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Membrane fluidity and epidermal growth factor receptor function in breast cancer

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A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science

January 1998

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Abbreviations

Adr	adriamycin
AEBS	antiestrogen binding sites
AF18	5-N-octadecanoyl aminofluorescein
APS	ammonium persulphate
AR	amphiregulin
ATP	adenosine trisphosphate
BSA	bovine serum albumin
BODIPY	dipyrrometheneboron difluoride
	4,4-difluoro-4-bora-3a,4a-diaza-s-in-diacene
cpm	counts per minute
DHIDCCFCS	dialysed heat inactivated dextran coated
	charcoal stripped fetal calf serum
D_L	lateral diffusion coefficient
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
E2	17β estradiol
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FRAP	fluorescence recovery after photobleaching
Kd	dissociation constant
LDL	low density lipoprotein
mdr	multi-drug resistance
MTT	dimethylthiazolyl-diphenyltetrazolium bromide
NDF	neu differentiation factor

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonylfluoride
PR	progesterone receptor
rpm	revolutions per minute
SAC	Staphylococcus aureus cells (Protein A cells)
sd	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SPM	sphingomyelin
TAM	tamoxifen
TEMED	N,N,N',N',tetramethylethylenediamine
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta

Abstract

The epidermal growth factor receptor (EGFR) has been implicated in the growth and development of a number of human cancers including breast cancer. The activity and expression of membrane associated proteins such as the EGFR may be modulated by the physical state of the membrane. Activation of the EGFR depends on the lateral diffusion and dimerisation of receptor monomers. Changes in membrane fluidity may therefore affect activation of the EGFR and hence growth signalling via this receptor.

Using the technique of fluorescence recovery after photobleaching, the lateral diffusion of number of fluorescent lipid probes was studied in three breast cancer cell lines (MCF-7, MDA-MB-231 and MCF-7AdrR). The antiestrogen drug tamoxifen which is widely used in the treatment of breast cancer, was demonstrated to decrease membrane fluidity in breast cancer cells e.g. in MCF-7 cells a 24 hour incubation with 10^{-6}M tamoxifen decreased the lateral diffusion coefficient of the AF18 probe from 203 $x10^{-10}cm^2/s$ to 15.4 $x10^{-10}cm^2/s$. Tamoxifen treatment had a general effect on membrane fluidity which was observed using different lipid probes and was not dependent on the estrogen receptor status of the cell line. Tamoxifen's ability to decrease membrane fluidity may be due to its ability to adopt a conformation which resembles the steroid nucleus. The steroids cholesterol, 17β-estradiol and the steroidal antiestrogen ICI182,780 all had a similar effect on membrane fluidity.

Tamoxifen has previously been reported to have several actions unrelated to inhibition of estrogen action. Tamoxifen can inhibit the growth of cells in the complete absence of estrogens and also inhibit cells which do not express the estrogen receptor. Tamoxifen can inhibit EGF-stimulated growth of MCF-7 breast cancer cells by a mechanism distinct from induction of TGFβ production. Tamoxifen had no effect on the cell surface expression or affinity of the EGFR and it did not inhibit the EGFR tyrosine kinase activity in isolated cell membranes. However, in intact cells tamoxifen treatment inhibited EGFR autophosphorylation. This effect could be due to

stabilisation of the membrane by tamoxifen and consequent reduction in the lateral diffusion of the EGF receptor, reducing dimerisation and autophosphorylation of the receptor. This would reduce growth stimulation initiated via the EGFR. Tamoxifen can be demonstrated to reduce EGFR mobility in A431 cells. These cells have elevated levels of EGFR which allow a sufficient level of signal for FRAP measurements of receptor mobility. It is therefore possible that tamoxifen's membrane effects contribute to its anticancer action by interfering with EGFR function.

The rate of internalisation of the EGFR was not altered by tamoxifen treatment which suggests that EGFR lateral diffusion is not a rate-limiting factor in receptor internalisation. Tamoxifen treatment also had no effect on the adriamycin sensitivity of the drug resistant cell line MCF-7AdrR.

