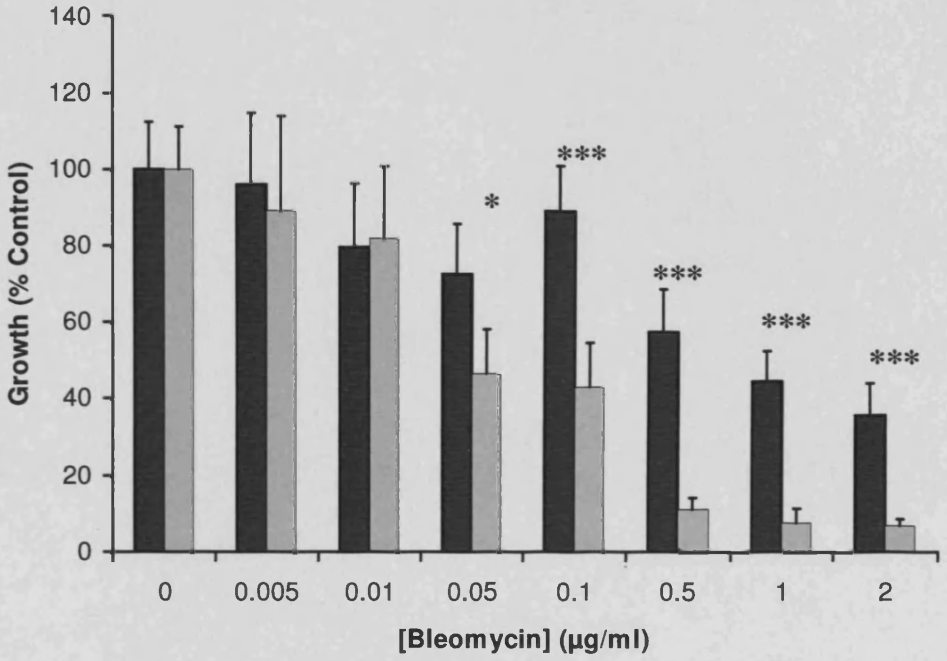


Figure 3.4.2 Sensitivity of wild-type t47d cells (■) and tamoxifen resistant t47d cells (t47d(TamR)) (■) to a seven day treatment with bleomycin. Bleomycin was added directly to the cell culture medium and refreshed on day 4. n=9. p values calculated from paired t-test comparing growth inhibition between t47d and t47d(TamR) cells caused by a given concentration of bleomycin * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars indicate standard deviation.

3.5 Drug uptake and efflux in tamoxifen resistant breast cancer cells.

The method by which MCF-7 cells become resistant to tamoxifen is not primarily reliant on tamoxifen efflux, but rather on the utilisation of alternative methods of growth factor signalling (see section 1.1.6.1). Despite this, changes in membrane composition or in the expression of proteins that regulate the uptake and efflux of drugs may accompany the development of tamoxifen resistance and could be responsible for the observed increase in sensitivity of tamoxifen resistant cells to particular drugs.

3.5.1 Direct measurement of drug uptake.

The uptake of doxorubicin was measured directly, as it was shown to effect MCF-7 and MCF-7(TamR) cells differently (particularly following short treatments with high concentrations, see fig 3.2.3 B) and also displays natural fluorescence making it readily detectable by flow cytometry. The amount of doxorubicin able to enter MCF-7 and MCF-7(TamR) cells during a four hour treatment was measured by flow cytometry, with a clear shift in the mean fluorescence intensity observable in the raw data following doxorubicin treatment (fig 3.5.1 A). Analysis of the data showed comparable uptake of doxorubicin in MCF-7 and MCF-7(TamR) at all concentrations studied (fig 3.5.1 B), including treatment lengths that were shown to have different effects on cell number between cell lines in previous experiments (fig 3.2.3). There was also no significant difference between the amount of doxorubicin taken up by MCF-7 and MCF-7(TamR) cells over a 24 hour period (fig 3.5.2 A, B).

3.5.2 Indirect measurements of drug uptake/efflux capacity.

The uptake of bleomycin is much harder to measure compared with naturally fluorescent molecules such as doxorubicin or molecules that are readily available in a radiolabelled form such as 5-fluorouracil. While it was not possible to directly measure bleomycin uptake, estimations of the rate of uptake can be made by measuring both the extent of DNA damage

3.5.1 Uptake of doxorubicin in tamoxifen resistant and sensitive MCF-7 cells over 4 hours.

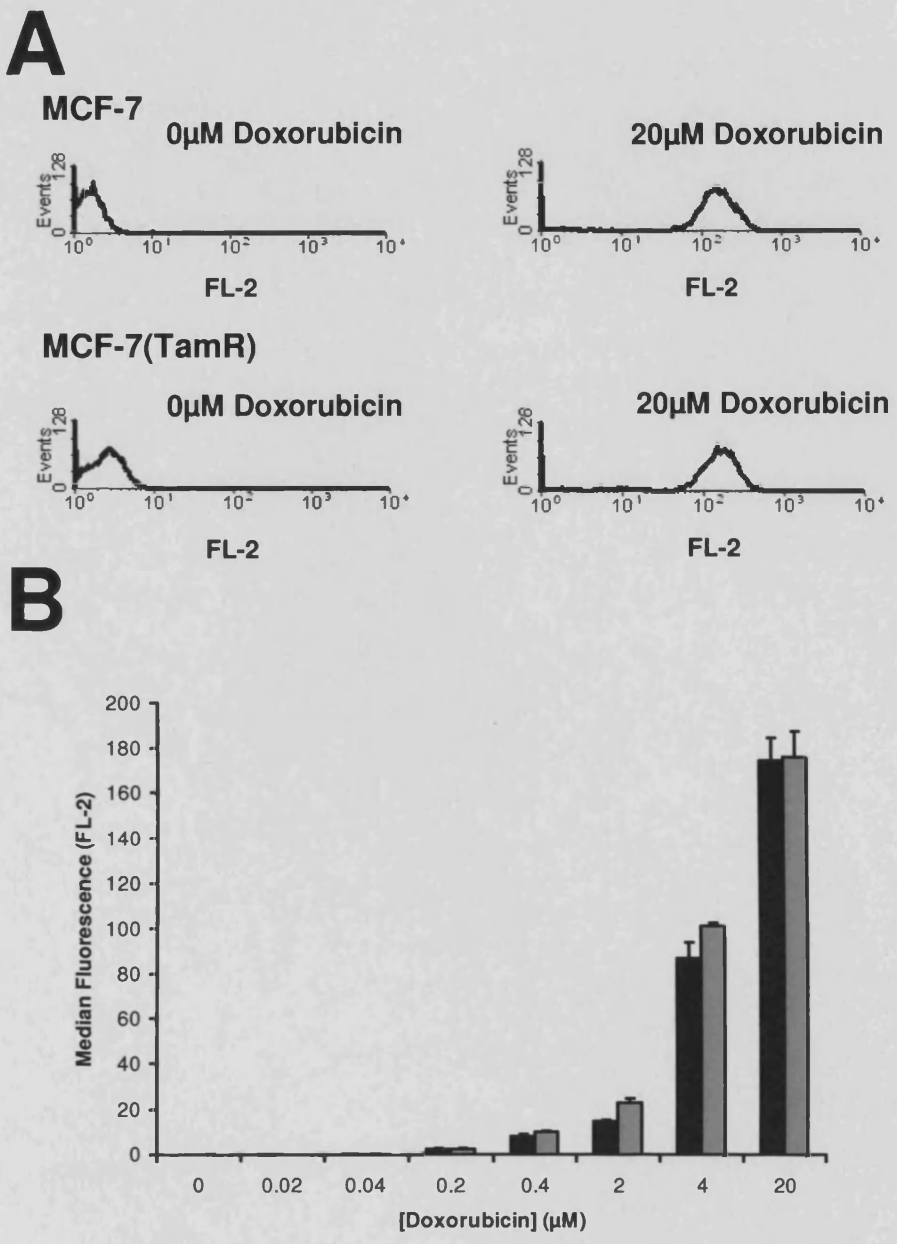


Figure 3.5.1 Uptake of doxorubicin by MCF-7 (■) and MCF-7(TamR) (■) after four hours in culture with the drug as measured by flow cytometry. Examples of distributions of FL-2 fluorescence for two concentrations of doxorubicin are shown in (A), median fluorescence for a wider range of concentrations shown in (B). n=6, error bars indicate standard deviation.

3.5.2 Uptake of doxorubicin in tamoxifen resistant and sensitive MCF-7 cells over 24 hours.

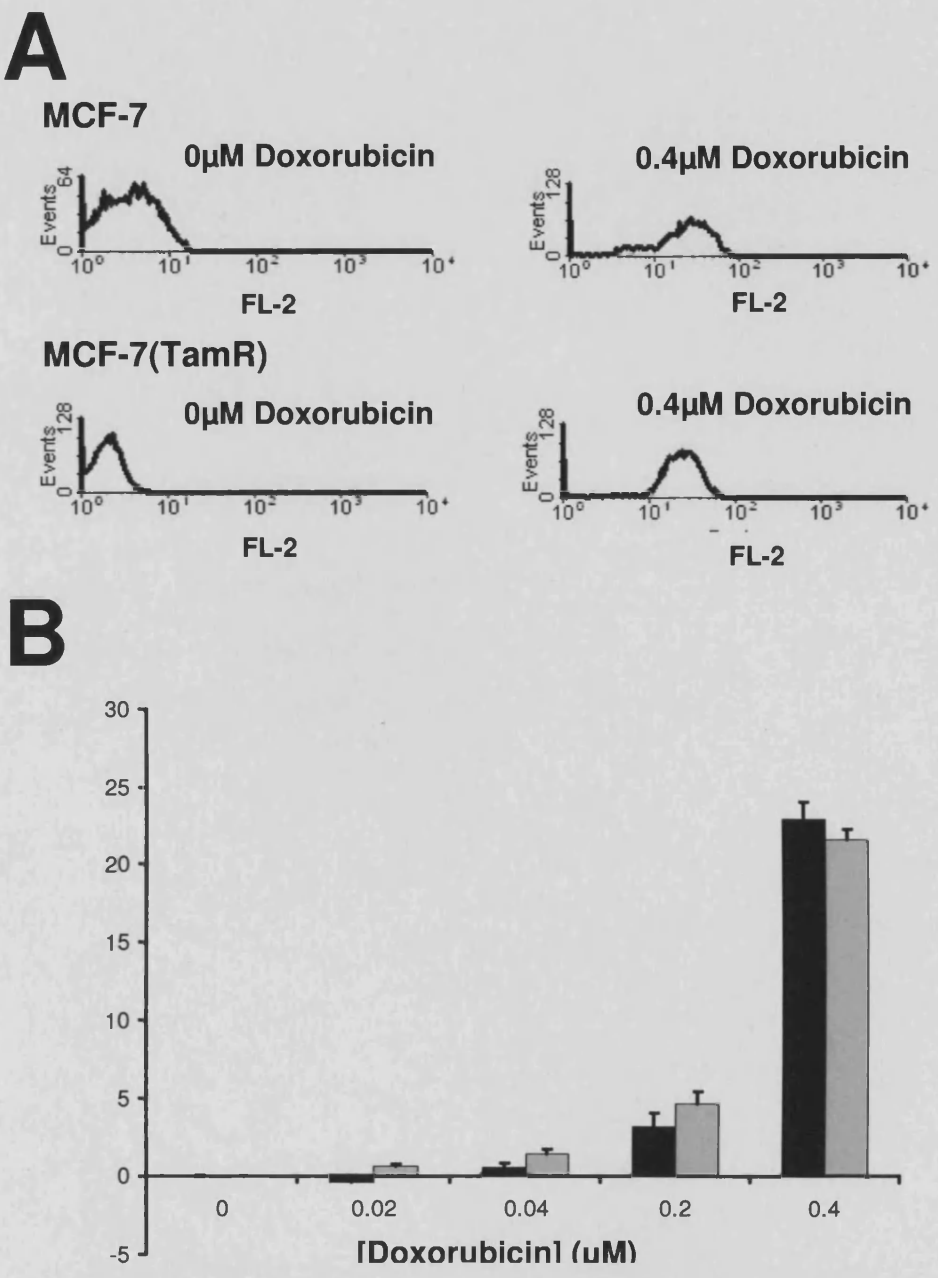


Figure 3.5.2 Uptake of doxorubicin by MCF-7 (■) and MCF-7(TamR) (■) after 24 hours in culture with the drug as measured by flow cytometry. Examples of distributions of FL-2 fluorescence for two concentrations of doxorubicin are shown in (A), median fluorescence for a wider range of concentrations shown in (B). n=6, error bars indicate standard deviation.

caused to both cell lines, and the rate of fluid phase endocytosis (explained fully in section 4.2).

Both cell lines readily took up the fluorescent marker of fluid phase endocytosis (10k dextran-FITC conjugate), and analysis of the median amount per cell taken up by each cell line showed no difference between MCF-7 and MCF-7(TamR) as measured by flow cytometry (fig 3.5.3). This data also revealed a slight difference in background FL-1 fluorescnce between the two cell lines, with untreated MCF-7(TamR) cells showing a very small decrease in autofluorescnce compared to MCF-7 cells (fig 3.5.3 A). In light of this, The background fluorescence from each cell line was subtracted from the values obtained from 10k dextran-FITC conjugate treated cells before calculating the extent of 10k dextran-FITC uptake (fig 3.5.3 B).

The neutral comet assay showed that bleomycin causes a measurable amout of DNA damage in the form of double stranded breaks to both cells lines. With the characteristic comet shaped cell debris being visible in treated cells following a seven day bleomycin treatment (fig 3.4.5 A). Comparison of the extent of damage sustained by MCF-7 and MCF-7(TamR) cells following a 7 day treatment with bleomycin at either 0.05µg/ml or 2µg/ml, resulted in no significant difference between the amount of DSBs detected in both both cell lines (fig 3.5.4 B).

3.5.3 Microarray analysis of drug efflux protein mRNA expression

Microarray analysis revealed that the mRNA expression of several proteins involved with drug efflux was altered with the development of tamoxifen resistance (fig 3.5.5). Levels of BCRP mRNA were downregulated following the development of tamoxifen resistance, with the level of expression in MCF-7(TamR) low enough to be declared absent. MDR1 mRNA was declared absent from both cell lines using two different probe sets. MRP1 mRNA was present in both cell lines, in equivalent

3.5.3 Measurement of rate of fluid phase endocytosis in MCF-7 and MCF-7(TamR) cells.

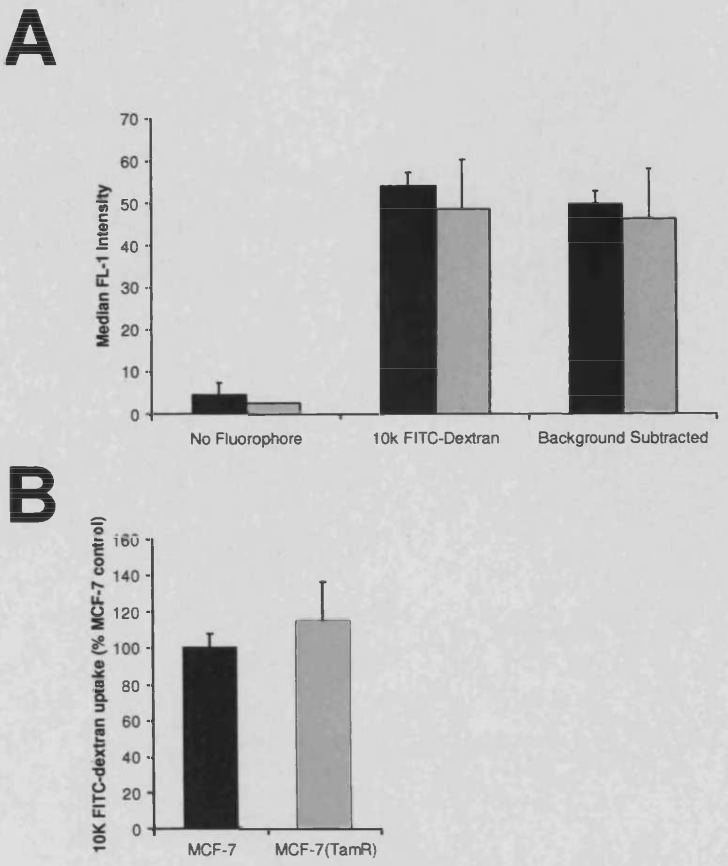


Figure 3.5.3. Rate of fluid phase endocytosis in MCF-7 and MCF-7(TamR) cells as measured by rate of 10k dextran-FITC uptake examined by flow cytometry. (A) A sample data set showing background levels of FL-1 fluorescence in MCF-7 (■) and MCF-7(TamR) (■) cells, and the change in FL-1 flourescence in both cell lines following 4hrs in culture with FITC-10k dextran conjugate. Background levels were subtracted and median fluorescence values normalised from three separate expierments to produce (B), a direct comparison of the rate of FITC-10k dextran uptake between MCF-7 and MCF-7(TamR) cells. n=9, error bars indicate standard deviation.

3.5.4 Measurement of DNA damage caused to MCF-7 and MCF-7(TamR) cells by bleomycin.

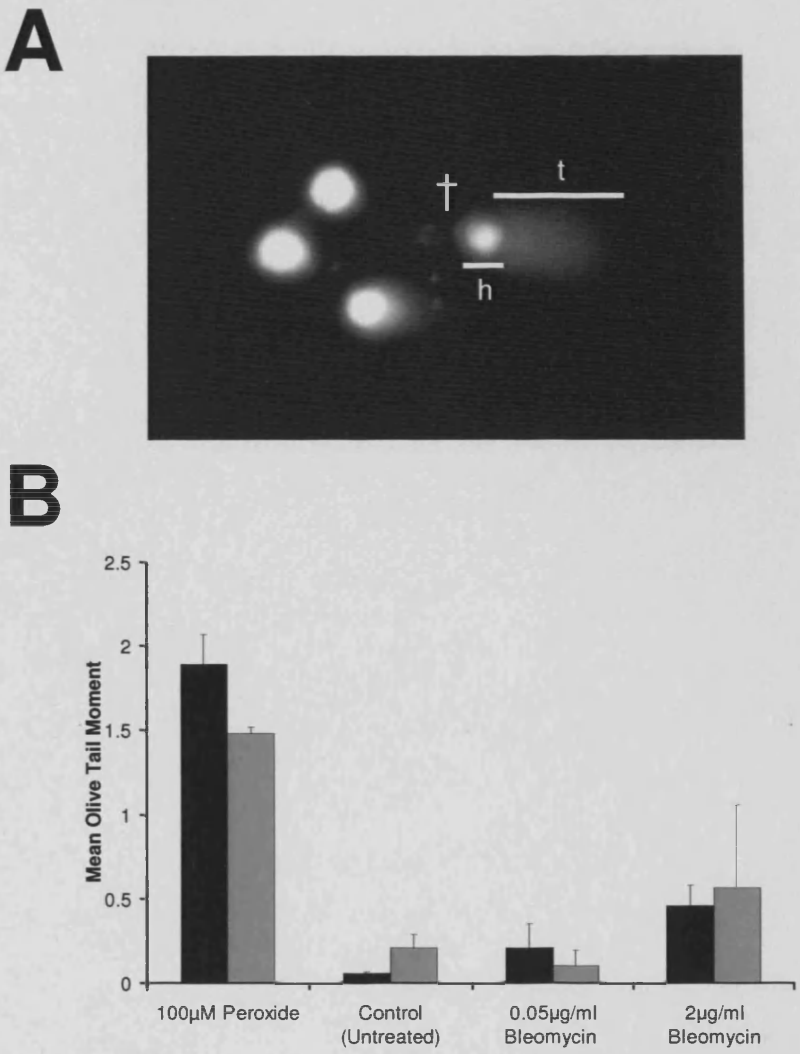
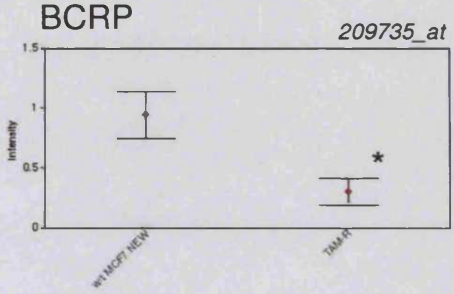


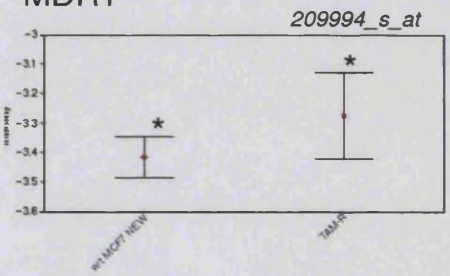
Figure 3.5.4 Measurement of double stranded breaks in DNA by neutral comet assay. (A) Example image (MCF-7 cells, 2µg/ml bleomycin) generated by comet assay as described in 2.7. † indicates cell which has sustained significant DNA damage. h and t indicate length of head and tail used to calculate tail moment. (B) Extent of DSB formation in MCF-7 (■) and MCF-7(TamR) (■) cells treated with bleomycin for seven days. A 30 minute treatment with hydrogen peroxide is included as a positive control. n=6, error bars indicate standard deviation.

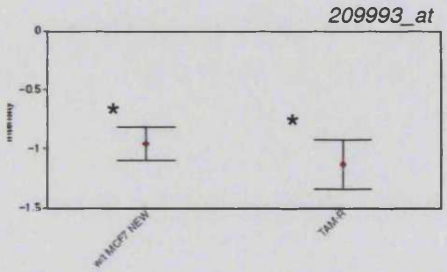
3.5.5 Microarray analysis of drug efflux pump mRNA expression in MCF-7 and MCF-7(TamR).

BCRP

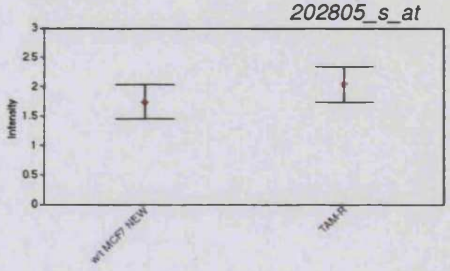


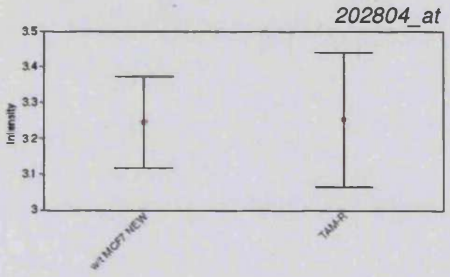
MDR1





MRP1





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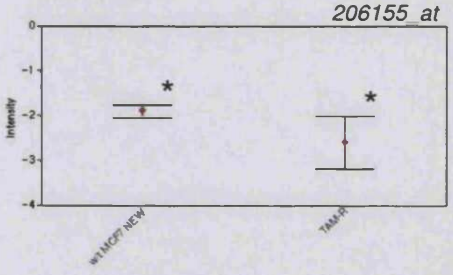

Figure 3.5.5. cont

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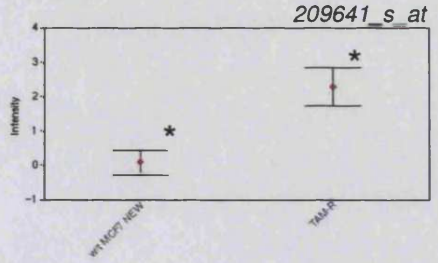
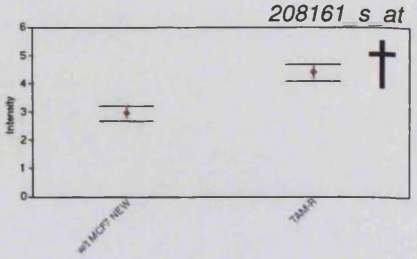
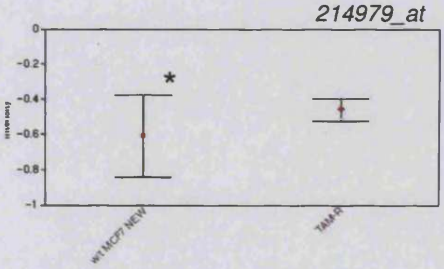
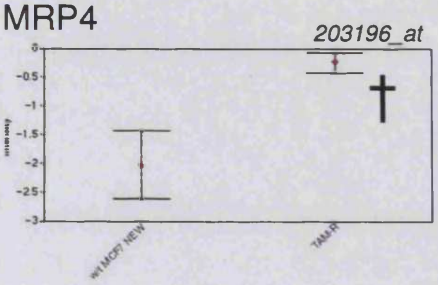






MRP4



MRP5

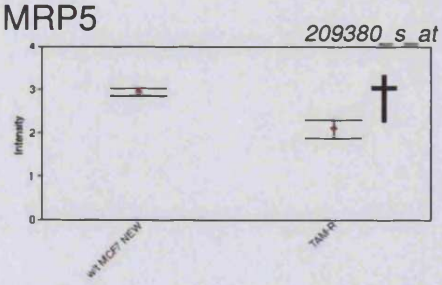
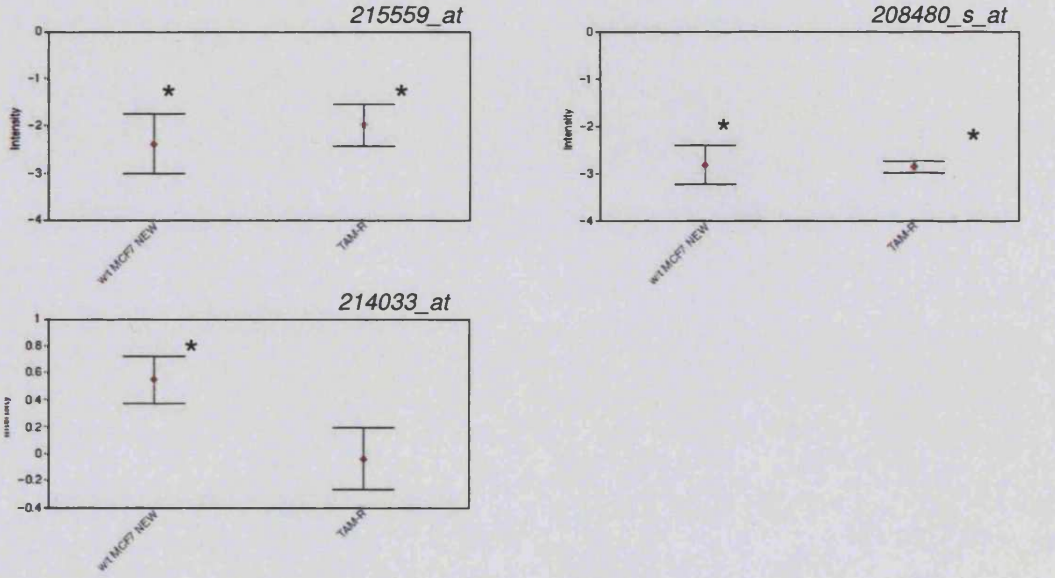


Figure 3.5.5. cont.

MRP6



MRP7

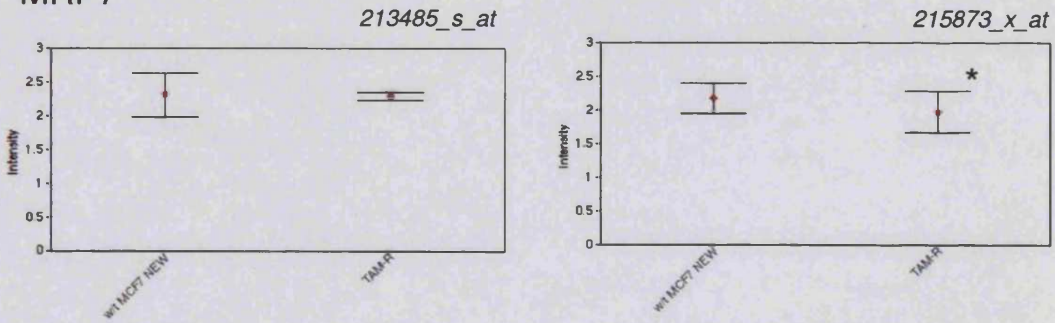


Figure 3.5.5. Cont

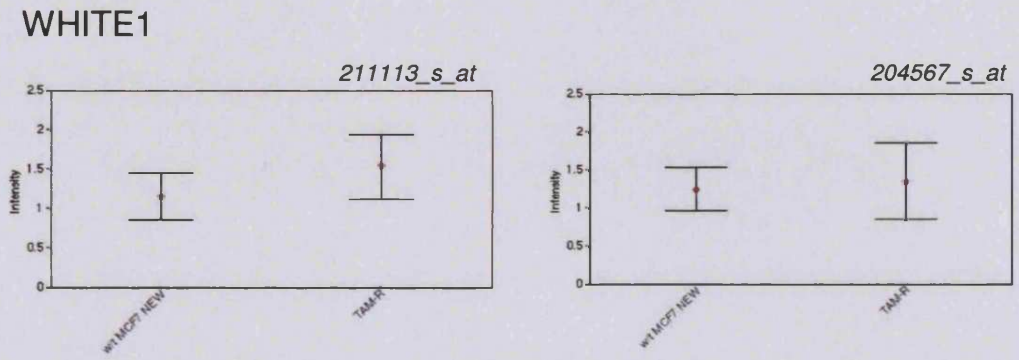


Figure 3.5.5. Analysis of mRNA expression levels of ten proteins concerned with drug efflux in MCF-7 and MCF-7(TamR) cells (for a summary of drug efflux genes, proteins and products.and substrates see section 1.4). * indicates a level of expression low enough to be declared absent by genesifter software. † indicates a significant difference ($p<0.05$)

amounts as measured by two separate probes. MRP2 mRNA was declared absent in both cell lines. MRP3 expression was shown to be increased in MCF-7(TamR). cells by two separate probes (*209641_s_at* and *208161_s_at*), a third probe (*214979_at*) did not generate a strong enough signal to measure accurately and was therefore declared absent by the analysis software. MRP4 mRNA was shown to be upregulated in MCF-7(TamR) cells, while MRP5 mRNA was shown to be downregulated. It was not possible to ascertain changes in the expression of MRP6 and MRP7, as the signal generated in both cases was very low with all probes. WHITE1 mRNA was detected in both cell lines, but no change in expression was observed between MCF-7 and MCF-7(TamR). The similar rate of doxorubicin uptake (described in 3.5.1, above) between MCF-7 and MCF-7(TamR) cells, combined with the similar rates of fluid-phase endocytosis and bleomycin-induced double stranded breaks in DNA (section 3.5.2) and the absence of any marked decrease in drug efflux pump mRNA expression suggest that changes in drug uptake or efflux are unlikely to be responsible for the changes in drug sensitivity that have been observed in MCF-7 and MCF-7(TamR) cells.

3.6 Apoptosis in tamoxifen resistant breast cancer.

3.6.1 Treatment of MCF-7 and MCF-7(TamR) with inducers of apoptosis.

There is a wealth of experimental evidence to suggest a link between the activity of the oestrogen receptor and the apoptotic threshold of breast cancer cells. Increased activation with oestrogens typically protects cells from cytotoxic treatments, with antioestrogens causing an increased propensity to apoptosis (discussed in detail in section 4.3). With this in mind, the readiness of both cell lines to undergo apoptosis was measured following treatment with camptothecin, a well studied inducer of apoptosis.

When challenged with camptothecin for 7 days, MCF-7(TamR) cells displayed a greatly increased sensitivity to the toxic effects of the drug in concentrations ≥1μM. At these concentrations the cell number of both cell lines was greatly reduced when compared to an untreated control, but while MCF-7 cells displayed a 70% reduction in growth, the number of MCF-7(TamR) cells surviving the treatment was so low as to be immeasurable (fig 3.6.1). While high concentrations of camptothecin appeared to have a greater effect on MCF-7(TamR) cells than MCF-7 cells, concentrations of camptothecin below 1μM had a similar effect on both cell lines, save for a single concentration (0.1μM), where the greatest reduction in growth was seen in MCF-7 cells.

3.6.2 bcl-2 levels and bleomycin sensitivity in MCF-7 derived cells.

The observed change in apoptosis between MCF-7 and MCF-7(TamR) cells following camptothecin treatment was consistent with the established link between oestrogen signalling and apoptosis. bcl-2 protein is well established as a mediator of apoptosis (see section 1.5.5), and its expression has been shown to be linked to oestrogen receptor signalling (discussed fully in section 4.3). To establish whether an antioestrogen mediated attenuation of bcl-2 expression was responsible for the acute

3.6.1 Camptothecin toxicity in MCF-7 and MCF-7(TamR) cells.

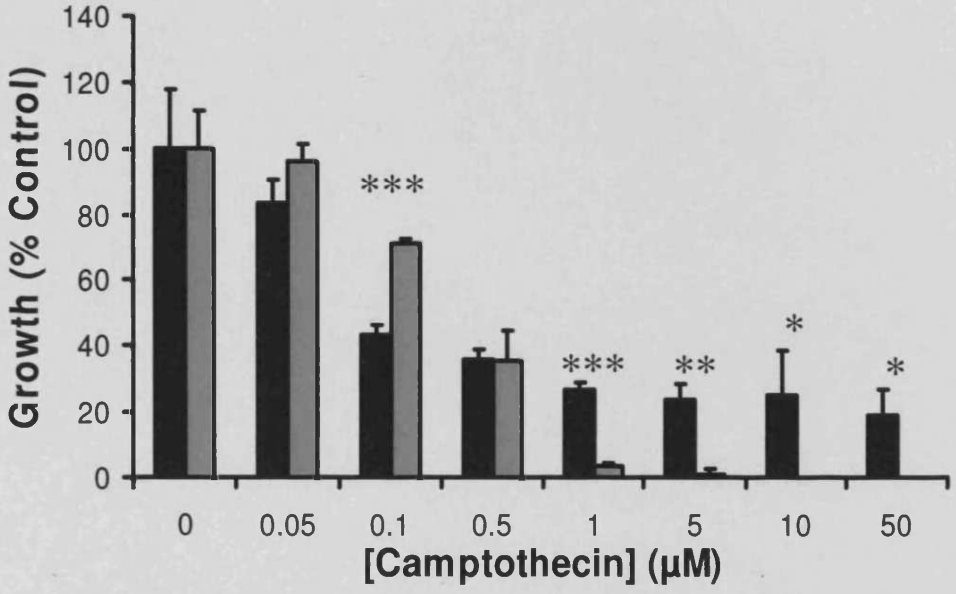


Figure 3.6.1 Growth of MCF-7 (■) and MCF-7(TamR) (■) cells cultured in media containing camptothecin for 7 days. n=6. p values calculated from paired t-test comparing growth inhibition between t47d and t47d(TamR) cells caused by a given concentration of camptothecin * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Error bars indicate standard deviation.

sensitivity of MCF-7(TamR) cells to bleomycin, levels of bcl-2 protein present in MCF-7 and MCF-7(TamR) cells was assayed, along with the levels of bcl-2 protein in two other cell lines which had also been derived from MCF-7.

Immunocytochemistry revealed a dramatic reduction in bcl-2 protein present in MCF-7(TamR) cells compared to MCF-7 cells. MCF-7 cells displayed clear dense staining in a high proportion of cells, whereas bcl-2 was undetectable in MCF-7(TamR) cells prepared in exactly the same manner. MCF-7(DR) cells, which are resistant to the growth inhibitory effects of both tamoxifen and gefitinib, also displayed very low levels of bcl-2 protein expression, similar to that observed in MCF-7(TamR). Another MCF-7 derived cell line, MCF-7(gfd), which is able to proliferate in the absence of external growth factor stimulation and as such can grow in media supplemented with serum in which the growth factors have been denatured by heat inactivation (see section 2.1.1), displayed high levels of bcl-2 protein expression, with a similar staining pattern to that seen in MCF-7 cells (fig 3.6.2).

When the sensitivity to bleomycin of these two new cell lines was tested, MCF-7(gfd) displayed the greatest resistance to a bleomycin treatment, with a 2µg/ml treatment only causing a 20% reduction in growth (compared to a 50% reduction observed in MCF-7 cells, and a 80% reduction seen in MCF-7(TamR) cells) (fig 3.6.3 A). The tamoxifen/gefitinib resistant MCF-7(DR) cell line displayed a much high sensitivity to the toxic effects of bleomycin, (almost identical to that previously seen in MCF-7(TamR)) with a 2µg/ml treatment causing a 80% reduction in growth, and a 0.05µg/ml treatment being required to reduce growth by 50%.(fig 3.6.3 B).

Once this circumstantial evidence linking the extent of bcl-2 signalling with bleomycin sensitivity had been established, the apoptotic effects of bleomycin on both MCF-7 and MCF-7(TamR) cells was studied using a fluorescently labelled marker of caspase activation (see section

3.6.2 Expression of bcl-2 in MCF-7 derived cell lines.

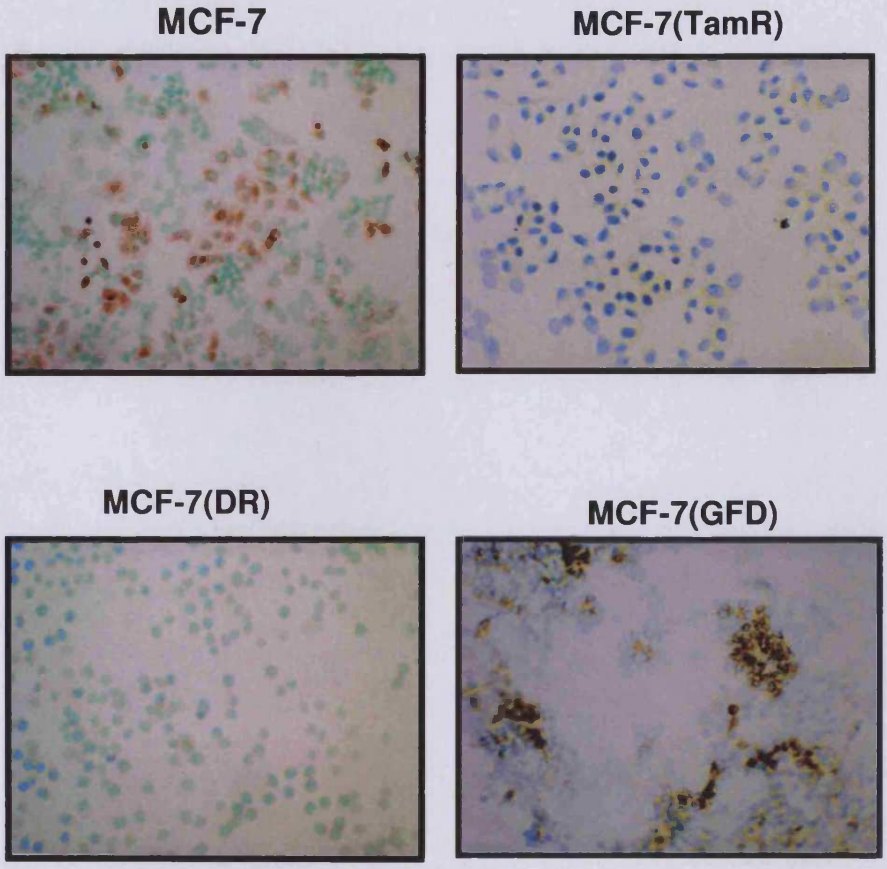


Figure 3.6.2. bcl-2 protein expression in wild-type (MCF-7), tamoxifen resistant (MCF-7(TamR)), tamoxifen and gefitinib resistant (MCF-7(DR)) and growth factor depletion resistant (MCF-7(gfd)) MCF-7 cells.

3.6.3 Sensitivity of MCF-7 derived cell lines to bleomycin.

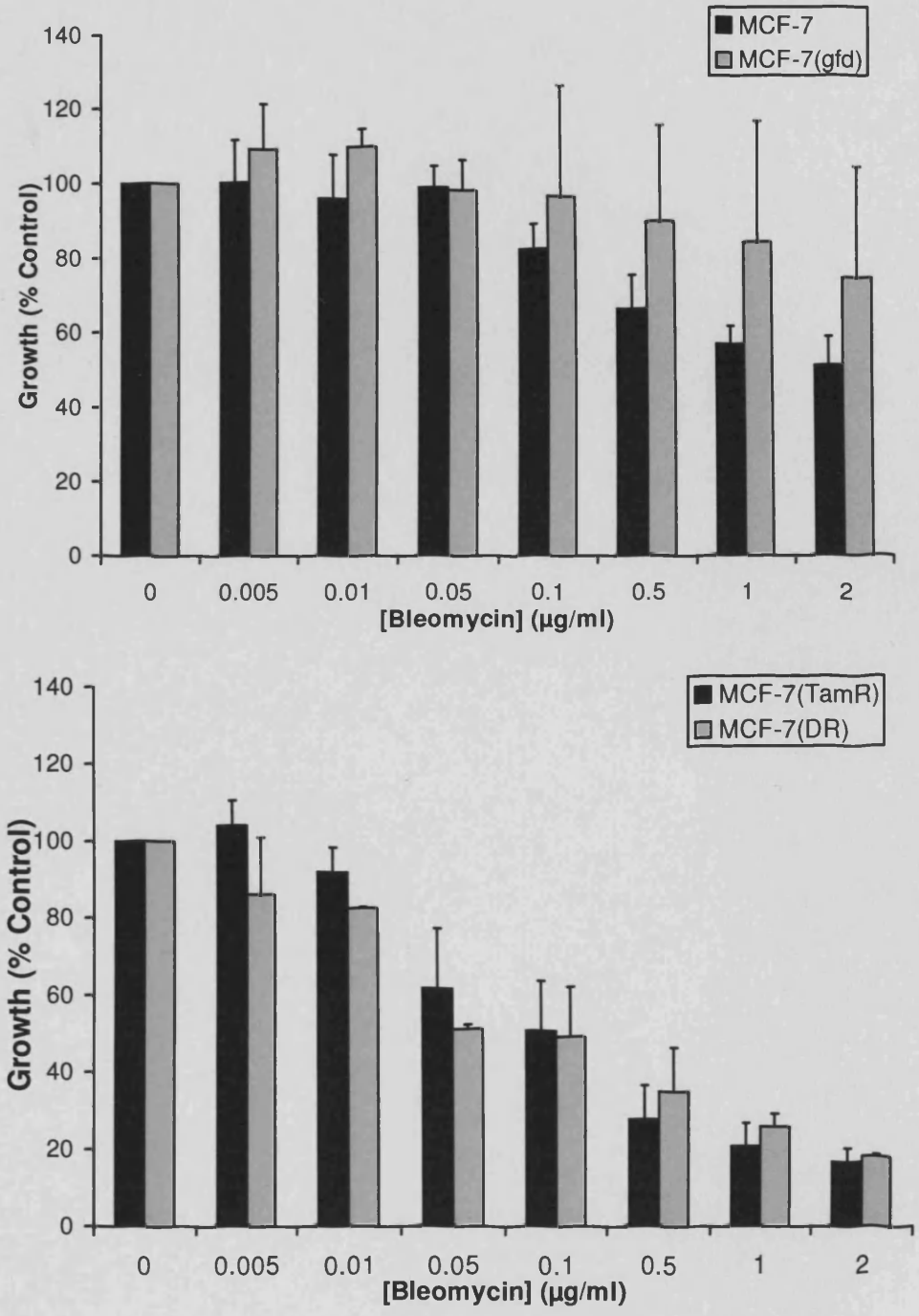


Figure 3.6.3 (A) Sensitivity of the growth factor deprived MCF-7 derived cell line MCF-7(gfd) to bleomycin c.f. wild type MCF-7 cells. (B). Sensitivity of the tamoxifen/gefitinib resistant cell line MCF-7(DR) to bleomycin c.f. tamoxifen resistant MCF-7 cells (MCF-7(TamR)). n=9, error bars indicate standard deviation.

1.6.11). Immediately following a 24-hour treatment with bleomycin, there was no observable increase in the extent of caspase activation as measured by flow cytometry. In both MCF-7 and MCF-7(TamR) the extent of caspase activation following treatment with either 1 or 10μg/ml bleomycin was comparable to that observed in bleomycin untreated cells treated with the fluorescent label (fig 3.6.4 A & B). While bleomycin did not increase caspase activation after a 24 hour treatment, at all times MCF-7(TamR) displayed a higher background level of caspase activation, with a greater proportion of cells exhibiting a fluorescence intensity above 10^2 (fig 3.6.4 A), resulting in a median intensity uniformly higher than that observed in MCF-7 cells (fig 3.6.4 B).

After a two day continuous treatment with an intermediate concentration of bleomycin (2μg/ml), known to have a differential effect on the growth of MCF-7 and MCF-7(TamR) cells, an equivalent increase in caspase activation was observed in both cell lines. Interestingly, untreated MCF-7(TamR) cells displayed a significantly higher degree of caspase activation than MCF-7 cells, suggesting that MCF-7(TamR) cells may posses more active apoptotic machinery than MCF-7 cells, even when unchallenged by a cytotoxic insult. Following 5 days in culture with or without 2μg/ml bleomycin, bleomycin treatment seemed to have little effect on the caspase activation of MCF-7 cells, while caspase activation was increased in MCF-7(TamR) following bleomycin treatment (fig 3.6.6 A & B). As in previous measurements at 24 hours and two days, the extent of background caspase activation was significantly higher in MCF-7(TamR) compared to MCF-7.