Chapter 1

Introduction

1. Breast Cancer

1. 1. Introduction

Breast cancer is the most common single cause of death in Western women between the ages of 35 and 54. The estimated lifetime risk in Scotland of developing the disease was 7.2% between 1985 and 1994 [Cancer Registration Statistics, 1996]. In Scotland each year, approximately 2600 new cases of the disease are diagnosed and there are 1250 deaths [Sharp, *et al*, 1993]. In 1994 this accounted for 24.4% of all cancers in women in Scotland [Cancer Registration Statistics, 1996]. In common with many other countries, the incidence of breast cancer in Scotland is increasing with a 12% rise over the period 1981 to 1990 [Sharp, *et al*, 1993]. Worldwide, the incidence of breast cancer varies in different countries - the lowest mortality rates are seen in East Asia (33 per 100,000 population in females aged 45 to 84 in 1986) compared with Western Europe which had a rate of 88 per 100,000 for the same period [Hoel, *et al*, 1992]. Breast cancer also occurs in males but is rare (typically less than 1% of cases). The maximum observed incidence in Scotland was 34 cases in 1976 since when the incidence has declined [Black, *et al*, 1995]

Cancers of the breast can be classified into a number of histological types [World Health Organisation., 1981]. Carcinoma in situ (or non-invasive carcinoma) in which the tumour has not breached the basement membrane can be subdivided into two types : intraductal and lobular carcinoma in situ. Patients with either of these types of tumour have a 20 to 30% chance of developing invasive carcinoma within 20 years of diagnosis [Bartow, 1995] Cancers where the basement membrane has been infiltrated are classified as invasive. The most common form is invasive, or infiltrating, ductal carcinoma which accounts for 65-80% of cases [Rosen, *et al*, 1996]. Paget's disease of the nipple is an uncommon variant of ductal carcinoma, either invasive or in situ, which spreads to involve the epidermis of the nipple or areola. The second most common form of invasive carcinoma (10%) is lobular

carcinoma [Bartow, 1995]. Finally, 5-10% of invasive carcinomas are designated medullary carcinoma. This type of carcinoma has the best prognosis of the invasive carcinomas [Robbins, *et al*, 1987].

Invasive breast cancers spread primarily via the lymphatic system to regional lymph nodes including axillary, internal mammary and supraclavicular lymph nodes. More distant metastases (blood-borne) are commonly found in the lungs and pleura, liver, bones, adrenal glands, brain and spleen [Bartow, 1995]. In a significant number of patients, the tumour has already metastasised at the time of diagnosis. Metastasis is usually the single most important criterion determining a patient's prognosis. The 5 year survival rate for a breast cancer patient with localised disease and no evidence of axillary lymph node involvement is around 80% but drops to around 30% when more than 10 lymph nodes are involved [Clark, 1996].

1. 2. Risk factors for breast cancer

Major risk factors for breast cancer established from epidemiological research appear to include early menarche and late menopause - a delay in menarche of 3 years from age 12 to 15 can half the risk of breast cancer as can a natural menopause at 45 rather than 55. Early menopause through ovarian ablation is similarly associated with a reduction in risk [Brinton,*et al*, 1988]. The age at which a woman has her first child is also a factor - a first child at 30 carries twice the risk associated with a first child at 18. Nulliparity is associated with increased risk although the risk is less than that associated with a first birth after 30 [MacMahon, *et al*, 1970; Ewetz, *et al*, 1990]. These factors all indicate an association with cyclic ovarian activity and consequent exposure to the known breast mitogen, estrogen which has been proposed to have a central etiological role in breast cancer [Fuqua, 1996].