3.6.4 Caspase activation in MCF-7 and MCF-7(TamR) following bleomycin treatment.

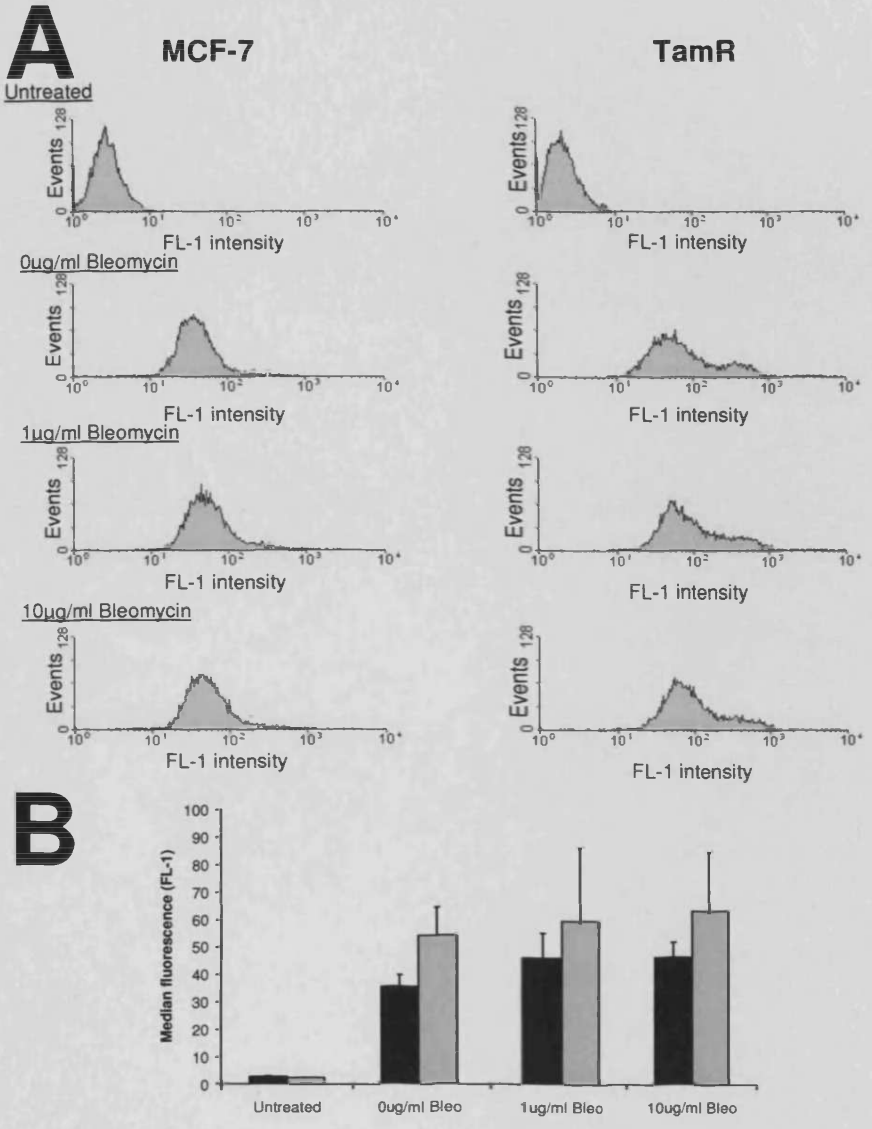


Figure 3.6.4 (A) Example histograms showing caspase activation in MCF-7 and MCF-7(TamR) cells as measured by caspACE™ staining and flow cytometry immediately after a 24 hour treatment with bleomycin. 'Untreated' refers to cells not treated with bleomycin or caspACE™ (B) Median data of MCF-7(■) and MCF-7(TamR) (■) treated cells as measured in section A. Bars indicate mean data (n=3). Error bars indicate standard deviation.

3.6.5 Caspase activation in MCF-7 and MCF-7(TamR) cells following a 2 day treatment with 2µg/ml bleomycin.

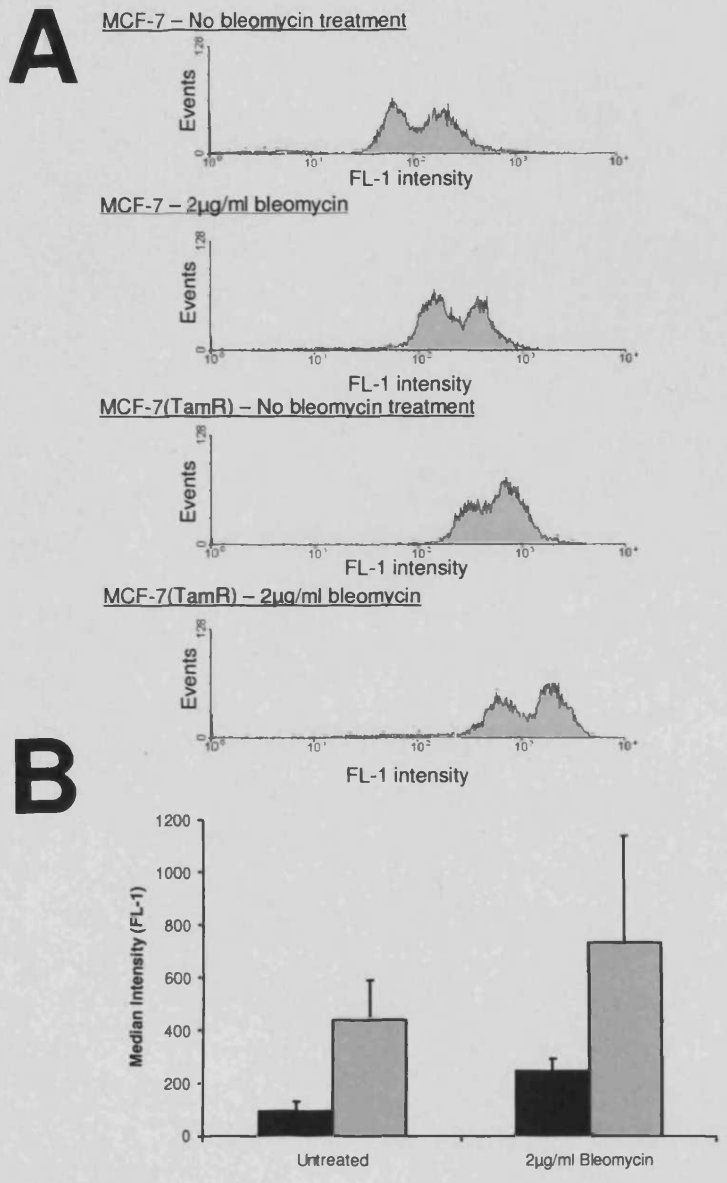


Figure 3.6.5 (A) Extent of caspase activation following a 2 day treatment with bleomycin in MCF-7 and MCF-7(TamR) cells as measured by caspACE™ staining and flow cytometry. (B) Median data for MCF-7(■) and MCF-7(TamR) (■) cells. n=3, error bars indicate standard deviation.

3.6.6 Caspase activation in MCF-7 and MCF-7(TamR) cells following a 5 day treatment with 2µg/ml bleomycin.

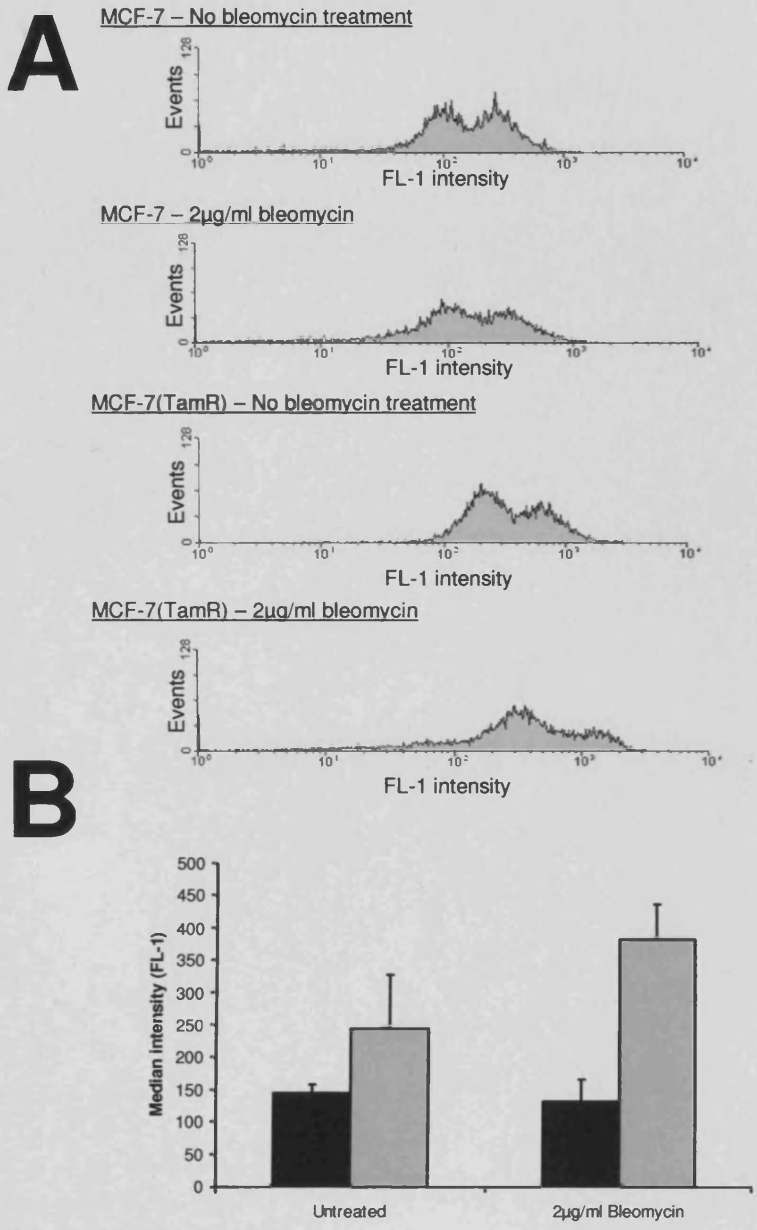


Figure 3.6.6 (A) Extent of caspase activation following a 5 day treatment with bleomycin in MCF-7 and MCF-7(TamR) cells as measured by caspACE™ staining and flow cytometry. (B) Median data for MCF-7(■) and MCF-7(TamR) (■) cells. n=3, error bars indicate standard deviation.

3.7 Modulation of bcl-2 expression

bcl-2 expression in MCF-7 cells was increased upon a 7 day treatment with oestradiol as measured by immunocytochemistry, with an increase in the number of cells stained as well as the density of staining. A 7 day treatment with the antioestrogens tamoxifen or faslodex reduced the levels of bcl-2 protein present in MCF-7 cells (fig 3.7.1). Treatment of MCF-7(TamR) cells with oestradiol or faslodex had little effect on bcl-2 protein expression compared with tamoxifen, which is routinely present in the culture media. Removal of tamoxifen from the cell culture media further reduced bcl-2 protein expression in MCF-7(TamR) cells (fig 3.7.2).

Culture of MCF-7 cells for 7 days with an antioestrogen present (either tamoxifen or faslodex) had little effect on bleomycin sensitivity, with tamoxifen or faslodex treated MCF-7 cells displaying similar sensitivity to MCF-7 treated with bleomycin alone. A 2µg/ml treatment of bleomycin was sufficient to reduce the growth rate of untreated, tamoxifen treated and faslodex treated MCF-7 cells by 50% (fig 3.7.3 A). Treatment with oestradiol appeared to sensitise MCF-7 cells to bleomycin, with a 50% reduction in growth being observed with a 0.5µg/ml treatment bleomycin. (fig 3.7.3.A). Tamoxifen, faslodex and oestrogen all had no effect on the sensitivity of MCF-7(TamR) cells to bleomycin, with the same sensitivity being observed in the presence or absence of tamoxifen, faslodex or oestradiol (fig 3.7.3).

Treatment of both MCF-7 and MCF-7(TamR) cells with Dharmafect™ transfection reagent caused a reduction in cell number that was comparable between cell lines. A full transfection treatment involving both transfection reagent and an siRNA (directed against either luciferase or bcl-2) also caused a similar reduction in cell number in both cell lines. siRNA directed against the non-endogenous luciferase gene had the same effect on cell number as siRNA directed against bcl-2 in both cell lines. These effects were observed in both single a 4 day transfection (fig 3.7.4 A) and a double 7 day transfection (fig 3.7.4 B). Despite having a marked

effect on cell number, transfection with a siRNA directed against bcl-2 caused a reduction in bcl-2 protein expression in MCF-7 cells as measured by immunocytochemistry, that was not observed in MCF-7 cells treated with an siRNA directed against luciferase (fig 3.7.5). This reduction in bcl-2 protein expression did not produce an accompanying increase in bleomycin sensitivity in MCF-7 cells, with cells treated with either 0.5μg/ml or 2μg/ml bleomycin displaying the same level of sensitivity regardless of whether they were transfected with an siRNA directed against luciferase or bcl-2. The same similarity in sensitivity was observed over both a 4 day (fig 3.7.6 A) and 7 day (fig 3.7.6 B) treatment.

3.7.1 Modulation of bcl-2 expression in MCF-7 cells by short term treatment with oestradiol and antioestrogens.

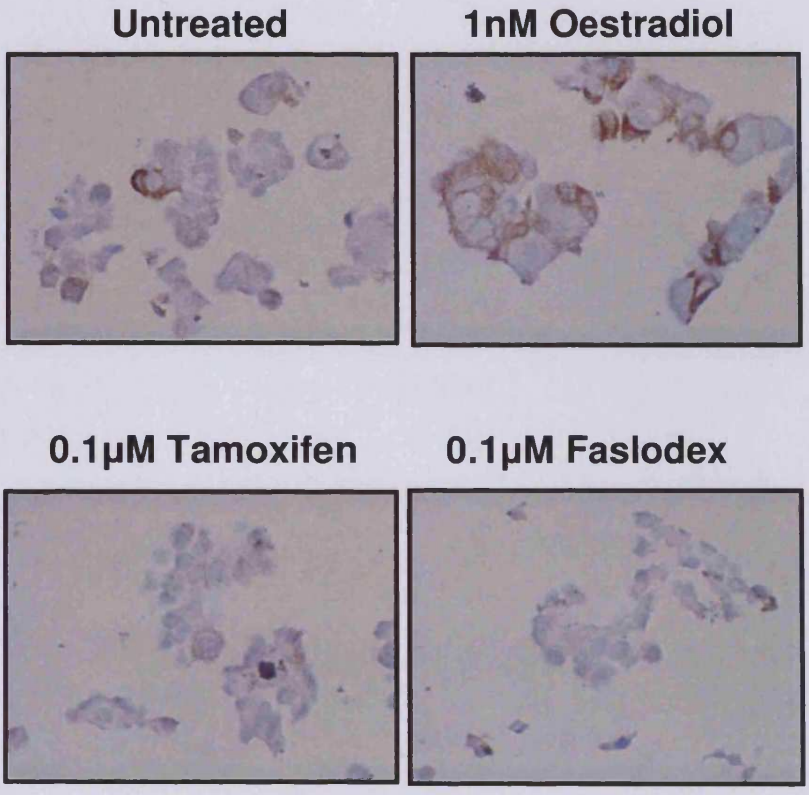


Figure 3.7.1. Measurement of bcl-2 protein expression by immunocytochemistry in MCF-7 cells treated for 7 days with the oestrogen, oestradiol, or the antiestrogens tamoxifen and faslodex.

3.7.2 Modulation of bcl-2 expression in MCF-7(TamR) cells by short term treatment with oestradiol and antioestrogens.

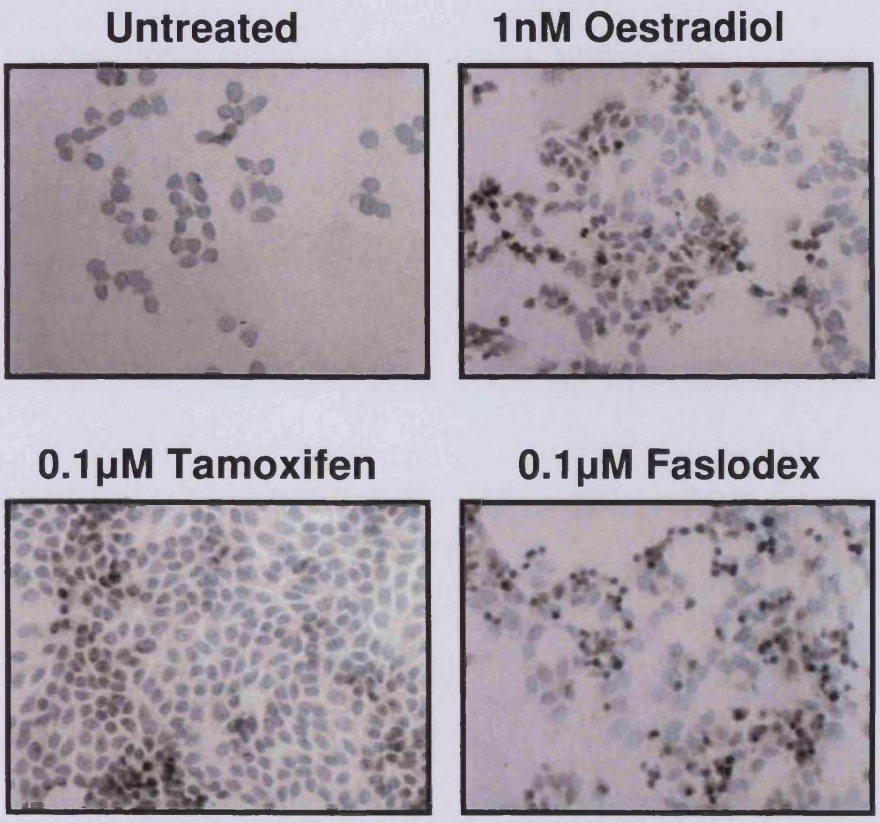


Figure 3.7.2. Measurement of bcl-2 protein expression by immunocytochemistry in MCF-7(TamR) cells treated for 7 days with the oestrogen, oestradiol, or the antiestrogens tamoxifen and faslodex.

3.7.3 Sensitivity of MCF-7 and MCF-7(TamR) cells treated with oestradiol and antioestrogens to bleomycin.

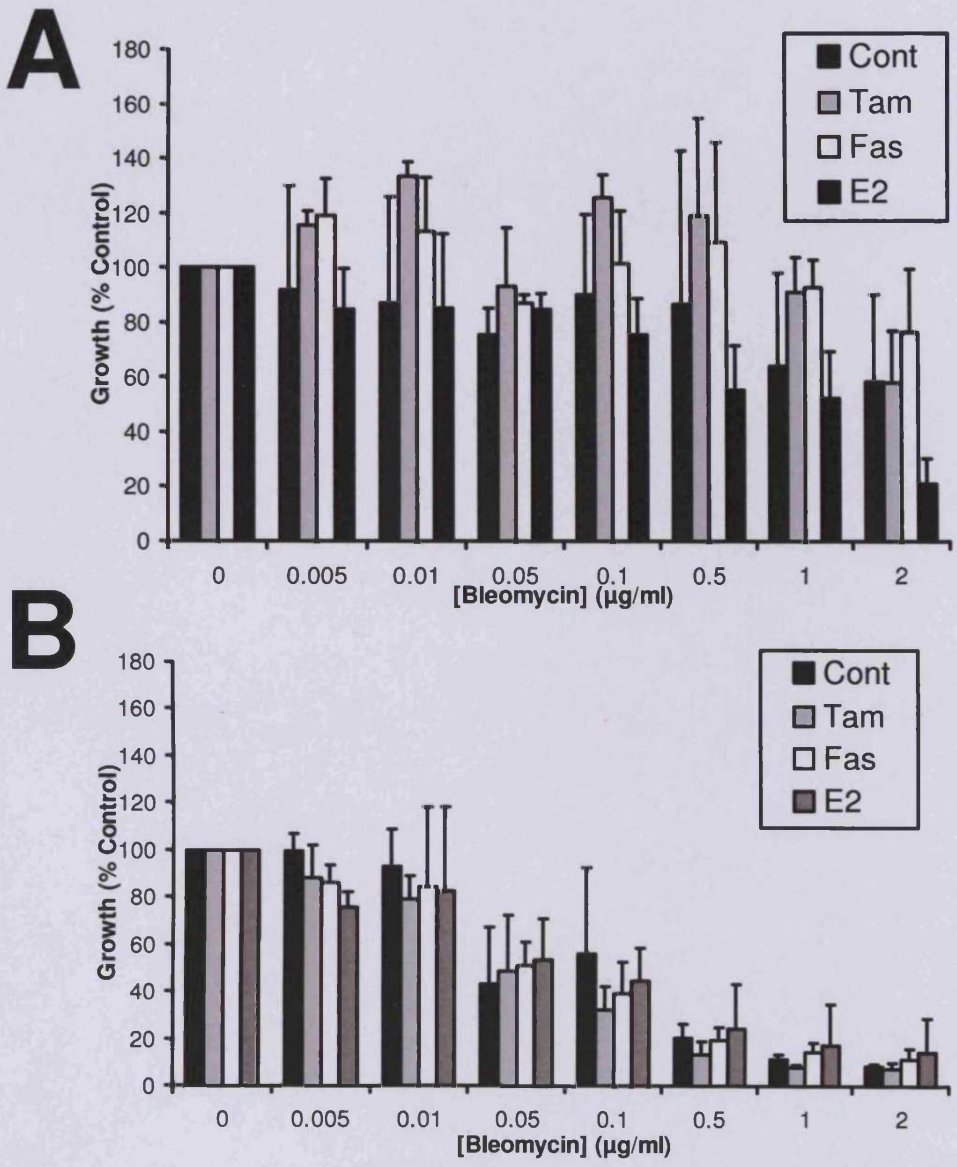


Figure 3.7.3 (A) Effect on growth of MCF-7 cells of a 7-day treatment with an oestrogen/antioestrogen and bleomycin. (B) Effect on growth of MCF-7(TamR) cells of a 7-day treatment with an oestrogen/antioestrogen and bleomycin.

3.7.4. Toxicity of transfection regimens to MCF-7 and MCF-7(TamR) cells.

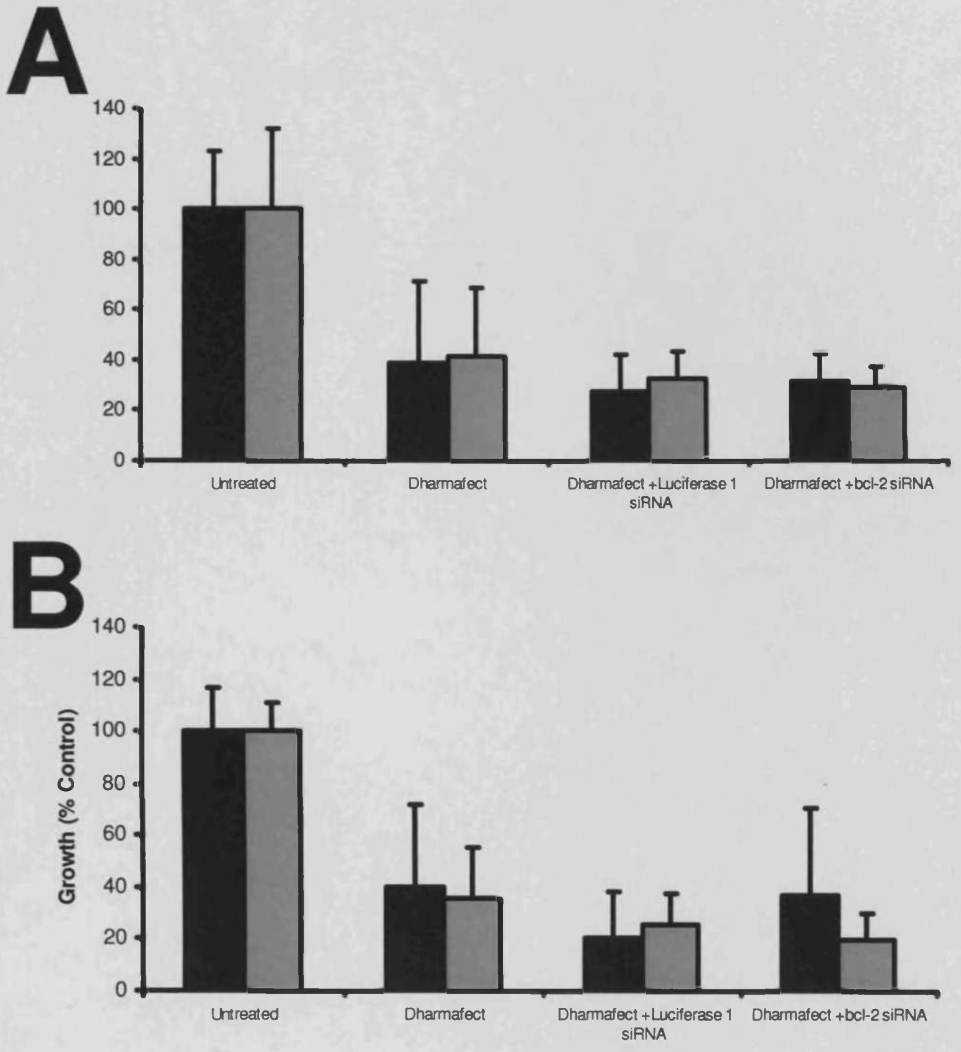
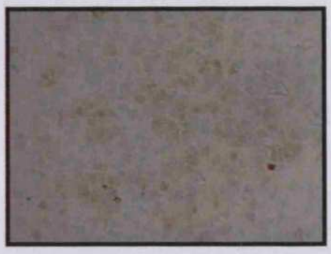


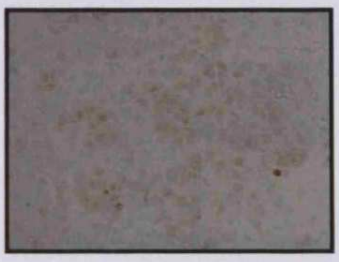
Figure 3.7.4 (A) Effect on growth of MCF-7 (■) and MCF-7(TamR) (■) cells of a single transfection regimen, comprising 4 days in culture with media containing Dharmafect™ and siRNA. (B) Effect on growth of MCF-7 (■) and MCF-7(TamR) (■) cells of a double transfection regimen, comprising 7 days in culture with media containing Dharmafect™ and siRNA, with media being changed on day 4.

3.7.5 Measurement of bcl-2 knockdown with siRNA in MCF-7 cells by immunocytochemistry.

Untreated



Luciferase siRNA transfected



bcl-2 siRNA transfected

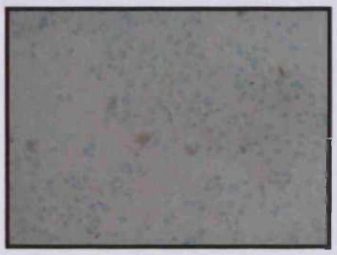


Figure 3.7.5 Observation of siRNA mediated bcl-2 protein knockdown in MCF-7 cells. Cells were treated with a mixture of four siRNAs directed against either bcl-2, or the non-endogenous luciferase mRNA and examined my immunocytochemistry.

3.7.6 Sensitivity of siRNA treated MCF-7 cells to bleomycin.

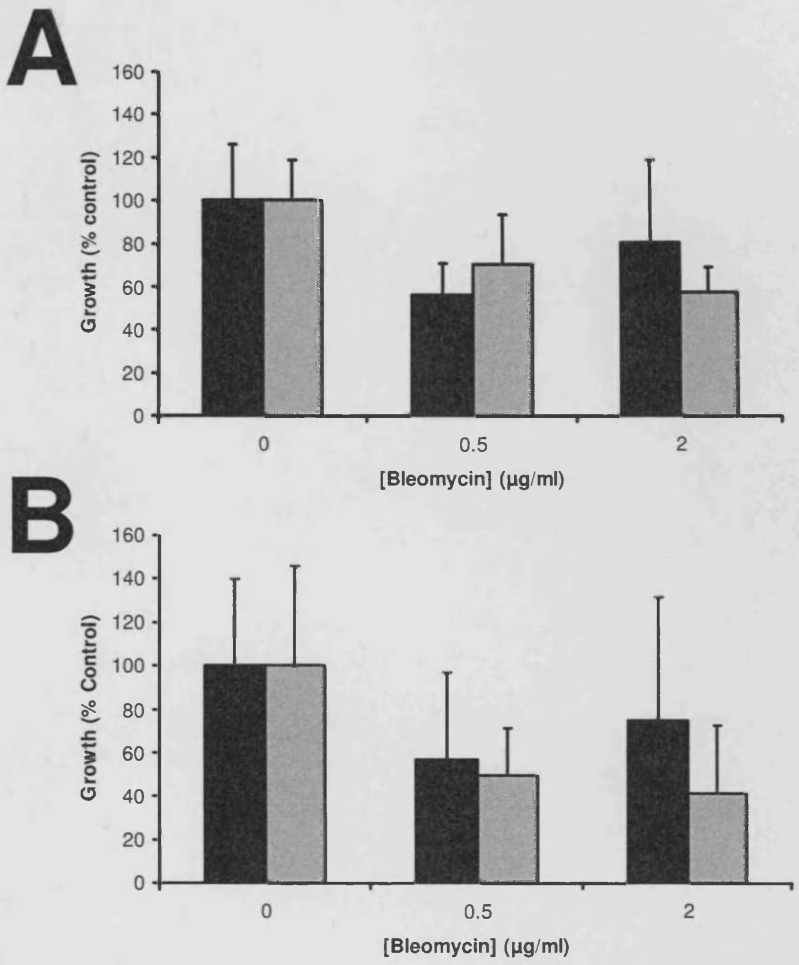


Figure 3.7.6 Sensitivity of MCF-7 cells treated with siRNA against luciferase (■) or bcl-2 (▒) to (A) A four day treatment with bleomycin. (B) A seven day treatment with bleomycin.

Chapter 4
Discussion and conclusions.

4.1 Changes in protein expression and drug sensitivity following the development of tamoxifen resistance.

Initial observation of the MCF-7/MCF-7(TamR) cell culture model (described in 2.1.1) revealed that tamoxifen resistance had developed (fig 3.1.2) and that the characteristics of interest previously reported by others (El-Zarruk & van den Berg, 1999) (Knowlden et al., 2003) (Gee et al., 2001) (Jones et al., 2004) were present. These included an increased rate of growth (fig 3.1.1.), increased EGFR expression and activation (fig 3.1.3), dependence on EGFR activity for growth (fig 3.1.6), and increased activation of downstream signalling molecules linked to proliferation and survival (fig 3.1.5). This demonstrated that MCF-7 / MCF-7(TamR) cells were a suitable model of tamoxifen resistance in which the role of EGFR in protection from cytotoxic agents could be studied.

The ability of the increased EGFR signalling to protect MCF-7(TamR) cells from the effects of a panel of cytotoxic drugs was carefully examined, and it was observed that increased levels of EGFR signalling offered cells no significant protection from any of the five drugs studied, and in the case of certain drugs (most clearly with bleomycin, but also to a lesser extent with other drugs, including etoposide), high-EGFR expressing tamoxifen resistant cells were significantly sensitised to killing with these agents. This observation is completely at odds with all previous studies, which link increased EGFR expression with drug or radioresistant phenotypes. There are many studies which have previously equated an increase in EGFR signalling during tumour development with a resistance to treatment with ionising radiation, for example EGFR signalling was found to be a strong determinant of tumour radioresponse in a panel of murine carcinomas (Akimoto et al., 1999) and in human glioblastomas (Barker et al., 2001), with strong EGFR expression correlating to poor response to radiotherapy in all cases. Artificially increasing EGFR levels by transfecting murine ovarian carcinoma cells with a human EGFR construct has also been shown to increase radioresistance (Liang et al., 2003). With this is mind it is of particular interest that tamoxifen resistant breast cancer cells displayed a

greatly increased sensitivity to bleomycin, and a smaller but pronounced increase in sensitivity to etoposide, as both of these drugs bring about a similar kind of DNA damage to cells as ionising radiation, namely the introduction of double stranded breaks into DNA.

Consistent with its well established role in the modulation of cell survival and apoptosis, increased AKT activity has also been linked with cytotoxic resistance. An inducible form of AKT has been shown to protect human myeloid cells from the cytotoxic effects of etoposide and AraC (1-ß-arabinofuranosylcytosine) (Grandage et al., 2005). A study concerned with assessing the therapeutic potential of the latent membrane protein (LMP1) of the Epstein-Barr virus (Mei et al., 2007) reported that following treatment of Epstein-Barr positive nasopharyngeal carcinoma C666-1 cells with an siRNA directed against LMP1, there was a decrease in AKT activation accompanied by an increase in sensitivity to bleomycin and cisplatin. This sensitivity to could be reversed by transfection with a constitutively active AKT construct, suggesting that in some cells, bleomycin sensitivity may be modulated by AKT activity. This does not appear to be the case in MCF-7(TamR) cells, which display a greatly increased sensitivity to bleomycin, along with increased levels of AKT activation.

This peculiar sensitivity of tamoxifen resistant MCF-7 cells in spite of their apparent increase in proliferative and pro-survival signalling was entirely the opposite of what was expected, and raises a number of questions concerning the changes that occur in breast cancer cells following the development of tamoxifen resistance. It was established that the extent of EGFR signalling appeared to have little effect on the sensitivity of either MCF-7 or MCF-7(TamR) cells to bleomycin or doxorubicin, as the addition of the EGFR inhibitor, AG1478 to cytotoxic treatments had no effect on the toxicity of these drugs to either cell line over a number of different treatment regimes (fig 3.3.1 & 3.3.2). Sensitisation to cytotoxics with EGFR inhibitors has been demonstrated in MCF-7 cells with certain cytotoxic/EGFR inhibitor combinations, such as cisplatin/gefitinib

(Friedmann et al., 2004), but it would appear that AG1478 does not cause significant sensitisation to doxorubicin or bleomycin following a 24 hour treatment, suggesting the observed extreme sensitivity to bleomycin is not likely to be caused by any changes in EGFR signalling that occur during the development of tamoxifen resistance. In addition to this, an alternative model of tamoxifen resistance using t47d cells as the parental cell line displays an identical response to bleomycin as in the MCF-7 based model, with the tamoxifen resistant derivative of t47d, (t47d(TamR)) displaying a greatly increased sensitivity

A probable explanation for the inability of increased EGFR levels in tamoxifen resistant breast cancer to protect against the cytotoxic effects of bleomycin is that that in addition to an increase in EGFR signalling, many other changes in cellular signalling pathways occur during the development of tamoxifen resistance, and that at least one of these changes is responsible for the marked increase in sensitivity to bleomycin. There are a number of possible candidate pathways that may become altered following the development of tamoxifen resistance and have an effect of bleomycin sensitivity, including changes in the ability of tamoxifen-resistant cells to take-up or expel drug molecules, differences in the ability of the cells to deal with the damage to DNA caused by cytotoxic drugs, or changes in the apoptotic machinery which may render cells particularly sensitive to attack with certain cytotoxics. All of these possibilities were subsequently examined and are discussed below.

4.2 Drug uptake and efflux and bleomycin sensitivity.

One explanation for the increase in drug sensitivity in MCF-7(TamR) cells is that in becoming tamoxifen resistant, either through changes in the cell membrane, or through proteins concerned with the uptake or efflux of drug molecules the rate at which molecules of bleomycin or other drugs are able to enter the cell is altered. While the method by which MCF-7 cells become resistant to tamoxifen is not generally reliant on tamoxifen efflux, but rather on the utilisation of alternative methods of growth factor signalling (see section 1.1.6.1) reduced intra-tumoural levels of tamoxifen have been observed in some cases of acquired tamoxifen resistance (Ring & Dowsett, 2004) suggesting that drug efflux may contribute to tamoxifen resistance in some cases. There is also evidence to suggest tamoxifen can modulate drug sensitivity by altering the activity of drug efflux pumps, for instance by causing formerly doxorubicin-resistant cell lines to become re-sensitised to treatment with doxorubicin (Ramu et al., 1984). This re-sensitising is thought to act through inhibition of the action of P-glycoprotein, either by direct interaction by tamoxifen (Callaghan & Higgins, 1995) or through more convoluted pathways involving interference with glycosphingolipid metabolism and glycosylation (Lavie et al., 1997). Tamoxifen treatment has also been shown to increase the amount of the cytotoxic daunorubicin taken up by a human lymphoma cell line (Berman et al., 1991).

In light of this, the uptake of doxorubicin, a naturally fluorescent cytotoxic, over both four and 24 hours was examined in both MCF-7 and MCF-7(TamR) cells by flow cytometry (figs 3.5.1 & 3.5.2) and was found in both cases to be very similar, with very little variation in uptake between cell lines at all time points and concentrations, in spite of the marked difference in toxicity of these treatments to MCF-7 and MCF-7(TamR) cells (fig 3.2.3 B). This would suggest that in the MCF-7 cell culture model of tamoxifen resistance long term tamoxifen treatment does not affect the rate of uptake of doxorubicin, making changes in uptake or efflux of drugs unlikely to be

responsible for the marked change in sensitivity observed in MCF-7(TamR) cells.

Microarray data was examined to study the effect of tamoxifen resistance on expression of several drug efflux pumps (fig 3.5.5), revealing several efflux pumps (MRP3 & MRP4) are upregulated at the mRNA level in MCF-7(TamR) whereas only MRP5 appeared to be downregulated. It should be noted that of the three efflux pumps for which a change in transcription levels is observed, only MRP3 is known to pump out any of the cytotoxics used in this study, being able to exclude doxorubicin, etoposide and cisplatin.

Due to it producing by far the largest difference in sensitivity between MCF-7 and MCF-7(TamR) it was of greatest interest to study the uptake of bleomycin and to ascertain whether comparable amounts of bleomycin were entering MCF-7 and MCF-7(TamR) cells. Bleomycin is a very large molecule, and is not a substrate of any known drug efflux pump. There are other methods by which bleomycin resistance can occur, usually attributed to an increase in expression of proteins that are either able to metabolise bleomycin into an inactive metabolite (Pei et al., 1995) or are involved with the repair of the DNA damaged caused by bleomycin such as Ape1 (Robertson et al., 2001), rather than increased efflux of the drug. This suggests further that the mechanisms involved in bleomycin sensitivity in tamoxifen resistant cells are unlikely to be connected with changes in the rate of drug uptake or efflux, but may lie in the ability of the cells to deal with bleomycin, or with the damage to DNA caused by bleomycin once it has entered the cell. In spite of this, it remained important to measure the uptake of bleomycin to ensure that the increased sensitivity to bleomycin was not a result of changes in uptake.

A number of methods have been used to measure bleomycin uptake in the past, but the majority of these were used several decades ago, and as such are reliant on reagents or apparatus that are either no longer

available, or prohibitively expensive. Bleomycin radiolabelled with ^{3}H (Roy & Horwitz, 1984), ^{13}C (Roy et al., 1981) and even ^{111}In (Grove et al., 1974) has been used in the past, but are no longer commercially available. Another possibility was to exploit the ability of the bleomycin molecule to chelate cobalt to manufacture a bleomycin molecule labelled with ^{57}Co, as described in more recent papers involving radiolabelled bleomycin (Poddevin et al., 1990). This method required too many specialised reagents and too much safety apparatus to be feasible within the remit of this study. A fluorescently labelled bleomycin derivative has also been reported (Aouida et al., 2004), but this is not currently commercially available, and consists of a purified form of one of the bleomycins (bleomycin-A5) conjugated to FITC. There is also at least one anti-bleomycin monoclonal antibody available, which could be used to detect bleomycin uptake by fluorescence microscopy.

The mechanism of uptake of bleomycin is poorly understood. It is known that the toxicity of bleomycin is greatly increased by the introduction of holes in the cell membrane by electroporation (Poddevin et al., 1991), demonstrating that the cell membrane presents a major barrier to bleomycin uptake. It has been proposed that bleomycin is taken up by cells in a receptor-mediated mechanism, and there is evidence to support the existence of a membrane-bound 'bleomycin receptor' in eukaryotic cells (Pron et al., 1993), and that bleomycin is able to enter the cell through receptor mediated endocytosis, with the amount of bleomycin entering a cell being directly correlated to the rate of membrane turnover and therefore fluid phase endocytosis (Pron et al., 1999). While changes in the amount of bleomycin-binding proteins on the cell membrane may account for the change in sensitivity to bleomycin observed during the development of tamoxifen resistance, the bleomycin-specific nature of this change would not account for the increases in sensitivity to doxorubicin, 5-fluorouracil and etoposide also seen in MCF-7(TamR). The proposed bleomycin-receptor protein is poorly characterised at present, and is generally only detected by exploiting its affinity to ^{57}Co labelled bleomycin, so examination of the ability

of MCF-7 and MCF-7(TamR) membranes to bind bleomycin was impossible for reasons previously discussed. It was however, possible to measure the rate of fluid-phase endocytosis in MCF-7 and MCF-7(TamR) and as such determine whether an increase in the rate of fluid phase endocytosis and therefore membrane turnover and bleomycin receptor and bleomycin uptake may contribute to bleomycin sensitivity in MCF-7(TamR) cells. The rate of fluid phase endocytosis as measured by flow cytometry was found to be equivalent in tamoxifen-sensitive MCF-7 cells and tamoxifen-resistant MCF-7(TamR) (fig 3.5.3).

While there was no robust method by which bleomycin uptake could be measured directly, it was possible to measure the effect which bleomycin has on the DNA of treated cells, namely the introduction of double stranded breaks. It was established, using the neutral comet assay, which provides a measurement of the number of double stranded breaks in DNA present in each cell measured, that over a seven day treatment with bleomycin either at 0.05µg/ml (roughly equivalent to the concentration of bleomycin required to reduce growth of MCF-7(TamR) cells by 50%) or 2µg/ml (roughly equivalent to the concentration of bleomycin required to reduce growth of MCF-7 cells by 50%), the mean amount of damage sustained by the nuclei of MCF-7 and MCF-7(TamR) cells is comparable at both concentrations (fig 3.5.4), suggesting that both cell lines receive the same amount of damage following bleomycin treatment.

This data does not prove conclusively that a change in bleomycin uptake is not responsible for the increased sensitivity of MCF-7(TamR) cells to bleomycin, but seems to strongly suggest that this may be the case. A change in the expression of the proposed bleomycin receptor remains a possible explanation for increased bleomycin sensitivity, but the evidence that both cell lines receive the same amount of damage from bleomycin treatment would not seem to back this up. Further characterisation of the bleomycin receptor may allow for closer examination of bleomycin uptake in MCF-7(TamR) cells at a later date. Should a monoclonal antibody directed

against bleomycin receptor become available, the extent of bleomycin receptor expression on MCF and MCF-7(TamR) cell membranes could easily be compared by immunocytochemistry or fluorescence microscopy.

4.3 Apoptosis and bleomycin sensitivty

The data discussed in 4.2 above suggests that a change in drug uptake or efflux is unlikely to be responsible for the change in drug sensitivity observed in MCF-7(TamR). Particular attention was paid to examination of the uptake of bleomycin, as MCF-7(TamR) cells appear to be particularly sensitive to this cytotoxic. MCF-7(TamR) cells also displayed a marked sensitivity to etoposide which also elicits its cytotoxic effect through the introduction of double stranded breaks in DNA albeit through an entirely different mechanism to bleomycin (see section 1.3). The fact that the largest increase in sensitivity between MCF-7 and MCF-7(TamR) is seen with drugs that cause double stranded breaks in DNA suggests that during the development of tamoxifen resistance, changes occur in the cellular machinery that is responsible for the detection and repair of double stranded breaks or for the execution of apoptosis where DNA is too severely damaged to be repaired. This argument is further supported by the observation that MCF-7(TamR) cells also displayed an increased sensitivity to camptothecin (fig 3.6.1), a topoisomerase I inhibitor and well-known inducer of apoptosis, which is known to cause double stranded breaks in DNA (Ryan et al., 1991).

Figure 3.5.4 shows that bleomycin treatments that cause equivalent levels of damage to the DNA of both MCF-7 and MCF-7(TamR) can elicit drastically different effects on cell number between the two cell lines. Concentrations of bleomycin (and therefore, presumably levels of DSBs in DNA) that are readily tolerated by MCF-7 cells can prove fatally toxic to MCF-7(TamR) cells. This could be attributed to decreased ability of MCF-7(TamR) cells to tolerate DSBs, or by an increased ability of MCF-7 cells to repair DSBs. The comet assays used to measure damage sustained by the cells were not sensitive enough to detect repair of the damage over time, but this may be possible using more sensitive techniques such as pulsed-field gel electrophoresis which is able to accurately resolve very large fragments of DNA and therefore quantify breaks in chromosomes. This

technique has been used extensively to accurately study the repair of DSBs (Kuhne et al., 2004; Rothkamm & Lobrich, 2003), but relies on specialised equipment which it was not possible to obtain during the study. Modulation of DNA repair by EGFR signalling has been previously reported (Friedmann et al., 2004), but this study found that EGFR inhibition had a detrimental effect on repair of DNA damage caused by etoposide and cisplatin. This would suggest that increased EGFR signalling may exert a small protective effect on MCF-7(TamR), but that other factors which change during the development of tamoxifen resistance may overshadow this protection with a sensitising effect.