A number of lifestyle factors such as diet and alcohol also contribute to the overall risk of breast cancer but their exact roles in its cause remain unclear [Daudt, *et al*, 1996]. The hypothesis of an association between diet and cancer incidence stems largely from comparisons of incidence of cancers in various parts of the world. Offspring of parents who move from a country with a low rate, to one with a high incidence, tend to acquire rates similar to the new country suggesting a role for environmental and lifestyle influences [Hunter, *et al*, 1996]. The role of dietary fat intake is unclear [Whittemore, 1993]. There is an observed association between per capita fat consumption and breast cancer incidence in developed countries [Boyle, *et al*, 1988]. However, recent studies show that in the USA, an increase in breast cancer incidence has occurred during a period of decreased fat consumption [Daudt, *et al*, 1996]. Obesity has been shown to be an independent risk factor for breast cancer. This may be of particular relevance in post menopausal women where adipose tissue is the major source of estrogens [Rose, 1986]. A number of studies have shown that increased alcohol intake is associated with an increased in risk of the disease which is possibly linked to an increase in endogenous estrogen levels [Willet, 1989; Daudt, *et al*, 1996].

Intake of vitamins A, C and E and selenium are all suggested to be protective against a variety of cancers including breast cancer. This may be related to the antioxidant effect of these compounds [Hunter, 1996] (section 5.5). Evidence suggests that consumption of green vegetable may be related to a reduced risk of breast cancer [Willett, 1995]. This may be due to antioxidant micronutrients or the presence of other constituents with a protective effect, or, may simply reflect a lower intake of fat or calories [Minchovicz, *et al*, 1990; Greenberg, *et al*, 1996]. The observed protective effect of a diet rich in soya may be due to its content of isoflavones such as genistein. These phytoestrogens have weak estrogen agonist activity which may interfere with endogenous estrogen [Daudt, *et al*, 1996]. Diet may also play a role in prolonged survival from breast cancer - certain dietary fatty

acids, in particular omega-3 fatty acids found in fish oils and gamma linolenic acid, have been found to inhibit growth of breast cell lines [Cantrill, *et al*, 1993].

Some breast cancers are associated with a family risk of the disease. A family history in a first degree relative is associated with an approximate doubling of the risk - this risk is even higher if both the mother and a sister have had breast cancer. Significant increases in this risk are found if the relative had bilateral disease or developed cancer at an early age [Brinton, *et al*, 1996]. Approximately 5% of all breast cancers are inherited forms of the disease with the majority of these associated with abnormalities in two genes - BRCA1 and BRCA2 [Daudt, *et al*, 1996]. Between 5 and 10% of women diagnosed under the age of 40 may have BRCA1 alterations [Blackwood, 1996]. In BRCA1 carriers, the risk of breast cancer is estimated to be 51% by the age of 40 and 85% by age 70.

1. 3. Prognostic and predictive factors in breast cancer

To determine the best treatment for any patient, the likelihood that the patient will have a recurrence of disease in the future and the potential efficacy of the available treatments on that tumour must be examined. Prognostic indicators give the disease free or overall survival in the absence of systemic adjuvant therapy while predictive factors give an indication of the response, or lack of response, to therapy [Clark, 1996]. A number of biomarkers are currently in use to classify individual patients. The standard prognostic factors currently in use for primary breast cancer include: axillary node status, histologic subtype, tumour size, tumour grade and estrogen and progesterone receptor status [Clark, 1996]. The presence or absence of metastatic involvement in the axillary lymph nodes is the single most powerful prognostic factor available for patients with primary breast cancer [Hynes, *et al*, 1994].

Many of the known risk factors for breast cancer indicate that a cumulative exposure to estrogen (and progesterone) plays an important causative role in breast

cancer (Section 1.2). The mitogenic effects of estrogen and progesterone on breast cancer cells in culture have been well documented [Dickson, *et al*, 1988]. Steroid receptor assays have become standard practice in the management of breast cancer- estrogen receptor (ER) positivity is generally indicative of a relatively good prognosis in primary breast cancer [Fuqua, 1996]. The ER status of a tumour is also used as a predictor of its response to endocrine therapy in advanced disease - 50% of ER positive patients have an objective response to this therapy compared with only 10% of ER negative cases [Klijn, *et al*, 1993]. However this leaves a large number of ER positive metastatic tumours which fail to respond to endocrine therapy while some ER negative do respond. There is also a relatively small difference (8-10%) in relapse free survival (RFS) between ER positive and ER negative tumours. Thus ER status, while useful, is a rather imperfect predictor of prognosis and response to therapy [Klijn, *et al*, 1993]. Progesterone receptor status in addition to ER status improves the ability to predict response to endocrine therapy - tumours with both steroid receptors have response rates higher than 70% [Fuqua, 1996].