Good candidates for the modulation of this sensitisation are proteins concerned with the regulation of apoptotic signals (in particular the bcl-2 family of apoptotic proteins, described in section 1.5.5). Oestrogens have been shown to upregulate bcl-2 expression in MCF-7 cells, leading to a protection to the cytotoxic effects of adriamycin (Teixeira et al., 1995), whereas treatment with tamoxifen has been shown to downregulate bcl-2 expression in MCF-7 cells (Zhang et al., 1999). Other antioestrogens, such as faslodex, have also been shown to affect bcl-2 expression in a manner similar to that observed with tamoxifen (Lim et al., 2001). There are also several studies that suggest the downregulation of bcl-2 expression is also observed following long term treatments with antioestrogens. A cell line derived from MCF-7 cells which displays resistance to the steroidal antioestrogen RU 58,668 also displayed a marked decrease in bcl-2 expression (Fog et al., 2005), and another study found that two separate MCF-7-derived cell lines resistant to antioestrogen treatment both showed an increased sensitivity to the vitamin D derived cytotoxic EB1089, attributed to a marked decrease in bcl-2 (Larsen et al., 2001).

Transcription of the bcl-2 gene is controlled by two promoters. The P_1 region is located 1.6kb upstream of the start codon, and is where the majority of transcription is initiated (Young & Korsmeyer, 1993). A second, minor promoter (P_2) is located 1.3kb downstream of P_1 (Miyashita et al.,

1994a). Neither of these promoters contain an ERE, but two EREs are present within the coding region of the bcl-2 gene, which have been shown to be responsible for the oestrogen regulation of bcl-2 transcription (Perillo et al., 2000).

In order to establish whether a tamoxifen-mediated change in bcl-2 expression was responsible for bleomycin sensitivity, the changes in bcl-2 expression were first measured in both MCF-7 and MCF-7(TamR) cell lines by immunocytochemistry. bcl-2 expression in MCF-7(TamR) appeared greatly reduced compared to MCF-7, suggesting that in common with the other cell culture models of antioestrogen resistance mentioned above, long-term antioestrogen treatment was causing a significant reduction in bcl-2 expression (fig 3.6.2). This reduction in bcl-2 expression provide an explaination for the observed increase in sensitivity to certain cytotoxic agents. The levels of expression of bcl-2 in MCF-7(DR), a cell line derived from MCF-7(TamR) which displays resistance to both tamoxifen and gefitinib (Jones et al., 2004), and was cultured in the presence of both of these compounds, and was found to be greatly reduced compared to MCF-7 cells. The very low levels of bcl-2 present were comparable to that observed in MCF-7(TamR) cells (fig 3.6.2), suggesting that bcl-2 downregulation occurs during the development of tamoxifen resistance, and bcl-2 remains downregulated as a second resistance to gefitinib develops. MCF-7(DR) also displayed sensitivity to bleomycin (fig 3.6.3 B). bcl-2 protein expression was also examined in a fourth MCF-7 derived cell line, MCF-7(gfd), which is resistant to long term oestrogen deprivation (Staka et al., 2005). In this cell line, bcl-2 expression levels were unchanged from the parental cell line (fig 3.6.2), and this correlated to a resistance to the cytotoxic effects of bleomycin (fig 3.6.3 A). This data suggests an inverse correlation between bcl-2 expression and bleomycin sensitivity.

In order to confirm that bleomycin was inducing apoptosis in the cells, the extent of caspase activation was measured using a commercially available fluorescent label for activated caspase. An increase in caspase

activation was seen in both cell lines for several days following a bleomycin treatment (figs 3.6.5 & 3.6.6) suggesting that bleomycin is causing apoptosis, and therefore changes in bcl-2 expression are likely to have an effect on the apoptotic response to these cells. Consistent with this, MCF-7(TamR) cells were observed to have higher levels of caspase activation than MCF-7 cells, even when not challenged with a cytotoxic (figs 3.6.4 , 3.6.5 & 3.6.6), giving weight to the argument that MCF-7(TamR) cells downregulate bcl-2 as a consequence of long term antioestrogen treatment, and that the lower levels of bcl-2 are responsible for a particular sensitivity to certain cytotoxic agents.

The majority of caspases (caspase 9, caspase 3, caspase 7) elicit their apoptotic effect after the mitochondrial stage of apoptosis (see figure 1.9). The role of bcl-2 as regulator of cytochrome c release would suggest that a decrease in bcl-2 should result in an increase in permeability of the mitochondrial membrane to cytochrome c, promote apoptosome formation, and cause an increase in caspase cleavage and therefore apoptosis. The data collected in this study fits this model, but there are several other experiments that could be carried out in order to better understand the role of bcl-2 signalling in bleomycin sensitivity. Further dissection of the apoptotic pathway could be achieved by measuring a number of variables, including the permeability of the mitochondrial membrane to cytochrome c, the amount of functional apoptosome present in each cell line and measurement of the level of activation of each individual caspase. This would enable the exact point in the apoptotic cascade that results in increased apoptosis in MCF-7(TamR) to be ascertained, and may uncover changes in other apoptotic signals that may occur as tamoxifen resistance develops. As regulation of mitochondrial permeability to cytochrome c is regulated by all members of the bcl-2 family and not just bcl-2, it would also be of interest to see how expression levels of a variety of bcl-2 family members, including pro-apoptotic, anti-apoptotic and BH3 proteins, change during the development of tamoxifen resistance.

4.4 Modulation of the bcl-2 signal and bleomycin sensitivty

4.4.1 Modulation of bcl-2 expression using oestrogens and antioestrogens

As previously discussed, many studies have reported changes in bcl-2 expression following treatment with oestrogens and antioestrogens (see section 1.5.10). In order to further understand the role of bcl-2 in the modulation of bleomycin sensitivity, bcl-2 levels were modulated in MCF-7 and MCF-7(TamR) cells using oestradiol, tamoxifen and faslodex. It was possible to increase bcl-2 protein expression in MCF-7 cells using short term treatments of estradiol, and short term treatments with tamoxifen and faslodex were also able to substantially reduce bcl-2 signalling in the same cells (fig 3.7.1). This effect was much less clear in MCF-7(TamR) cells, which only express a very low level of bcl-2 to begin with. It appeared that estradiol and faslodex were unable to modulate bcl-2 expression, with similar levels of bcl-2 being observed in cells cultured in these drugs as those cultured in tamoxifen. Withdrawal of tamoxifen however, appeared to reduce bcl-2 expression further (fig 3.7.2). The fact that bcl-2 levels in MCF-7 cells exhibited the expected response to oestrogens and antioestrogens, while MCF-7(TamR) cells did not could be attributed to a number of factors. Levels of bcl-2 expression are much lower in MCF-7(TamR) cells, making the detection of slight changes in expression much more difficult to detect.

Modulation of the bcl-2 signal by treatment with antioestrogens did not have the expected effect on bleomycin sensitivity. In MCF-7 cells neither of the antioestrogens further sensitised cells to the toxic effects of bleomycin, despite displaying reduced bcl-2 signalling, and estradiol appeared to sensitise rather than protect cells from very high concentrations of bleomycin (fig 3.7.3 A).. The sensitivity of MCF-7(TamR) cells to bleomycin was entirely unaffected by estradiol, tamoxifen or faslodex (fig 3.7.3 B). This lack of correlation between bcl-2 expression and bleomycin sensitivity could be attributed to one of many other effects estradiol has on MCF-7 cells. The massive increase in growth rate (Katzenellenbogen et al., 1987) observed in MCF-7 cells treated with

oestradiol which could easily mask the expected change in bleomycin sensitivity, and the many other changes in intracellular signalling which occur upon treatment with oestrogens and antioestrogen will undoubtedly have affected the expression and activation of many other molecules in addition to bcl-2.

4.4.2 Modulation of bcl-2 expression using siRNA

In order to directly modulate bcl-2 signalling, without affecting other pathways concerned with proliferation or survival, an siRNA was used to knock down bcl-2 expression in MCF-7 cells. Initial experiments proved that the transfection procedure was traumatic to cells, with a severe reduction in growth rate being observed in both MCF-7 and MCF-7(TamR) cells following transfection with siRNA directed against bcl-2 or siRNA directed against the non-endogenous luciferase gene (fig 3.7.4). While the cells were undoubtedly affected by the transfection, both cell lines were affected to the same extent, suggesting that whatever is responsible for the difference in bleomycin sensitivity observed between MCF-7 and MCF-7(TamR) cells does not cause a difference in sensitivity to the transfection procedure. The toxicity of the luciferase siRNA transfection also shows that it is the transfection procedure, and not the lack of bcl-2 that is causing the reduction in cell number.

The transfection regime produced a knockdown of bcl-2 expression in MCF-7 cells that was not observed in MCF-7 cells treated with siRNA targeting luciferase. This demonstrates that the stress caused by the transfection procedure is not causing a reduction in bcl-2 levels as a response to the toxicity of the transfection.

When transfected and treated with bleomycin, bcl-2 siRNA treated MCF-7 cells displayed no more sensitivity than luciferase siRNA treated cells, despite having lower levels of bcl-2 (fig 3.7.6). While this seems to suggest that a reduction in bcl-2 is unlikely to be the cause of bleomycin sensitivity in MCF-7(TamR) cells, a number of points need to be considered

before the theory is dismissed. Firstly, the traumatic effect of the transfection on the cells may possibly affect the response of the cells to bleomycin. The observation that a luciferase-siRNA transfected MCF-7 cells displayed an entirely different sensitivity to bleomycin that that observed in normal MCF-7 cells suggest that the trauma of siRNA transfection causes a change in the cellular response to cytotoxics, and as such may obscure any changes in bleomycin sensitivity that bcl-2 knockdown may facilitate. A far less traumatic approach would be the development of a stably transfected cell lined derived from MCF-7(TamR) which inducibly expresses bcl-2. This would provide an extremely useful tool for further understanding the role of bcl-2 in bleomycin sensitivity.

4.5 Conclusions and further work.

The data obtained in the study suggests that the use of cytotoxics, in particular bleomycin may be a viable strategy for the treatment of tamoxifen resistant breast cancer. Current strategies proposed for the treatment of tamoxifen resistant breast cancer are generally either geared towards the blockade of alternative growth factor signalling pathways such as EGFR family members or insulin-like growth factor (Jones et al., 2004), or by attempting to create an 'oestrogen free' environment where no oestrogens or SERMs are present, or able to stimulate tumour growth. This can be achieved through the use of aromatase inhibitors to limit production of oestrogens, or through use of fulvestrant to destroy the oestrogen receptor reviewed in (Patel et al., 2007). The observed increased sensitivity of tamoxifen resistant breast cancer cells in culture represents a potential new cytotoxic approach to the treatment of tamoxifen resistant breast cancer which may accompany or replace those methods described above. In order to further develop bleomycin treatment as a plausible treatment for tamoxifen resistant breast cancer, further work will need to be undertaken to both properly understand the signalling changes which underpin the phenomenon, and to establish whether the same bleomycin sensitivity is observed in a clinical environment, and with a dose of bleomycin that is physiologically appropriate.

These data suggest that a change in the apoptotic signalling in tamoxifen resistant cells underpins the observed phenomenon of bleomycin sensitivity. Changes in the level of EGFR signalling, or in the rate of drug uptake do not seem to be responsible for the sensitivity of tamoxifen resistant cells to bleomycin. This could be demonstrated with further study into the extent of DSB formation by bleomycin using a more sensitive technique such as pulsed-field gel electrophoresis. Such a technique would also allow repair kinetics to be studied. It would be of great interest to learn if tamoxifen resistant cells are any less proficient at repairing their DSBs than their original tamoxifen sensitive cell lines.

The change in bleomycin sensitivity looks likely to be caused by a change in signalling molecules or apoptotic machinery lying downstream of the damage event. bcl-2 looks to be a good candidate, being downregulated in tamoxifen resistant cells and having a key role in the prevention of apoptosis, but attempts to alter levels of bcl-2 and examine the effect on bleomycin cytotoxicity did not show any clear correlation. Many bcl-2 family members have been shown to be under oestrogen regulation in certain cell types. A study in neurones has shown that the proapoptotic bcl-2 family member bim is downregulated by oestrogen signalling, while the anti-apoptotic bcl-2 family member bcl-w is upregulated (Yao et al., 2007). Studies with antioestrogens have also demonstrated downregulation of anti-, and upregulation of pro-apoptotic bcl-2 family members (Mcl-1 and BimS respectively) following tamoxifen treatment in myelomas (Gauduchon et al., 2005). It is quite possible that the downregulation of bcl-2 expression caused by siRNA or antioestrogen treatment is of little consequence to bleomycin sensitivity, and that it is changes in the expression of other bcl-2 family members that cause changes in the apoptotic machinery of tamoxifen resistant cells. A full scale study of the changes in expression of bcl-2 family members at both mRNA and protein level may reveal which bcl-2 family members are altered, and then further studies with inhibitors or siRNAs would allow candidate proteins to be firmly implicated. This may also allow a protein marker for tumours that are likely to be adversely affected by bleomycin to be identified.

Chapter 5
References.

Abbott, D.W., Thompson, M.E., Robinson-Benion, C., Tomlinson, G., Jensen, R.A. & Holt, J.T. (1999). BRCA1 expression restores radiation resistance in BRCA1-defective cancer cells through enhancement of transcription-coupled DNA repair. *J Biol Chem*, **274,** 18808-12.

Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X. & Akey, C.W. (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell*, **9,** 423-32.

Adachi, N., Ishino, T., Ishii, Y., Takeda, S. & Koyama, H. (2001). DNA ligase IV-deficient cells are more resistant to ionizing radiation in the absence of Ku70: Implications for DNA double-strand break repair. *Proc Natl Acad Sci U S A*, **98,** 12109-13.

Akhtar, S., Dunnion, D., Poyner, D., Ackroyd, J., Bibby, M. & Double, J. (2002). Sequence and chemistry requirements for a novel aptameric oligonucleotide inhibitor of EGF receptor tyrosine kinase activity. *Biochem Pharmacol*, **63,** 2187-95.

Akimoto, T., Hunter, N.R., Buchmiller, L., Mason, K., Ang, K.K. & Milas, L. (1999). Inverse relationship between epidermal growth factor receptor expression and radiocurability of murine carcinomas. *Clin Cancer Res*, **5,** 2884-90.

Al-Hazzaa, A., Bowen, I.D., Randerson, P. & Birchall, M.A. (2005). The effect of ZD1839 (Iressa), an epidermal growth factor receptor tyrosine kinase inhibitor, in combination with cisplatin, on apoptosis in SCC-15 cells. *Cell Prolif*, **38,** 77-86.

Alimonti, J.B., Shi, L., Baijal, P.K. & Greenberg, A.H. (2001). Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. *J Biol Chem*, **276,** 6974-82.

Allen, C., Halbrook, J. & Nickoloff, J.A. (2003). Interactive competition between homologous recombination and non-homologous end joining. *Mol Cancer Res*, **1,** 913-20.

Allen, J.D. & Schinkel, A.H. (2002). Multidrug resistance and pharmacological protection mediated by the breast cancer resistance protein (BCRP/ABCG2). *Mol Cancer Ther*, **1,** 427-34.

Ambudkar, S.V., Sauna, Z.E., Gottesman, M.M. & Szakacs, G. (2005). A novel way to spread drug resistance in tumor cells: functional intercellular transfer of P-glycoprotein (ABCB1). *Trends Pharmacol Sci*, **26,** 385-7.

Amorino, G.P., Mikkelsen, R.B., Valerie, K. & Schmidt-Ullrich, R.K. (2003). Dominant-negative cAMP-responsive element-binding protein inhibits proliferating cell nuclear antigen and DNA repair, leading to increased cellular radiosensitivity. *J Biol Chem*, **278,** 29394-9.

Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R. & Martinou, J.C. (1997). Inhibition of Bax channel-forming activity by Bcl-2. *Science*, **277,** 370-2.

Antonsson, B., Montessuit, S., Sanchez, B. & Martinou, J.C. (2001). Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem*, **276,** 11615-23.

Anzick, S.L., Kononen, J., Walker, R.L., Azorsa, D.O., Tanner, M.M., Guan, X.Y., Sauter, G., Kallioniemi, O.P., Trent, J.M. & Meltzer, P.S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science*, **277,** 965-8.

Aouida, M., Leduc, A., Wang, H. & Ramotar, D. (2004). Characterization of a transport and detoxification pathway for the antitumour drug bleomycin in Saccharomyces cerevisiae. *Biochem J*, **384,** 47-58.

Arcamone, F., Animati, F., Capranico, G., Lombardi, P., Pratesi, G., Manzini, S., Supino, R. & Zunino, F. (1997). New developments in antitumor anthracyclines. *Pharmacol Ther*, **76,** 117-24.

Arcamone, F., Cassinelli, G., Fantini, G., Grein, A., Orezzi, P., Pol, C. & Spalla, C. (1969). Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from S. peucetius var. caesius. *Biotechnol Bioeng*, **11,** 1101-10.

Assikis, V.J., Neven, P., Jordan, V.C. & Vergote, I. (1996). A realistic clinical perspective of tamoxifen and endometrial carcinogenesis. *Eur J Cancer*, **32A,** 1464-76.

Bae, S.S., Choi, J.H., Oh, Y.S., Perry, D.K., Ryu, S.H. & Suh, P.G. (2001). Proteolytic cleavage of epidermal growth factor receptor by caspases. *FEBS Lett*, **491,** 16-20.

Bandyopadhyay, D., Mandal, M., Adam, L., Mendelsohn, J. & Kumar, R. (1998). Physical interaction between epidermal growth factor receptor and DNA-dependent protein kinase in mammalian cells. *J Biol Chem*, **273,** 1568-73.

Barker, F.G., 2nd, Simmons, M.L., Chang, S.M., Prados, M.D., Larson, D.A., Sneed, P.K., Wara, W.M., Berger, M.S., Chen, P., Israel, M.A. & Aldape, K.D. (2001). EGFR overexpression and radiation response in glioblastoma multiforme. *Int J Radiat Oncol Biol Phys*, **51,** 410-8.

Baselga, J., Norton, L., Masui, H., Pandiella, A., Coplan, K., Miller, W.H., Jr. & Mendelsohn, J. (1993). Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. *J Natl Cancer Inst*, **85,** 1327-33.

Baulida, J., Kraus, M.H., Alimandi, M., Di Fiore, P.P. & Carpenter, G. (1996). All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J Biol Chem*, **271,** 5251-7.

Beatson, G. (1896). On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. *Lancet*, **ii,** 104-7.

Belenkov, A.I., Paiement, J.P., Panasci, L.C., Monia, B.P. & Chow, T.Y. (2002). An antisense oligonucleotide targeted to human Ku86 messenger RNA sensitizes M059K malignant glioma cells to ionizing radiation, bleomycin, and etoposide but not DNA cross-linking agents. *Cancer Res*, **62,** 5888-96.

Belinsky, M.G., Chen, Z.S., Shchaveleva, I., Zeng, H. & Kruh, G.D. (2002). Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). *Cancer Res*, **62,** 6172-7.

Benhar, M., Engelberg, D. & Levitzki, A. (2002). Cisplatin-induced activation of the EGF receptor. *Oncogene*, **21,** 8723-31.

Benz, C.C., Scott, G.K., Sarup, J.C., Johnson, R.M., Tripathy, D., Coronado, E., Shepard, H.M. & Osborne, C.K. (1993). Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res Treat*, **24,** 85-95.

Berman, E., Adams, M., Duigou-Osterndorf, R., Godfrey, L., Clarkson, B. & Andreeff, M. (1991). Effect of tamoxifen on cell lines displaying the multidrug-resistant phenotype. *Blood*, **77,** 818-25.

Bernstein, C., Bernstein, H., Payne, C.M. & Garewal, H. (2002). DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. *Mutat Res*, **511,** 145-78.

Britton, D.J., Hutcheson, I.R., Knowlden, J.M., Barrow, D., Giles, M., McClelland, R.A., Gee, J.M. & Nicholson, R.I. (2006). Bidirectional cross talk between ERalpha and EGFR signalling pathways regulates tamoxifen-resistant growth. *Breast Cancer Res Treat*, **96,** 131-46.

Bromberg, K.D., Burgin, A.B. & Osheroff, N. (2003). A two-drug model for etoposide action against human topoisomerase IIalpha. *J Biol Chem*, **278,** 7406-12.

Brooks, S.C., Locke, E.R. & Soule, H.D. (1973). Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. *J Biol Chem*, **248,** 6251-3.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J. & Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, **96,** 857-68.

Brunner, N., Frandsen, T.L., Holst-Hansen, C., Bei, M., Thompson, E.W., Wakeling, A.E., Lippman, M.E. & Clarke, R. (1993). MCF7/LCC2: a 4-hydroxytamoxifen resistant human breast cancer variant that retains sensitivity to the steroidal antiestrogen ICI 182,780. *Cancer Res*, **53,** 3229-32.

Bulun, S.E., Price, T.M., Aitken, J., Mahendroo, M.S. & Simpson, E.R. (1993). A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. *J Clin Endocrinol Metab*, **77,** 1622-8.

Bunone, G., Briand, P.A., Miksicek, R.J. & Picard, D. (1996). Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *Embo J*, **15,** 2174-83.

Callaghan, R. & Higgins, C.F. (1995). Interaction of tamoxifen with the multidrug resistance P-glycoprotein. *Br J Cancer*, **71,** 294-9.

Canbay, E. (2003). Erb-B2 homodimerization inhibits MUC1 transcription in cultured human mammary epithelial cells. *Cell Biol Int*, **27,** 477-81.

Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B. & Siliciano, J.D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*, **281,** 1677-9.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. & Reed, J.C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science*, **282,** 1318-21.

Carmichael, A.R. & Bates, T. (2004). Obesity and breast cancer: a review of the literature. *Breast*, **13,** 85-92.

Carpenter, G., Lembach, K.J., Morrison, M.M. & Cohen, S. (1975). Characterization of the binding of 125-I-labeled epidermal growth factor to human fibroblasts. *J Biol Chem*, **250,** 4297-304.

Carraway, K.L., Perez, A., Idris, N., Jepson, S., Arango, M., Komatsu, M., Haq, B., Price-Schiavi, S.A., Zhang, J. & Carraway, C.A. (2002).

Muc4/sialomucin complex, the intramembrane ErbB2 ligand, in cancer and epithelia: to protect and to survive. *Prog Nucleic Acid Res Mol Biol*, **71,** 149-85.

Castoria, G., Migliaccio, A., Bilancio, A., Di Domenico, M., de Falco, A., Lombardi, M., Fiorentino, R., Varricchio, L., Barone, M.V. & Auricchio, F. (2001). PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *Embo J*, **20,** 6050-9.

Chavez-MacGregor, M., Elias, S.G., Onland-Moret, N.C., van der Schouw, Y.T., Van Gils, C.H., Monninkhof, E., Grobbee, D.E. & Peeters, P.H. (2005). Postmenopausal breast cancer risk and cumulative number of menstrual cycles. *Cancer Epidemiol Biomarkers Prev*, **14,** 799-804.

Chen, Z., Yuhanna, I.S., Galcheva-Gargova, Z., Karas, R.H., Mendelsohn, M.E. & Shaul, P.W. (1999). Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest*, **103,** 401-6.

Cheng, E.H., Kirsch, D.G., Clem, R.J., Ravi, R., Kastan, M.B., Bedi, A., Ueno, K. & Hardwick, J.M. (1997). Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science*, **278,** 1966-8.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T. & Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell*, **8,** 705-11.

Chipuk, J.E., Kuwana, T., Bouchier-Hayes, L., Droin, N.M., Newmeyer, D.D., Schuler, M. & Green, D.R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science*, **303,** 1010-4.

Chou, J.J., Li, H., Salvesen, G.S., Yuan, J. & Wagner, G. (1999). Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell*, **96,** 615-24.

Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Pomatico, G., De Placido, S., Bianco, A.R. & Tortora, G. (2000). Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res*, **6,** 2053-63.

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem*, **237,** 1555-62.

Cohen, S., Ushiro, H., Stoscheck, C. & Chinkers, M. (1982). A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. *J Biol Chem*, **257,** 1523-31.

Cohen, S.S., Flaks, J.G., Barner, H.D., Loeb, M.R. & Lichtenstein, J. (1958). The Mode Of Action Of 5-Fluorouracil And Its Derivatives. *Proc Natl Acad Sci U S A*, **44,** 1004-12.

Cole, S.P., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M. & Deeley, R.G. (1992). Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science*, **258,** 1650-4.

Cole, S.P., Sparks, K.E., Fraser, K., Loe, D.W., Grant, C.E., Wilson, G.M. & Deeley, R.G. (1994). Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res*, **54,** 5902-10.

Contessa, J.N., Reardon, D.B., Todd, D., Dent, P., Mikkelsen, R.B., Valerie, K., Bowers, G.D. & Schmidt-Ullrich, R.K. (1999). The inducible expression of dominant-negative epidermal growth factor receptor-CD533 results in radiosensitization of human mammary carcinoma cells. *Clin Cancer Res*, **5,** 405-11.

Coopman, P., Garcia, M., Brunner, N., Derocq, D., Clarke, R. & Rochefort, H. (1994). Anti-proliferative and anti-estrogenic effects of ICI 164,384 and ICI 182,780 in 4-OH-tamoxifen-resistant human breast-cancer cells. *Int J Cancer*, **56,** 295-300.

Cosulich, S.C., Horiuchi, H., Zerial, M., Clarke, P.R. & Woodman, P.G. (1997). Cleavage of rabaptin-5 blocks endosome fusion during apoptosis. *Embo J*, **16,** 6182-91.

Coussens, L., Yang-Feng, T.L., Liao, Y.C., Chen, E., Gray, A., McGrath, J., Seeburg, P.H., Libermann, T.A., Schlessinger, J., Francke, U. & et al. (1985). Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science*, **230,** 1132-9.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. & Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, **91,** 231-41.

Delbaldo, C., Faivre, S. & Raymond, E. (2003). [Epidermal growth factor inhibitors]. *Rev Med Interne*, **24,** 372-83.

Dent, P., Reardon, D.B., Park, J.S., Bowers, G., Logsdon, C., Valerie, K. & Schmidt-Ullrich, R. (1999). Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. *Mol Biol Cell*, **10,** 2493-506.

Dickstein, B.M., Wosikowski, K. & Bates, S.E. (1995). Increased resistance to cytotoxic agents in ZR75B human breast cancer cells transfected

with epidermal growth factor receptor. *Mol Cell Endocrinol*, **110,** 205-11.

Diel, P., Smolnikar, K. & Michna, H. (1999). The pure antiestrogen ICI 182780 is more effective in the induction of apoptosis and down regulation of BCL-2 than tamoxifen in MCF-7 cells. *Breast Cancer Res Treat*, **58,** 87-97.

Dijkers, P.F., Medema, R.H., Lammers, J.W., Koenderman, L. & Coffer, P.J. (2000). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol*, **10,** 1201-4.

Dikic, I. (2003). Mechanisms controlling EGF receptor endocytosis and degradation. *Biochem Soc Trans*, **31,** 1178-81.

Dittmann, K., Mayer, C., Fehrenbacher, B., Schaller, M., Raju, U., Milas, L., Chen, D.J., Kehlbach, R. & Rodemann, H.P. (2005a). Radiation-induced epidermal growth factor receptor nuclear import is linked to activation of DNA-dependent protein kinase. *J Biol Chem*, **280,** 31182-9.

Dittmann, K., Mayer, C. & Rodemann, H.P. (2005b). Inhibition of radiation-induced EGFR nuclear import by C225 (Cetuximab) suppresses DNA-PK activity. *Radiother Oncol*, **76,** 157-61.

Dixit, M., Yang, J.L., Poirier, M.C., Price, J.O., Andrews, P.A. & Arteaga, C.L. (1997). Abrogation of cisplatin-induced programmed cell death in human breast cancer cells by epidermal growth factor antisense RNA. *J Natl Cancer Inst*, **89,** 365-73.

Dore, A.S., Drake, A.C., Brewerton, S.C. & Blundell, T.L. (2004). Identification of DNA-PK in the arthropods. Evidence for the ancient ancestry of vertebrate non-homologous end-joining. *DNA Repair (Amst)*, **3,** 33-41.

Doyle, L.A., Yang, W., Abruzzo, L.V., Krogmann, T., Gao, Y., Rishi, A.K. & Ross, D.D. (1998). A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A*, **95,** 15665-70.

Duan, R., Porter, W. & Safe, S. (1998). Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. *Endocrinology*, **139,** 1981-90.

Dumitrescu, R.G. & Cotarla, I. (2005). Understanding breast cancer risk -- where do we stand in 2005? *J Cell Mol Med*, **9,** 208-21.

El-Rayes, B.F. & LoRusso, P.M. (2004). Targeting the epidermal growth factor receptor. *Br J Cancer*, **91,** 418-24.

El-Zarruk, A.A. & van den Berg, H.W. (1999). The anti-proliferative effects of tyrosine kinase inhibitors towards tamoxifen-sensitive and tamoxifen-resistant human breast cancer cell lines in relation to the expression of epidermal growth factor receptors (EGF-R) and the inhibition of EGF-R tyrosine kinase. *Cancer Lett*, **142,** 185-93.

Eliopoulos, A.G., Kerr, D.J., Herod, J., Hodgkins, L., Krajewski, S., Reed, J.C. & Young, L.S. (1995). The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. *Oncogene*, **11,** 1217-28.

Ellis, A.G., Doherty, M.M., Walker, F., Weinstock, J., Nerrie, M., Vitali, A., Murphy, R., Johns, T.G., Scott, A.M., Levitzki, A., McLachlan, G., Webster, L.K., Burgess, A.W. & Nice, E.C. (2006). Preclinical analysis of the analinoquinazoline AG1478, a specific small molecule inhibitor of EGF receptor tyrosine kinase. *Biochem Pharmacol*, **71,** 1422-34.

Ellis, H.M. & Horvitz, H.R. (1986). Genetic control of programmed cell death in the nematode C. elegans. *Cell*, **44,** 817-29.

Eskes, R., Desagher, S., Antonsson, B. & Martinou, J.C. (2000). Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol*, **20,** 929-35.

Fan, S., Meng, Q., Gao, B., Grossman, J., Yadegari, M., Goldberg, I.D. & Rosen, E.M. (2000). Alcohol stimulates estrogen receptor signaling in human breast cancer cell lines. *Cancer Res*, **60,** 5635-9.

Fichtinger-Schepman, A.M., van der Veer, J.L., den Hartog, J.H., Lohman, P.H. & Reedijk, J. (1985). Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. *Biochemistry*, **24,** 707-13.

Finnie, N.J., Gottlieb, T.M., Blunt, T., Jeggo, P.A. & Jackson, S.P. (1995). DNA-dependent protein kinase activity is absent in xrs-6 cells: implications for site-specific recombination and DNA double-strand break repair. *Proc Natl Acad Sci U S A*, **92,** 320-4.

Finucane, D.M., Bossy-Wetzel, E., Waterhouse, N.J., Cotter, T.G. & Green, D.R. (1999). Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem*, **274,** 2225-33.

Fischer, O.M., Hart, S., Gschwind, A., Prenzel, N. & Ullrich, A. (2004). Oxidative and osmotic stress signaling in tumor cells is mediated by ADAM proteases and heparin-binding epidermal growth factor. *Mol Cell Biol*, **24,** 5172-83.

Fog, C.K., Christensen, I.J. & Lykkesfeldt, A.E. (2005). Characterization of a human breast cancer cell line, MCF-7/RU58R-1, resistant to the pure antiestrogen RU 58,668. *Breast Cancer Res Treat*, **91,** 133-44.

Folprecht, G., Lutz, M.P., Schoffski, P., Seufferlein, T., Nolting, A., Pollert, P. & Kohne, C.H. (2006). Cetuximab and irinotecan/5-fluorouracil/folinic acid is a safe combination for the first-line treatment of patients with epidermal growth factor receptor expressing metastatic colorectal carcinoma. *Ann Oncol*, **17,** 450-6.

Fresno Vara, J.A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C. & Gonzalez-Baron, M. (2004). PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev*, **30,** 193-204.

Friedenson, B. (2005). BRCA1 and BRCA2 pathways and the risk of cancers other than breast or ovarian. *MedGenMed*, **7,** 60.

Friedmann, B., Caplin, M., Hartley, J.A. & Hochhauser, D. (2004). Modulation of DNA repair in vitro after treatment with chemotherapeutic agents by the epidermal growth factor receptor inhibitor gefitinib (ZD1839). *Clin Cancer Res*, **10,** 6476-86.

Galcheva-Gargova, Z., Konstantinov, K.N., Wu, I.H., Klier, F.G., Barrett, T. & Davis, R.J. (1996). Binding of zinc finger protein ZPR1 to the epidermal growth factor receptor. *Science*, **272,** 1797-802.

Gauduchon, J., Gouilleux, F., Maillard, S., Marsaud, V., Renoir, J.M. & Sola, B. (2005). 4-Hydroxytamoxifen inhibits proliferation of multiple myeloma cells in vitro through down-regulation of c-Myc, up-regulation of p27Kip1, and modulation of Bcl-2 family members. *Clin Cancer Res*, **11,** 2345-54.

Gee, J., Hutcheson, I., Knowlden, J., Barrow, D., Harper, M.E., Jones, H.E., Wakeling, A.E. & Nicholson, R.I. (2001). The EGFR-selective tyrosine kinase inhibitor ZD1839 ("Iressa") is an effective inhibitor of tamoxifen resistant breast cancer growth. *Eur J Cancer*, **37,** S261.

Gell, D. & Jackson, S.P. (1999). Mapping of protein-protein interactions within the DNA-dependent protein kinase complex. *Nucleic Acids Res*, **27,** 3494-502.

Gewirtz, D.A. (1999). A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol*, **57,** 727-41.

Ginsberg, S.J. & Comis, R.L. (1982). The pulmonary toxicity of antineoplastic agents. *Semin Oncol*, **9,** 34-51.

Goldstein, N.I., Prewett, M., Zuklys, K., Rockwell, P. & Mendelsohn, J. (1995). Biological efficacy of a chimeric antibody to the epidermal

growth factor receptor in a human tumor xenograft model. *Clin Cancer Res*, **1,** 1311-8.

Grandage, V.L., Gale, R.E., Linch, D.C. & Khwaja, A. (2005). PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, Mapkinase and p53 pathways. *Leukemia*, **19,** 586-94.

Grant, C.E., Valdimarsson, G., Hipfner, D.R., Almquist, K.C., Cole, S.P. & Deeley, R.G. (1994). Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res*, **54,** 357-61.

Green, D.R. & Reed, J.C. (1998). Mitochondria and apoptosis. *Science*, **281,** 1309-12.

Gross, A., Jockel, J., Wei, M.C. & Korsmeyer, S.J. (1998). Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *Embo J*, **17,** 3878-85.

Grove, R.B., Reba, R.C., Eckelman, W.C. & Goodyear, M. (1974). Clinical evaluation of radiolabeled bleomycin (BLEO) for tumor detection. *J Nucl Med*, **15,** 386-90.

Guo, A., Marinaro, W., Hu, P. & Sinko, P.J. (2002). Delineating the contribution of secretory transporters in the efflux of etoposide using Madin-Darby canine kidney (MDCK) cells overexpressing P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP1), and canalicular multispecific organic anion transporter (cMOAT). *Drug Metab Dispos*, **30,** 457-63.

Guo, Y., Kotova, E., Chen, Z.S., Lee, K., Hopper-Borge, E., Belinsky, M.G. & Kruh, G.D. (2003). MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl)adenine. *J Biol Chem*, **278,** 29509-14.

Guzman, R.C., Yang, J., Rajkumar, L., Thordarson, G., Chen, X. & Nandi, S. (1999). Hormonal prevention of breast cancer: mimicking the protective effect of pregnancy. *Proc Natl Acad Sci U S A*, **96,** 2520-5.

Hackel, P.O., Zwick, E., Prenzel, N. & Ullrich, A. (1999). Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol*, **11,** 184-9.

Haga, S., Hinoshita, E., Ikezaki, K., Fukui, M., Scheffer, G.L., Uchiumi, T. & Kuwano, M. (2001). Involvement of the multidrug resistance protein 3 in drug sensitivity and its expression in human glioma. *Jpn J Cancer Res*, **92,** 211-9.

Haigler, H.T., McKanna, J.A. & Cohen, S. (1979). Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J Cell Biol*, **81,** 382-95.

Hall, J.M., Lee, M.K., Newman, B., Morrow, J.E., Anderson, L.A., Huey, B. & King, M.C. (1990). Linkage of early-onset familial breast cancer to chromosome 17q21. *Science*, **250,** 1684-9.

Hamadeh, M.J., Devries, M.C. & Tarnopolsky, M.A. (2005). Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. *J Clin Endocrinol Metab*, **90,** 3592-9.

Han, J., Sabbatini, P., Perez, D., Rao, L., Modha, D. & White, E. (1996a). The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev*, **10,** 461-77.

Han, Y., Caday, C.G., Nanda, A., Cavenee, W.K. & Huang, H.J. (1996b). Tyrphostin AG 1478 preferentially inhibits human glioma cells expressing truncated rather than wild-type epidermal growth factor receptors. *Cancer Res*, **56,** 3859-61.

Harari, P.M. (2004). Epidermal growth factor receptor inhibition strategies in oncology. *Endocr Relat Cancer*, **11,** 689-708.

Harkin, D.P., Bean, J.M., Miklos, D., Song, Y.H., Truong, V.B., Englert, C., Christians, F.C., Ellisen, L.W., Maheswaran, S., Oliner, J.D. & Haber, D.A. (1999). Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. *Cell*, **97,** 575-86.

Haugh, J.M., Schooler, K., Wells, A., Wiley, H.S. & Lauffenburger, D.A. (1999). Effect of epidermal growth factor receptor internalization on regulation of the phospholipase C-gamma1 signaling pathway. *J Biol Chem*, **274,** 8958-65.

Hengartner, M.O., Ellis, R.E. & Horvitz, H.R. (1992). Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. *Nature*, **356,** 494-9.

Herbst, R.S. (2004). Review of epidermal growth factor receptor biology. *Int J Radiat Oncol Biol Phys*, **59,** 21-6.

Hinoshita, E., Uchiumi, T., Taguchi, K., Kinukawa, N., Tsuneyoshi, M., Maehara, Y., Sugimachi, K. & Kuwano, M. (2000). Increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein 2, in human colorectal carcinomas. *Clin Cancer Res*, **6,** 2401-7.

Hiscox, S., Jiang, W.G., Obermeier, K., Taylor, K., Morgan, L., Burmi, R., Barrow, D. & Nicholson, R.I. (2006). Tamoxifen resistance in MCF7

cells promotes EMT-like behaviour and involves modulation of beta-catenin phosphorylation. *Int J Cancer*, **118,** 290-301.

Hiscox, S., Morgan, L., Green, T.P., Barrow, D., Gee, J. & Nicholson, R.I. (2005). Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. *Breast Cancer Res Treat*, 1-12.

Holbro, T., Beerli, R.R., Maurer, F., Koziczak, M., Barbas, C.F., 3rd & Hynes, N.E. (2003). The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci U S A*, **100,** 8933-8.

Hollenberg, M.D. & Cuatrecasas, P. (1975). Insulin and epidermal growth factor. Human fibroblast receptors related to deoxyribonucleic acid synthesis and amino acid uptake. *J Biol Chem*, **250,** 3845-53.

Howell, A., Robertson, J.F., Quaresma Albano, J., Aschermannova, A., Mauriac, L., Kleeberg, U.R., Vergote, I., Erikstein, B., Webster, A. & Morris, C. (2002). Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol*, **20,** 3396-403.

Huang, D.C. & Strasser, A. (2000). BH3-Only proteins-essential initiators of apoptotic cell death. *Cell*, **103,** 839-42.

Huang, H.S., Nagane, M., Klingbeil, C.K., Lin, H., Nishikawa, R., Ji, X.D., Huang, C.M., Gill, G.N., Wiley, H.S. & Cavenee, W.K. (1997a). The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J Biol Chem*, **272,** 2927-35.

Huang, L.C., Clarkin, K.C. & Wahl, G.M. (1996). Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest. *Proc Natl Acad Sci U S A*, **93,** 4827-32.

Huang, S., Armstrong, E.A., Benavente, S., Chinnaiyan, P. & Harari, P.M. (2004). Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): combining anti-EGFR antibody with tyrosine kinase inhibitor. *Cancer Res*, **64,** 5355-62.

Huang, S.M. & Harari, P.M. (2000). Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin Cancer Res*, **6,** 2166-74.

Huang, Z., Hankinson, S.E., Colditz, G.A., Stampfer, M.J., Hunter, D.J., Manson, J.E., Hennekens, C.H., Rosner, B., Speizer, F.E. & Willett,

W.C. (1997b). Dual effects of weight and weight gain on breast cancer risk. *Jama*, **278,** 1407-11.

Hughes, E.N., Engelsberg, B.N. & Billings, P.C. (1992). Purification of nuclear proteins that bind to cisplatin-damaged DNA. Identity with high mobility group proteins 1 and 2. *J Biol Chem*, **267,** 13520-7.

Hussain, M., Beale, G., Hughes, M. & Akhtar, S. (2002). Co-delivery of an antisense oligonucleotide and 5-fluorouracil using sustained release poly (lactide-co-glycolide) microsphere formulations for potential combination therapy in cancer. *Int J Pharm*, **234,** 129-38.

Ingraham, H.A., Tseng, B.Y. & Goulian, M. (1980). Mechanism for exclusion of 5-fluorouracil from DNA. *Cancer Res*, **40,** 998-1001.

Jackson, S.P. & Jeggo, P.A. (1995). DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK. *Trends Biochem Sci*, **20,** 412-5.

Jackson, T.A., Richer, J.K., Bain, D.L., Takimoto, G.S., Tung, L. & Horwitz, K.B. (1997). The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. *Mol Endocrinol*, **11,** 693-705.

Jamieson, E.R. & Lippard, S.J. (1999). Structure, Recognition, and Processing of Cisplatin-DNA Adducts. *Chem Rev*, **99,** 2467-98.

Janicke, R.U., Sprengart, M.L., Wati, M.R. & Porter, A.G. (1998). Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem*, **273,** 9357-60.

Jensen, E. & Jacobson, H. (1960). Basic guides to the mechanism of estrogen action. *Recent Prog Horm Res*, 387-414.

Joensuu, H., Ejlertsen, B., Lonning, P.E. & Rutqvist, L.E. (2005). Aromatase inhibitors in the treatment of early and advanced breast cancer. *Acta Oncol*, **44,** 23-31.

Johnson, S.W., Perez, R.P., Godwin, A.K., Yeung, A.T., Handel, L.M., Ozols, R.F. & Hamilton, T.C. (1994). Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. *Biochem Pharmacol*, **47,** 689-97.

Jones, H.E., Goddard, L., Gee, J.M., Hiscox, S., Rubini, M., Barrow, D., Knowlden, J.M., Williams, S., Wakeling, A.E. & Nicholson, R.I. (2004). Insulin-like growth factor-I receptor signalling and acquired resistance to gefitinib (ZD1839; Iressa) in human breast and prostate cancer cells. *Endocr Relat Cancer*, **11,** 793-814.

Jones, S.L., Hickson, I.D., Harris, A.L. & Harnett, P.R. (1994). Repair of cisplatin-DNA adducts by protein extracts from human ovarian carcinoma. *Int J Cancer*, **59,** 388-93.

Jordan, N.J., Gee, J.M., Barrow, D., Wakeling, A.E. & Nicholson, R.I. (2004). Increased constitutive activity of PKB/Akt in tamoxifen resistant breast cancer MCF-7 cells. *Breast Cancer Res Treat*, **87,** 167-80.

Juliano, R.L. & Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta*, **455,** 152-62.

Juo, P., Woo, M.S., Kuo, C.J., Signorelli, P., Biemann, H.P., Hannun, Y.A. & Blenis, J. (1999). FADD is required for multiple signaling events downstream of the receptor Fas. *Cell Growth Differ*, **10,** 797-804.

Jurgensmeier, J.M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D. & Reed, J.C. (1998). Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci U S A*, **95,** 4997-5002.

Karran, P. (2000). DNA double strand break repair in mammalian cells. *Curr Opin Genet Dev*, **10,** 144-50.

Karunagaran, D., Tzahar, E., Beerli, R.R., Chen, X., Graus-Porta, D., Ratzkin, B.J., Seger, R., Hynes, N.E. & Yarden, Y. (1996). ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *Embo J*, **15,** 254-64.

Kashishian, A., Douangpanya, H., Clark, D., Schlachter, S.T., Eary, C.T., Schiro, J.G., Huang, H., Burgess, L.E., Kesicki, E.A. & Halbrook, J. (2003). DNA-dependent protein kinase inhibitors as drug candidates for the treatment of cancer. *Mol Cancer Ther*, **2,** 1257-64.

Katzenellenbogen, B.S., Kendra, K.L., Norman, M.J. & Berthois, Y. (1987). Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res*, **47,** 4355-60.

Kennedy, S.G., Kandel, E.S., Cross, T.K. & Hay, N. (1999). Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. *Mol Cell Biol*, **19,** 5800-10.

Kim, C.H., Park, S.J. & Lee, S.H. (2002). A targeted inhibition of DNA-dependent protein kinase sensitizes breast cancer cells following ionizing radiation. *J Pharmacol Exp Ther*, **303,** 753-9.

Kim, H.E., Du, F., Fang, M. & Wang, X. (2005). Formation of apoptosome is initiated by cytochrome c-induced dATP hydrolysis and subsequent nucleotide exchange on Apaf-1. *Proc Natl Acad Sci U S A*, **102,** 17545-50.

Kim, H.P., Lee, J.Y., Jeong, J.K., Bae, S.W., Lee, H.K. & Jo, I. (1999). Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. *Biochem Biophys Res Commun*, **263,** 257-62.