Since none of these factors alone or in combination separates completely patients who are cured by local therapy from those whose cancer will recur, newer biomarkers must be examined for their significance. Since evidence exists that breast epithelial cells are under growth control of members of the EGFR family, this protein has been examined for its prognostic value. A number of studies have reported that this protein is indeed an indicator of poor prognosis and can be used to predict lack of response to endocrine therapy [Klijn, *et al*, 1993] (Section 3.).

1. 4. Treatment of breast cancer

A number of options are available in the treatment of breast cancer including surgery, radiation, chemotherapy and endocrine therapy. Treatment for localised breast cancer usually involves lumpectomy either alone or in conjunction with

radiotherapy. Data shows that patients treated in this way have the same five year survival rate as patients treated by total mastectomy for localised disease [Morrow, *et al*, 1996]. Adjuvant therapy is used after primary surgery to kill or inhibit clinically occult micro-metastases. In cases where the disease has metastasised to the axillary lymph nodes, chemotherapy is used. The most widely used form is a combination (CMF) of cyclophosphamide, methotrexate and fluorouracil although a number of alternative treatments exist. Endocrine therapy may involve the use of anti-estrogens such as tamoxifen (Section 5), aromatase inhibitors which block an enzyme involved in estrogen synthesis, luteinizing hormone-releasing hormone analogues or progestins [Bajetta, *et al*, 1994]. Antiestrogens are considered the treatment of first choice for metastatic disease in post-menopausal patients or for adjuvant therapy. Selection of the appropriate treatment for individual patients whether chemotherapy or endocrine therapy is critical for the successful treatment of the disease.

1.5. Drug resistance in breast cancer

A major problem in the successful treatment of cancer, including breast cancer, is the phenomenon of drug resistance. Resistance to chemotherapy may be intrinsic where the tumour fails to respond when initially treated, or acquired as in tumours where there is a good response to chemotherapy initially, but eventually resistance develops, not only to the original drug used in treatment, but also to a wide variety of other drugs [Bosch, *et al*, 1996]. The drugs to which resistance is acquired do not necessarily have a common structure or intracellular target. They include anthracyclines, taxanes, vinca alkaloids, epipodophyllotoxins and mitoxantrone [Blackwood, 1996]. Such broad spectrum resistance to chemotherapy is called multi-drug resistance and efforts to reverse this phenomenon have important clinical implications for breast cancer patients.

Multi-drug resistance and was first reported in chinese hamster lung cells in 1970 [Biedler, *et al*, 1970]. In 1976, it was reported that multi-drug resistance was associated with a 170,000 molecular weight surface glycoprotein, p-glycoprotein, and that the degree of expression of this protein correlated with the degree of resistance [Juliano, *et al*, 1976]. P-glycoprotein, or p170, has since been characterised as an ATP-dependent transmembrane pump which acts to prevent intracellular accumulation of a variety of chemotherapeutic drugs [Horio, *et al*, 1988]. There is large body of evidence to support the theory that overexpression of this protein is responsible for drug resistance. Increased protein expression correlates well with the degree of drug resistance in a number of cell lines and it is the most consistent phenotypic alteration found in the condition [Kartner, *et al*, 1985]. Transfection of the gene for p-glycoprotein is sufficient to confer multidrug resistance on a drug sensitive cell line [Gros, *et al*, 1986]. Elevated levels of the protein are found in tumours of every histological type and also in normal tissue such as the adrenal gland suggesting its normal physiological function may be related to the packaging and secretion of physiologically important substances [Bosch, *et al*, 1996].