Kim, S., Prichard, C.N., Younes, M.N., Yazici, Y.D., Jasser, S.A., Bekele, B.N. & Myers, J.N. (2006). Cetuximab and irinotecan interact synergistically to inhibit the growth of orthotopic anaplastic thyroid carcinoma xenografts in nude mice. *Clin Cancer Res*, **12,** 600-7.

King, C.R., Kasprzyk, P.G., Fischer, P.H., Bird, R.E. & Turner, N.A. (1996). Preclinical testing of an anti-erbB-2 recombinant toxin. *Breast Cancer Res Treat*, **38,** 19-25.

Kirkin, V., Joos, S. & Zornig, M. (2004). The role of Bcl-2 family members in tumorigenesis. *Biochim Biophys Acta*, **1644,** 229-49.

Klapper, L.N., Vaisman, N., Hurwitz, E., Pinkas-Kramarski, R., Yarden, Y. & Sela, M. (1997). A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. *Oncogene*, **14,** 2099-109.

Klapper, L.N., Waterman, H., Sela, M. & Yarden, Y. (2000). Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer Res*, **60,** 3384-8.

Klein-Hitpass, L., Schorpp, M., Wagner, U. & Ryffel, G.U. (1986). An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. *Cell*, **46,** 1053-61.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R. & Newmeyer, D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*, **275,** 1132-6.

Knowlden, J.M., Hutcheson, I.R., Jones, H.E., Madden, T., Gee, J.M., Harper, M.E., Barrow, D., Wakeling, A.E. & Nicholson, R.I. (2003). Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology*, **144,** 1032-44.

Koike, M. (2002). Dimerization, translocation and localization of Ku70 and Ku80 proteins. *J Radiat Res (Tokyo)*, **43,** 223-36.

Koizumi, F., Kanzawa, F., Ueda, Y., Koh, Y., Tsukiyama, S., Taguchi, F., Tamura, T., Saijo, N. & Nishio, K. (2004). Synergistic interaction between the EGFR tyrosine kinase inhibitor gefitinib ("Iressa") and the DNA topoisomerase I inhibitor CPT-11 (irinotecan) in human colorectal cancer cells. *Int J Cancer*, **108,** 464-72.

Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T.J., Kirschner, M.W., Koths, K., Kwiatkowski, D.J. & Williams, L.T. (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science*, **278,** 294-8.

Kraus, M.H., Issing, W., Miki, T., Popescu, N.C. & Aaronson, S.A. (1989). Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. *Proc Natl Acad Sci U S A*, **86,** 9193-7.

Krishnamachary, N. & Center, M.S. (1993). The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. *Cancer Res*, **53,** 3658-61.

Kruh, G.D. & Belinsky, M.G. (2003). The MRP family of drug efflux pumps. *Oncogene*, **22,** 7537-52.

Kuhne, M., Riballo, E., Rief, N., Rothkamm, K., Jeggo, P.A. & Lobrich, M. (2004). A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity. *Cancer Res*, **64,** 500-8.

Kuiper, G.G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. & Gustafsson, J.A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology*, **138,** 863-70.

Kumar, V. & Chambon, P. (1988). The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell*, **55,** 145-56.

Kuo, M.T. (1981). Preferential damage of active chromatin by bleomycin. *Cancer Res*, **41,** 2439-43.

Kuwana, T., Smith, J.J., Muzio, M., Dixit, V., Newmeyer, D.D. & Kornbluth, S. (1998). Apoptosis induction by caspase-8 is amplified through the mitochondrial release of cytochrome c. *J Biol Chem*, **273,** 16589-94.

Larsen, S.S., Heiberg, I. & Lykkesfeldt, A.E. (2001). Anti-oestrogen resistant human breast cancer cell lines are more sensitive towards treatment with the vitamin D analogue EB1089 than parent MCF-7 cells. *Br J Cancer*, **84,** 686-90.

Lavie, Y., Cao, H., Volner, A., Lucci, A., Han, T.Y., Geffen, V., Giuliano, A.E. & Cabot, M.C. (1997). Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. *J Biol Chem*, **272,** 1682-7.

Lee, K.J., Jovanovic, M., Udayakumar, D., Bladen, C.L. & Dynan, W.S. (2004). Identification of DNA-PKcs phosphorylation sites in XRCC4

and effects of mutations at these sites on DNA end joining in a cell-free system. *DNA Repair (Amst)*, **3,** 267-76.

Lees-Miller, S.P. & Meek, K. (2003). Repair of DNA double strand breaks by non-homologous end joining. *Biochimie*, **85,** 1161-73.

Levchenko, A., Mehta, B.M., Niu, X., Kang, G., Villafania, L., Way, D., Polycarpe, D., Sadelain, M. & Larson, S.M. (2005). Intercellular transfer of P-glycoprotein mediates acquired multidrug resistance in tumor cells. *Proc Natl Acad Sci U S A*, **102,** 1933-8.

Levin, E.R. (1999). Cellular Functions of the Plasma Membrane Estrogen Receptor. *Trends Endocrinol Metab*, **10,** 374-377.

Li, H., Zhu, H., Xu, C.J. & Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*, **94,** 491-501.

Liang, K., Ang, K.K., Milas, L., Hunter, N. & Fan, Z. (2003). The epidermal growth factor receptor mediates radioresistance. *Int J Radiat Oncol Biol Phys*, **57,** 246-54.

Lieber, M.R., Ma, Y., Pannicke, U. & Schwarz, K. (2003). Mechanism and regulation of human non-homologous DNA end-joining. *Nat Rev Mol Cell Biol*, **4,** 712-20.

Lim, K.B., Ng, C.Y., Ong, C.K., Ong, C.S., Tran, E., Nguyen, T.T., Chan, G.M. & Huynh, H. (2001). Induction of apoptosis in mammary gland by a pure anti-estrogen ICI 182780. *Breast Cancer Res Treat*, **68,** 127-38.

Lin, W.C., Lin, F.T. & Nevins, J.R. (2001). Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation. *Genes Dev*, **15,** 1833-44.

Lippman, M., Bolan, G. & Huff, K. (1976). The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. *Cancer Res*, **36,** 4595-601.

Lips, J. & Kaina, B. (2001). DNA double-strand breaks trigger apoptosis in p53-deficient fibroblasts. *Carcinogenesis*, **22,** 579-85.

Liu, D., Aguirre Ghiso, J., Estrada, Y. & Ossowski, L. (2002). EGFR is a transducer of the urokinase receptor initiated signal that is required for in vivo growth of a human carcinoma. *Cancer Cell*, **1,** 445-57.

Liu, L.F. (1989). DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem*, **58,** 351-75.

Liu, M.L., Xu, X., Rang, W.Q., Li, Y.J. & Song, H.P. (2004). Influence of ovariectomy and 17beta-estradiol treatment on insulin sensitivity,

lipid metabolism and post-ischemic cardiac function. *Int J Cardiol*, **97,** 485-93.

Long, B.H., Musial, S.T. & Brattain, M.G. (1985). Single- and double-strand DNA breakage and repair in human lung adenocarcinoma cells exposed to etoposide and teniposide. *Cancer Res*, **45,** 3106-12.

Lorkowski, S. & Cullen, P. (2002). ABCG Subfamily of human ATP-binding cassette proteins. *Pure Appl Chem*, **74,** 2057-2081.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A. & Jacks, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362,** 847-9.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C. & Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell*, **94,** 481-90.

Macklon, N.S., Stouffer, R.L., Giudice, L.C. & Fauser, B.C. (2006). The science behind 25 years of ovarian stimulation for IVF. *Endocr Rev*.

MacMahon, B., Cole, P., Lin, T.M., Lowe, C.R., Mirra, A.P., Ravnihar, B., Salber, E.J., Valaoras, V.G. & Yuasa, S. (1970). Age at first birth and breast cancer risk. *Bull World Health Organ*, **43,** 209-21.

Marmorstein, L.Y., Ouchi, T. & Aaronson, S.A. (1998). The BRCA2 gene product functionally interacts with p53 and RAD51. *Proc Natl Acad Sci U S A*, **95,** 13869-74.

Marsden, V.S., O'Connor, L., O'Reilly, L.A., Silke, J., Metcalf, D., Ekert, P.G., Huang, D.C., Cecconi, F., Kuida, K., Tomaselli, K.J., Roy, S., Nicholson, D.W., Vaux, D.L., Bouillet, P., Adams, J.M. & Strasser, A. (2002). Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature*, **419,** 634-7.

Marshall, F. & Jolley, W. (1906). The ovary as an organ of internal secretion. *Phil Trans R Soc*, 91-141.

Mayo, L.D., Turchi, J.J. & Berberich, S.J. (1997). Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res*, **57,** 5013-6.

McGrath, T. & Center, M.S. (1987). Adriamycin resistance in HL60 cells in the absence of detectable P-glycoprotein. *Biochem Biophys Res Commun*, **145,** 1171-6.

Mei, Y.P., Zhou, J.M., Wang, Y., Huang, H., Deng, R., Feng, G.K., Zeng, Y.X. & Zhu, X.F. (2007). Silencing of LMP1 induces cell cycle arrest and enhances chemosensitivity through inhibition of AKT signaling

pathway in EBV-positive nasopharyngeal carcinoma cells. *Cell Cycle*, **6,** 1379-85.

Meier, R. & Hemmings, B.A. (1999). Regulation of protein kinase B. *J Recept Signal Transduct Res*, **19,** 121-8.

Merot, Y., Metivier, R., Penot, G., Manu, D., Saligaut, C., Gannon, F., Pakdel, F., Kah, O. & Flouriot, G. (2004). The relative contribution exerted by AF-1 and AF-2 transactivation functions in estrogen receptor alpha transcriptional activity depends upon the differentiation stage of the cell. *J Biol Chem*, **279,** 26184-91.

Meves, A., Stock, S.N., Beyerle, A., Pittelkow, M.R. & Peus, D. (2001). H(2)O(2) mediates oxidative stress-induced epidermal growth factor receptor phosphorylation. *Toxicol Lett*, **122,** 205-14.

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E. & Auricchio, F. (1996). Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *Embo J*, **15,** 1292-300.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W. & et al. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*, **266,** 66-71.

Milner, J., Ponder, B., Hughes-Davies, L., Seltmann, M. & Kouzarides, T. (1997). Transcriptional activation functions in BRCA2. *Nature*, **386,** 772-3.

Mir, L.M., Tounekti, O. & Orlowski, S. (1996). Bleomycin: revival of an old drug. *Gen Pharmacol*, **27,** 745-8.

Miyashita, T., Harigai, M., Hanada, M. & Reed, J.C. (1994a). Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res*, **54,** 3131-5.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B. & Reed, J.C. (1994b). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene*, **9,** 1799-805.

Modjtahedi, H., Komurasaki, T., Toyoda, H. & Dean, C. (1998). Anti-EGFR monoclonal antibodies which act as EGF, TGF alpha, HB-EGF and BTC antagonists block the binding of epiregulin to EGFR-expressing tumours. *Int J Cancer*, **75,** 310-6.

Monaghan, P., Robertson, D., Amos, T.A., Dyer, M.J., Mason, D.Y. & Greaves, M.F. (1992). Ultrastructural localization of bcl-2 protein. *J Histochem Cytochem*, **40,** 1819-25.

Moras, D. & Gronemeyer, H. (1998). The nuclear receptor ligand-binding domain: structure and function. *Curr Opin Cell Biol*, **10,** 384-91.

Mosselman, S., Polman, J. & Dijkema, R. (1996). ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett*, **392,** 49-53.

Nagane, M., Levitzki, A., Gazit, A., Cavenee, W.K. & Huang, H.J. (1998). Drug resistance of human glioblastoma cells conferred by a tumor-specific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3-like proteases. *Proc Natl Acad Sci U S A*, **95,** 5724-9.

Nagane, M., Narita, Y., Mishima, K., Levitzki, A., Burgess, A.W., Cavenee, W.K. & Huang, H.J. (2001). Human glioblastoma xenografts overexpressing a tumor-specific mutant epidermal growth factor receptor sensitized to cisplatin by the AG1478 tyrosine kinase inhibitor. *J Neurosurg*, **95,** 472-9.

Nagata, S. & Golstein, P. (1995). The Fas death factor. *Science*, **267,** 1449-56.

Nagy, P., Arndt-Jovin, D.J. & Jovin, T.M. (2003). Small interfering RNAs suppress the expression of endogenous and GFP-fused epidermal growth factor receptor (erbB1) and induce apoptosis in erbB1-overexpressing cells. *Exp Cell Res*, **285,** 39-49.

Nakata, E., Hunter, N., Mason, K., Fan, Z., Ang, K.K. & Milas, L. (2004). C225 antiepidermal growth factor receptor antibody enhances the efficacy of docetaxel chemoradiotherapy. *Int J Radiat Oncol Biol Phys*, **59,** 1163-73.

Nicholson, R.I. & Johnston, S.R. (2005). Endocrine therapy--current benefits and limitations. *Breast Cancer Res Treat*, **93 Suppl 1,** S3-10.

Nicholson, R.I., McClelland, R.A., Finlay, P., Eaton, C.L., Gullick, W.J., Dixon, A.R., Robertson, J.F., Ellis, I.O. & Blamey, R.W. (1993). Relationship between EGF-R, c-erbB-2 protein expression and Ki67 immunostaining in breast cancer and hormone sensitivity. *Eur J Cancer*, **29A,** 1018-23.

O'Malley, B.W., McGuire, W.L. & Middleton, P.A. (1968). Altered gene expression during differentiation: population changes in hybridizable RNA after stimulation of the chick oviduct with oestrogen. *Nature*, **218,** 1249-51.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T. & Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*, **288,** 1053-8.

Ohmichi, M., Tasaka, K., Kurachi, H. & Murata, Y. (2005). Molecular mechanism of action of selective estrogen receptor modulator in target tissues. *Endocr J*, **52,** 161-7.

Okayasu, R., Suetomi, K., Yu, Y., Silver, A., Bedford, J.S., Cox, R. & Ullrich, R.L. (2000). A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse. *Cancer Res*, **60,** 4342-5.

Olayioye, M.A., Neve, R.M., Lane, H.A. & Hynes, N.E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. *Embo J*, **19,** 3159-67.

Osaku, M., Ueda, M., Ando, N., Shinozawa, Y., Hirota, N., Shimizu, N. & Abe, O. (1991). Targeted killing of squamous carcinoma cells by a monoclonal antibody-peplomycin conjugate which recognizes the EGF receptor. *Anticancer Res*, **11,** 1951-6.

Osaku, M., Ueda, M., Miyakawa, T., Toyoda, H., Uesato, K., Yamada, Y., Asanuma, F., Ando, N., Ozawa, S., Kitagawa, Y. & Kitajima, M. (2001). Correlation between EGF receptor expression and peplomycin cytotoxicity in squamous cell carcinoma cell lines. *Oncol Rep*, **8,** 855-60.

Osborne, C.K. & Schiff, R. (2005). Aromatase inhibitors: future directions. *J Steroid Biochem Mol Biol*, **95,** 183-7.

Pace, P., Taylor, J., Suntharalingam, S., Coombes, R.C. & Ali, S. (1997). Human estrogen receptor beta binds DNA in a manner similar to and dimerizes with estrogen receptor alpha. *J Biol Chem*, **272,** 25832-8.

Pal, T., Permuth-Wey, J., Betts, J.A., Krischer, J.P., Fiorica, J., Arango, H., LaPolla, J., Hoffman, M., Martino, M.A., Wakeley, K., Wilbanks, G., Nicosia, S., Cantor, A. & Sutphen, R. (2005). BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases. *Cancer*, **104,** 2807-16.

Pan, G., Ni, J., Wei, Y.F., Yu, G., Gentz, R. & Dixit, V.M. (1997a). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science*, **277,** 815-8.

Pan, G., O'Rourke, K., Chinnaiyan, A.M., Gentz, R., Ebner, R., Ni, J. & Dixit, V.M. (1997b). The receptor for the cytotoxic ligand TRAIL. *Science*, **276,** 111-3.

Park, S.J., Armstrong, S., Kim, C.H., Yu, M., Robertson, K., Kelley, M.R. & Lee, S.H. (2005). Lack of EGF receptor contributes to drug sensitivity of human germline cells. *Br J Cancer*, **92,** 334-41.

Parker, R.J., Eastman, A., Bostick-Bruton, F. & Reed, E. (1991). Acquired cisplatin resistance in human ovarian cancer cells is associated with

enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. *J Clin Invest*, **87,** 772-7.

Partik, G., Hochegger, K., Schorkhuber, M. & Marian, B. (1999). Inhibition of epidermal-growth-factor-receptor-dependent signalling by tyrphostins A25 and AG1478 blocks growth and induces apoptosis in colorectal tumor cells in vitro. *J Cancer Res Clin Oncol*, **125,** 379-88.

Patel, R.R., Sharma, C.G. & Jordan, V.C. (2007). Optimizing the antihormonal treatment and prevention of breast cancer. *Breast Cancer*, **14,** 113-22.

Pegram, M.D., Lipton, A., Hayes, D.F., Weber, B.L., Baselga, J.M., Tripathy, D., Baly, D., Baughman, S.A., Twaddell, T., Glaspy, J.A. & Slamon, D.J. (1998). Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J Clin Oncol*, **16,** 2659-71.

Pei, Z., Calmels, T.P., Creutz, C.E. & Sebti, S.M. (1995). Yeast cysteine proteinase gene ycp1 induces resistance to bleomycin in mammalian cells. *Mol Pharmacol*, **48,** 676-81.

Perillo, B., Sasso, A., Abbondanza, C. & Palumbo, G. (2000). 17beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. *Mol Cell Biol*, **20,** 2890-901.

Petros, A.M., Medek, A., Nettesheim, D.G., Kim, D.H., Yoon, H.S., Swift, K., Matayoshi, E.D., Oltersdorf, T. & Fesik, S.W. (2001). Solution structure of the antiapoptotic protein bcl-2. *Proc Natl Acad Sci U S A*, **98,** 3012-7.

Petros, A.M., Olejniczak, E.T. & Fesik, S.W. (2004). Structural biology of the Bcl-2 family of proteins. *Biochim Biophys Acta*, **1644,** 83-94.

Pfahl, M. (1993). Nuclear receptor/AP-1 interaction. *Endocr Rev*, **14,** 651-8.

Pietras, R.J., Poen, J.C., Gallardo, D., Wongvipat, P.N., Lee, H.J. & Slamon, D.J. (1999). Monoclonal antibody to HER-2/neureceptor modulates repair of radiation-induced DNA damage and enhances radiosensitivity of human breast cancer cells overexpressing this oncogene. *Cancer Res*, **59,** 1347-55.

Plowman, G.D., Culouscou, J.M., Whitney, G.S., Green, J.M., Carlton, G.W., Foy, L., Neubauer, M.G. & Shoyab, M. (1993). Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc Natl Acad Sci U S A*, **90,** 1746-50.

Poddevin, B., Belehradek, J., Jr. & Mir, L.M. (1990). Stable [57Co]-bleomycin complex with a very high specific radioactivity for use at very low concentrations. *Biochem Biophys Res Commun*, **173,** 259-64.

Poddevin, B., Orlowski, S., Belehradek, J., Jr. & Mir, L.M. (1991). Very high cytotoxicity of bleomycin introduced into the cytosol of cells in culture. *Biochem Pharmacol*, **42 Suppl,** S67-75.

Pratt, W.B. & Toft, D.O. (1997). Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev*, **18,** 306-60.

Premkumar, D.R., Arnold, B. & Pollack, I.F. (2006). Cooperative inhibitory effect of ZD1839 (Iressa) in combination with 17-AAG on glioma cell growth. *Mol Carcinog*, **45,** 288-301.

Pron, G., Belehradek, J., Jr. & Mir, L.M. (1993). Identification of a plasma membrane protein that specifically binds bleomycin. *Biochem Biophys Res Commun*, **194,** 333-7.

Pron, G., Mahrour, N., Orlowski, S., Tounekti, O., Poddevin, B., Belehradek, J., Jr. & Mir, L.M. (1999). Internalisation of the bleomycin molecules responsible for bleomycin toxicity: a receptor-mediated endocytosis mechanism. *Biochem Pharmacol*, **57,** 45-56.

Pu, Y.S., Hsieh, M.W., Wang, C.W., Liu, G.Y., Huang, C.Y., Lin, C.C., Guan, J.Y., Lin, S.R. & Hour, T.C. (2006). Epidermal growth factor receptor inhibitor (PD168393) potentiates cytotoxic effects of paclitaxel against androgen-independent prostate cancer cells. *Biochem Pharmacol*, **71,** 751-60.

Pugazhenthi, S., Nesterova, A., Sable, C., Heidenreich, K.A., Boxer, L.M., Heasley, L.E. & Reusch, J.E. (2000). Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem*, **275,** 10761-6.

Ramu, A., Glaubiger, D. & Fuks, Z. (1984). Reversal of acquired resistance to doxorubicin in P388 murine leukemia cells by tamoxifen and other triparanol analogues. *Cancer Res*, **44,** 4392-5.

Ranson, M. (2004). Epidermal growth factor receptor tyrosine kinase inhibitors. *Br J Cancer*, **90,** 2250-5.

Ring, A. & Dowsett, M. (2004). Mechanisms of tamoxifen resistance. *Endocr Relat Cancer*, **11,** 643-58.

Robertson, K.A., Bullock, H.A., Xu, Y., Tritt, R., Zimmerman, E., Ulbright, T.M., Foster, R.S., Einhorn, L.H. & Kelley, M.R. (2001). Altered expression of Ape1/ref-1 in germ cell tumors and overexpression in

NT2 cells confers resistance to bleomycin and radiation. *Cancer Res*, **61,** 2220-5.

Robey, R.W., Honjo, Y., Morisaki, K., Nadjem, T.A., Runge, S., Risbood, M., Poruchynsky, M.S. & Bates, S.E. (2003). Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. *Br J Cancer*, **89,** 1971-8.

Rodrigues, G.A., Falasca, M., Zhang, Z., Ong, S.H. & Schlessinger, J. (2000). A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. *Mol Cell Biol*, **20,** 1448-59.

Rojas, E., Lopez, M.C. & Valverde, M. (1999). Single cell gel electrophoresis assay: methodology and applications. *J Chromatogr B Biomed Sci Appl*, **722,** 225-54.

Rooney, S., Alt, F.W., Lombard, D., Whitlow, S., Eckersdorff, M., Fleming, J., Fugmann, S., Ferguson, D.O., Schatz, D.G. & Sekiguchi, J. (2003). Defective DNA repair and increased genomic instability in Artemis-deficient murine cells. *J Exp Med*, **197,** 553-65.

Rosen, E.M., Fan, S., Pestell, R.G. & Goldberg, I.D. (2003). BRCA1 gene in breast cancer. *J Cell Physiol*, **196,** 19-41.

Rothkamm, K., Kuhne, M., Jeggo, P.A. & Lobrich, M. (2001). Radiation-induced genomic rearrangements formed by nonhomologous end-joining of DNA double-strand breaks. *Cancer Res*, **61,** 3886-93.

Rothkamm, K. & Lobrich, M. (2003). Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. *Proc Natl Acad Sci U S A*, **100,** 5057-62.

Roux, P.P., Richards, S.A. & Blenis, J. (2003). Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. *Mol Cell Biol*, **23,** 4796-804.

Roy, S.N. & Horwitz, S.B. (1984). Characterization of the association of radiolabeled bleomycin A2 with HeLa cells. *Cancer Res*, **44,** 1541-6.

Roy, S.N., Orr, G.A., Brewer, C.F. & Horwitz, S.B. (1981). Chemical synthesis of radiolabeled bleomycin A2 and its binding to DNA. *Cancer Res*, **41,** 4471-7.

Rudel, T. & Bokoch, G.M. (1997). Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science*, **276,** 1571-4.

Ruff, M., Gangloff, M., Wurtz, J.M. & Moras, D. (2000). Estrogen receptor transcription and transactivation: Structure-function relationship in

DNA- and ligand-binding domains of estrogen receptors. *Breast Cancer Res*, **2,** 353-9.

Runic, R., Lockwood, C.J., LaChapelle, L., Dipasquale, B., Demopoulos, R.I., Kumar, A. & Guller, S. (1998). Apoptosis and Fas expression in human fetal membranes. *J Clin Endocrinol Metab*, **83,** 660-6.

Russo, J., Balogh, G.A., Chen, J., Fernandez, S.V., Fernbaugh, R., Heulings, R., Mailo, D.A., Moral, R., Russo, P.A., Sheriff, F., Vanegas, J.E., Wang, R. & Russo, I.H. (2006). The concept of stem cell in the mammary gland and its implication in morphogenesis, cancer and prevention. *Front Biosci*, **11,** 151-72.

Russo, J., Moral, R., Balogh, G.A., Mailo, D. & Russo, I.H. (2005). The protective role of pregnancy in breast cancer. *Breast Cancer Res*, **7,** 131-42.

Ryan, A.J., Squires, S., Strutt, H.L. & Johnson, R.T. (1991). Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistent double strand breaks in replicating DNA. *Nucleic Acids Res*, **19,** 3295-300.

Safavy, A., Bonner, J.A., Waksal, H.W., Buchsbaum, D.J., Gillespie, G.Y., Khazaeli, M.B., Arani, R., Chen, D.T., Carpenter, M. & Raisch, K.P. (2003). Synthesis and biological evaluation of paclitaxel-C225 conjugate as a model for targeted drug delivery. *Bioconjug Chem*, **14,** 302-10.

Sakahira, H., Enari, M. & Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*, **391,** 96-9.

Salomon, D.S., Brandt, R., Ciardiello, F. & Normanno, N. (1995). Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol*, **19,** 183-232.

Salvesen, G.S. & Dixit, V.M. (1997). Caspases: intracellular signaling by proteolysis. *Cell*, **91,** 443-6.

Sam, J.W., Takahashi, S., Lippai, I., Peisach, J. & Rousseau, D.L. (1998). Sequence-specific changes in the metal site of ferric bleomycin induced by the binding of DNA. *J Biol Chem*, **273,** 16090-7.

Savage, C.R., Jr., Hash, J.H. & Cohen, S. (1973). Epidermal growth factor. Location of disulfide bonds. *J Biol Chem*, **248,** 7669-72.

Savage, C.R., Jr., Inagami, T. & Cohen, S. (1972). The primary structure of epidermal growth factor. *J Biol Chem*, **247,** 7612-21.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H. & Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *Embo J*, **17,** 1675-87.

Schinkel, A.H. & Jonker, J.W. (2003). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev*, **55,** 3-29.

Schinkel, A.H., Kemp, S., Dolle, M., Rudenko, G. & Wagenaar, E. (1993). N-glycosylation and deletion mutants of the human MDR1 P-glycoprotein. *J Biol Chem*, **268,** 7474-81.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. *Cell*, **103,** 211-25.

Seelig, A. (1998). A general pattern for substrate recognition by P-glycoprotein. *Eur J Biochem*, **251,** 252-61.

Sharma, R., Beith, J. & Hamilton, A. (2005). Systematic review of LHRH agonists for the adjuvant treatment of early breast cancer. *Breast*, **14,** 181-91.

She, Q.B., Solit, D.B., Ye, Q., O'Reilly, K.E., Lobo, J. & Rosen, N. (2005). The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. *Cancer Cell*, **8,** 287-97.

Shibue, T. & Taniguchi, T. (2006). BH3-only proteins: integrated control point of apoptosis. *Int J Cancer*, **119,** 2036-43.

Shieh, S.Y., Ikeda, M., Taya, Y. & Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell*, **91,** 325-34.

Shin, D.M., Donato, N.J., Perez-Soler, R., Shin, H.J., Wu, J.Y., Zhang, P., Lawhorn, K., Khuri, F.R., Glisson, B.S., Myers, J., Clayman, G., Pfister, D., Falcey, J., Waksal, H., Mendelsohn, J. & Hong, W.K. (2001). Epidermal growth factor receptor-targeted therapy with C225 and cisplatin in patients with head and neck cancer. *Clin Cancer Res*, **7,** 1204-13.

Shinohara, A., Ogawa, H., Matsuda, Y., Ushio, N., Ikeo, K. & Ogawa, T. (1993). Cloning of human, mouse and fission yeast recombination genes homologous to RAD51 and recA. *Nat Genet*, **4,** 239-43.

Shintani, S., Li, C., Mihara, M., Terakado, N., Yano, J., Nakashiro, K. & Hamakawa, H. (2003). Enhancement of tumor radioresponse by combined treatment with gefitinib (Iressa, ZD1839), an epidermal growth factor receptor tyrosine kinase inhibitor, is accompanied by inhibition of DNA damage repair and cell growth in oral cancer. *Int J Cancer*, **107,** 1030-7.

Sibghatullah, Husain, I., Carlton, W. & Sancar, A. (1989). Human nucleotide excision repair in vitro: repair of pyrimidine dimers, psoralen and cisplatin adducts by HeLa cell-free extract. *Nucleic Acids Res*, **17,** 4471-84.

Simpson, E., Rubin, G., Clyne, C., Robertson, K., O'Donnell, L., Jones, M. & Davis, S. (2000). The role of local estrogen biosynthesis in males and females. *Trends Endocrinol Metab*, **11,** 184-8.

Simpson, E.R. & Davis, S.R. (2001). Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives. *Endocrinology*, **142,** 4589-94.

Singh, N.P., McCoy, M.T., Tice, R.R. & Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*, **175,** 184-91.

Singletary, K.W. & Gapstur, S.M. (2001). Alcohol and breast cancer: review of epidemiologic and experimental evidence and potential mechanisms. *Jama*, **286,** 2143-51.

Sipples, R. (2006). Common Side Effects of Anti-EGFR Therapy: Acneform Rash. *Semin Oncol Nurs*, **22,** 28-34.

Sirotnak, F.M., Zakowski, M.F., Miller, V.A., Scher, H.I. & Kris, M.G. (2000). Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res*, **6,** 4885-92.

Smith-Warner, S.A., Spiegelman, D., Yaun, S.S., van den Brandt, P.A., Folsom, A.R., Goldbohm, R.A., Graham, S., Holmberg, L., Howe, G.R., Marshall, J.R., Miller, A.B., Potter, J.D., Speizer, F.E., Willett, W.C., Wolk, A. & Hunter, D.J. (1998). Alcohol and breast cancer in women: a pooled analysis of cohort studies. *Jama*, **279,** 535-40.

Sohn, J.W., Lee, S.Y., Lee, S.J., Kim, E.J., Cha, S.I., Kim, C.H., Lee, J.T., Jung, T.H. & Park, J.Y. (2006). MDR1 polymorphisms predict the response to etoposide-cisplatin combination chemotherapy in small cell lung cancer. *Jpn J Clin Oncol*, **36,** 137-41.

Soltoff, S.P., Carraway, K.L., 3rd, Prigent, S.A., Gullick, W.G. & Cantley, L.C. (1994). ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. *Mol Cell Biol*, **14,** 3550-8.

Staka, C.M., Nicholson, R.I. & Gee, J.M. (2005). Acquired resistance to oestrogen deprivation: role for growth factor signalling kinases/oestrogen receptor cross-talk revealed in new MCF-7X model. *Endocr Relat Cancer*, **12 Suppl 1,** S85-97.

Stea, B., Falsey, R., Kislin, K., Patel, J., Glanzberg, H., Carey, S., Ambrad, A.A., Meuillet, E.J. & Martinez, J.D. (2003). Time and dose-

dependent radiosensitization of the glioblastoma multiforme U251 cells by the EGF receptor tyrosine kinase inhibitor ZD1839 ('Iressa'). *Cancer Lett*, **202,** 43-51.

Stein, B. & Yang, M.X. (1995). Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta. *Mol Cell Biol*, **15,** 4971-9.

Stoll, B.A. (2000). Adiposity as a risk determinant for postmenopausal breast cancer. *Int J Obes Relat Metab Disord*, **24,** 527-33.

Tada, Y., Wada, M., Migita, T., Nagayama, J., Hinoshita, E., Mochida, Y., Maehara, Y., Tsuneyoshi, M., Kuwano, M. & Naito, S. (2002). Increased expression of multidrug resistance-associated proteins in bladder cancer during clinical course and drug resistance to doxorubicin. *Int J Cancer*, **98,** 630-5.

Taira, N., Doihara, H., Oota, T., Hara, F., Shien, T., Takahashi, H., Yoshitomi, S., Ishibe, Y. & Shimizu, N. (2006). Gefitinib, an epidermal growth factor receptor blockade agent, shows additional or synergistic effects on the radiosensitivity of esophageal cancer cells in vitro. *Acta Med Okayama*, **60,** 25-34.

Tanaka, M., Itai, T., Adachi, M. & Nagata, S. (1998). Downregulation of Fas ligand by shedding. *Nat Med*, **4,** 31-6.

Taylor, J.M., Mitchell, W.M. & Cohen, S. (1972). Epidermal growth factor. Physical and chemical properties. *J Biol Chem*, **247,** 5928-34.

Teixeira, C., Reed, J.C. & Pratt, M.A. (1995). Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res*, **55,** 3902-7.

Teraishi, F., Kagawa, S., Watanabe, T., Tango, Y., Kawashima, T., Umeoka, T., Nisizaki, M., Tanaka, N. & Fujiwara, T. (2005). ZD1839 (Gefitinib, 'Iressa'), an epidermal growth factor receptor-tyrosine kinase inhibitor, enhances the anti-cancer effects of TRAIL in human esophageal squamous cell carcinoma. *FEBS Lett*, **579,** 4069-75.

Thangaraju, M., Kaufmann, S.H. & Couch, F.J. (2000). BRCA1 facilitates stress-induced apoptosis in breast and ovarian cancer cell lines. *J Biol Chem*, **275,** 33487-96.

Thomas, D.B. (1984). Do hormones cause breast cancer? *Cancer*, **53,** 595-604.

Thompson, D., Szabo, C.I., Mangion, J., Oldenburg, R.A., Odefrey, F., Seal, S., Barfoot, R., Kroeze-Jansema, K., Teare, D., Rahman, N., Renard, H., Mann, G., Hopper, J.L., Buys, S.S., Andrulis, I.L., Senie, R., Daly, M.B., West, D., Ostrander, E.A., Offit, K., Peretz, T., Osorio, A.,

Benitez, J., Nathanson, K.L., Sinilnikova, O.M., Olah, E., Bignon, Y.J., Ruiz, P., Badzioch, M.D., Vasen, H.F., Futreal, A.P., Phelan, C.M., Narod, S.A., Lynch, H.T., Ponder, B.A., Eeles, R.A., Meijers-Heijboer, H., Stoppa-Lyonnet, D., Couch, F.J., Eccles, D.M., Evans, D.G., Chang-Claude, J., Lenoir, G., Weber, B.L., Devilee, P., Easton, D.F., Goldgar, D.E. & Stratton, M.R. (2002). Evaluation of linkage of breast cancer to the putative BRCA3 locus on chromosome 13q21 in 128 multiple case families from the Breast Cancer Linkage Consortium. *Proc Natl Acad Sci U S A*, **99,** 827-31.

Turchi, J.J. & Henkels, K. (1996). Human Ku autoantigen binds cisplatin-damaged DNA but fails to stimulate human DNA-activated protein kinase. *J Biol Chem*, **271,** 13861-7.

Turton, N.J., Judah, D.J., Riley, J., Davies, R., Lipson, D., Styles, J.A., Smith, A.G. & Gant, T.W. (2001). Gene expression and amplification in breast carcinoma cells with intrinsic and acquired doxorubicin resistance. *Oncogene*, **20,** 1300-6.

Tzukerman, M.T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M.G., Stein, R.B., Pike, J.W. & McDonnell, D.P. (1994). Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol*, **8,** 21-30.

Umezawa, H., Maeda, K., Takeuchi, T. & Okami, Y. (1966). New antibiotics, bleomycin A and B. *J Antibiot (Tokyo)*, **19,** 200-9.

Valerie, K. & Povirk, L.F. (2003). Regulation and mechanisms of mammalian double-strand break repair. *Oncogene*, **22,** 5792-812.

van Agthoven, T., van Agthoven, T.L., Portengen, H., Foekens, J.A. & Dorssers, L.C. (1992). Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res*, **52,** 5082-8.

van der Burg, B., Rutteman, G.R., Blankenstein, M.A., de Laat, S.W. & van Zoelen, E.J. (1988). Mitogenic stimulation of human breast cancer cells in a growth factor-defined medium: synergistic action of insulin and estrogen. *J Cell Physiol*, **134,** 101-8.

van Heemst, D., Brugmans, L., Verkaik, N.S. & van Gent, D.C. (2004). End-joining of blunt DNA double-strand breaks in mammalian fibroblasts is precise and requires DNA-PK and XRCC4. *DNA Repair (Amst)*, **3,** 43-50.

Vaux, D.L., Cory, S. & Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*, **335,** 440-2.

Wang, J., Lobito, A.A., Shen, F., Hornung, F., Winoto, A. & Lenardo, M.J. (2000a). Inhibition of Fas-mediated apoptosis by the B cell antigen receptor through c-FLIP. *Eur J Immunol*, **30,** 155-63.

Wang, Q. & Greene, M.I. (2005). EGFR enhances Survivin expression through the phosphoinositide 3 (PI-3) kinase signaling pathway. *Exp Mol Pathol*, **79,** 100-7.

Wang, S., Guo, M., Ouyang, H., Li, X., Cordon-Cardo, C., Kurimasa, A., Chen, D.J., Fuks, Z., Ling, C.C. & Li, G.C. (2000b). The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest. *Proc Natl Acad Sci U S A*, **97,** 1584-8.

Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S.J. & Qin, J. (2000c). BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev*, **14,** 927-39.

Ward, W.H., Cook, P.N., Slater, A.M., Davies, D.H., Holdgate, G.A. & Green, L.R. (1994). Epidermal growth factor receptor tyrosine kinase. Investigation of catalytic mechanism, structure-based searching and discovery of a potent inhibitor. *Biochem Pharmacol*, **48,** 659-66.

Warmuth, I., Harth, Y., Matsui, M.S., Wang, N. & DeLeo, V.A. (1994). Ultraviolet radiation induces phosphorylation of the epidermal growth factor receptor. *Cancer Res*, **54,** 374-6.

Wassermann, K., Markovits, J., Jaxel, C., Capranico, G., Kohn, K.W. & Pommier, Y. (1990). Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II. *Mol Pharmacol*, **38,** 38-45.

Wei, M.C., Lindsten, T., Mootha, V.K., Weiler, S., Gross, A., Ashiya, M., Thompson, C.B. & Korsmeyer, S.J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev*, **14,** 2060-71.

Weihua, Z., Andersson, S., Cheng, G., Simpson, E.R., Warner, M. & Gustafsson, J.A. (2003). Update on estrogen signaling. *FEBS Lett*, **546,** 17-24.

Welshons, W.V., Wolf, M.F., Murphy, C.S. & Jordan, V.C. (1988). Estrogenic activity of phenol red. *Mol Cell Endocrinol*, **57,** 169-78.

Wen, L.P., Fahrni, J.A., Troie, S., Guan, J.L., Orth, K. & Rosen, G.D. (1997). Cleavage of focal adhesion kinase by caspases during apoptosis. *J Biol Chem*, **272,** 26056-61.

Wessler, S., Otto, C., Wilck, N., Stangl, V. & Fritzemeier, K.H. (2005). Identification of estrogen receptor ligands leading to activation of

non-genomic signaling pathways while exhibiting only weak transcriptional activity. *J Steroid Biochem Mol Biol.*

Wiley, H.S. & Burke, P.M. (2001). Regulation of receptor tyrosine kinase signaling by endocytic trafficking. *Traffic*, **2,** 12-8.

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. & Youle, R.J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol*, **139,** 1281-92.

Wong, A.J., Ruppert, J.M., Bigner, S.H., Grzeschik, C.H., Humphrey, P.A., Bigner, D.S. & Vogelstein, B. (1992). Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A*, **89,** 2965-9.

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C. & Micklem, G. (1995). Identification of the breast cancer susceptibility gene BRCA2. *Nature*, **378,** 789-92.

Wooster, R., Neuhausen, S.L., Mangion, J., Quirk, Y., Ford, D., Collins, N., Nguyen, K., Seal, S., Tran, T., Averill, D. & et al. (1994). Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science*, **265,** 2088-90.

Worthylake, R., Opresko, L.K. & Wiley, H.S. (1999). ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors. *J Biol Chem*, **274,** 8865-74.

Wosikowski, K., Schuurhuis, D., Kops, G.J., Saceda, M. & Bates, S.E. (1997). Altered gene expression in drug-resistant human breast cancer cells. *Clin Cancer Res*, **3,** 2405-14.

Wurzer, J.C., Tallarida, R.J. & Sirover, M.A. (1994). New mechanism of action of the cancer chemotherapeutic agent 5-fluorouracil in human cells. *J Pharmacol Exp Ther*, **269,** 39-43.

Xia, F., Taghian, D.G., DeFrank, J.S., Zeng, Z.C., Willers, H., Iliakis, G. & Powell, S.N. (2001). Deficiency of human BRCA2 leads to impaired homologous recombination but maintains normal nonhomologous end joining. *Proc Natl Acad Sci U S A*, **98,** 8644-9.

Yacoub, A., McKinstry, R., Hinman, D., Chung, T., Dent, P. & Hagan, M.P. (2003). Epidermal growth factor and ionizing radiation up-regulate the DNA repair genes XRCC1 and ERCC1 in DU145 and LNCaP prostate carcinoma through MAPK signaling. *Radiat Res*, **159,** 439-52.

Yao, M., Nguyen, T.V. & Pike, C.J. (2007). Estrogen regulates Bcl-w and Bim expression: role in protection against beta-amyloid peptide-induced neuronal death. *J Neurosci*, **27,** 1422-33.

Yarden, Y. (2001). The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *Eur J Cancer*, **37 Suppl 4,** S3-8.

Yarden, Y. & Schlessinger, J. (1987a). Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. *Biochemistry*, **26,** 1443-51.

Yarden, Y. & Schlessinger, J. (1987b). Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. *Biochemistry*, **26,** 1434-42.

Ying, M., Agrawal, A. & Cheung, K.-L. (2005). The 'other half' of breast cancer: A review of male breast cancer. *Jmhg*, **2,** 406-413.

Young, R.L. & Korsmeyer, S.J. (1993). A negative regulatory element in the bcl-2 5'-untranslated region inhibits expression from an upstream promoter. *Mol Cell Biol*, **13,** 3686-97.

Yu, X., Sharma, K.D., Takahashi, T., Iwamoto, R. & Mekada, E. (2002). Ligand-independent dimer formation of epidermal growth factor receptor (EGFR) is a step separable from ligand-induced EGFR signaling. *Mol Biol Cell*, **13,** 2547-57.

Yu, Y., Wang, W., Ding, Q., Ye, R., Chen, D., Merkle, D., Schriemer, D., Meek, K. & Lees-Miller, S.P. (2003). DNA-PK phosphorylation sites in XRCC4 are not required for survival after radiation or for V(D)J recombination. *DNA Repair (Amst)*, **2,** 1239-52.

Zelcer, N., Saeki, T., Reid, G., Beijnen, J.H. & Borst, P. (2001). Characterization of drug transport by the human multidrug resistance protein 3 (ABCC3). *J Biol Chem*, **276,** 46400-7.

Zhang, G.J., Kimijima, I., Onda, M., Kanno, M., Sato, H., Watanabe, T., Tsuchiya, A., Abe, R. & Takenoshita, S. (1999). Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-X(L), without alteration of p53 protein levels. *Clin Cancer Res*, **5,** 2971-7.

Zhao, H.J., Hosoi, Y., Miyachi, H., Ishii, K., Yoshida, M., Nemoto, K., Takai, Y., Yamada, S., Suzuki, N. & Ono, T. (2000). DNA-dependent protein kinase activity correlates with Ku70 expression and radiation sensitivity in esophageal cancer cell lines. *Clin Cancer Res*, **6,** 1073-8.

Zheng, Z.S., Chen, R.Z. & Prystowsky, J.H. (1993). UVB radiation induces phosphorylation of the epidermal growth factor receptor, decreases

EGF binding and blocks EGF induction of ornithine decarboxylase gene expression in SV40-transformed human keratinocytes. *Exp Dermatol*, **2,** 257-65.

Zhivotovsky, B. & Kroemer, G. (2004). Apoptosis and genomic instability. *Nat Rev Mol Cell Biol*, **5,** 752-62.

Zhou, H., Kim, Y.S., Peletier, A., McCall, W., Earp, H.S. & Sartor, C.I. (2004). Effects of the EGFR/HER2 kinase inhibitor GW572016 on EGFR- and HER2-overexpressing breast cancer cell line proliferation, radiosensitization, and resistance. *Int J Radiat Oncol Biol Phys*, **58,** 344-52.

Zhu, X.F., Liu, Z.C., Xie, B.F., Li, Z.M., Feng, G.K., Yang, D. & Zeng, Y.X. (2001). EGFR tyrosine kinase inhibitor AG1478 inhibits cell proliferation and arrests cell cycle in nasopharyngeal carcinoma cells. *Cancer Lett*, **169,** 27-32.

Ziegler, R.G., Hoover, R.N., Pike, M.C., Hildesheim, A., Nomura, A.M., West, D.W., Wu-Williams, A.H., Kolonel, L.N., Horn-Ross, P.L., Rosenthal, J.F. & Hyer, M.B. (1993). Migration patterns and breast cancer risk in Asian-American women. *J Natl Cancer Inst*, **85,** 1819-27.