Some expression of p-glycoprotein can be found in normal breast epithelial cells [Cordon-Cardo, *et al*, 1989]. In breast cancer, p-glycoprotein is expressed at high levels by between 20 and 70% of cancers although some studies failed to detect high levels of expression of either the protein or RNA [Bosch, *et al*, 1996]. Previously treated cancers are consistently more likely to express high levels of p-glycoprotein [Salmon,*et al*, 1989]. Expression of p-glycoprotein may be associated with a loss of expression of steroid receptors and increased epidermal growth factor expression [Meyers, *et al*, 1986; Vickers, *et al*, 1988; Dickstein, *et al*, 1993].

2. Epidermal Growth Factor and its receptor

2. 1. Epidermal growth factor

Epidermal growth factor (EGF) was first isolated in 1962 in an experiment to extract nerve growth factor (NGF) from mouse submaxillary glands. A fraction of extract from these glands, which was negative for NGF activity, was found to induce premature incisor eruption and eyelid opening when injected into newborn mice. The factor responsible was isolated and purified and found to be a small, heat stable polypeptide [Cohen, 1962]. These effects were due to stimulation of epidermal growth and keratinization [Cohen, *et al*, 1963]. The protein, named epidermal growth factor, is highly abundant in male mouse submaxillary gland where it accounts for 0.5% of the total protein content [Carpenter, *et al*, 1979]. The human form of EGF was isolated from urine and was named urogastrone since it was initially characterised as an agent which inhibited gastric acid secretion [Gregory, 1975]. Daily urinary excretion of the protein can exceed 50µg/ml and is significantly higher in females, particularly in those taking oral contraceptives or during pregnancy [Dailey, *et al*, 1978]. The human and mouse forms of EGF differ in molecular weight and immunological properties, but compete for receptor binding and have similar biological activities [Carpenter, *et al*, 1979]. EGF has since been isolated from many tissues and most body fluids including milk [Boonstra, *et al*, 1995]. In mouse the predominant site of EGF synthesis is the submaxillary glands although secondary sites are evident after removal of these glands. Expression has been detected in the submaxillary gland, the kidney and (in humans) Bruners gland in the gut, but the exact site of synthesis is uncertain [Carpenter, 1985].

Mouse EGF is a 53 amino acid chain (MW 6045) with 3 intramolecular disulphide bonds. Human EGF has a similar amino acid sequence to the mouse form but has a smaller molecular weight (MW 5400) [Schlessinger, *et al*, 1983]. The disulphide bonds are required for biological activity and their position is highly conserved

among different species and with related peptides such as TGFα [Carpenter, *et al*, 1990]. cDNA cloning revealed that the peptide is produced as a larger 130KDa transmembrane precursor of approximately 1200 residues which is subsequently processed by proteases to produce the mature growth factor. In addition to EGF, this preproEGF contains seven EGF-like domains - the function of these is unknown [Boonstra, *et al*, 1995]

EGF has a wide range of biological effects which have been demonstrated in different systems both in culture and in vivo. At a cellular level, EGF is a potent mitogen for a number of different cell types [Carpenter, *et al*, 1979]. In addition to its stimulatory effects on the proliferation of the epidermis and corneal epithelium, liver, lung and kidney are also stimulated. Of relevance to breast cancer is its stimulatory role in mammary tissue which has been demonstrated both in cell lines and in vivo [Osborne, *et al*, 1980; Gabelman, *et al*, 1992].

EGF produces a number of cellular responses which precede cell division. These include phosphorylation of many cellular proteins including the EGFR itself, increased ion and nutrient transport, changes in cytoskeletal organisation and in cell morphology. EGF also induces expression of a number of genes such as c-myc and c-fos and stimulates biosynthesis of fibronectin and keratin [Schlessinger, *et al*, 1983; Harris, *et al*, 1988]. In a number of cell types including keratinocytes, EGF mediates chemotaxis and migration [McCawley, *et al*, 1997].

2. 2. EGF in the breast

Several studies have indicated that EGF has an important role in regulating the growth of the mammary epithelium: EGF has been detected in milk, breast cyst fluid and breast tumour tissue [Nickell, *et al*, 1983; Zweibel, *et al*, 1986; Connolly, *et al*, 1988]. mRNA for EGF and its receptor are found in normal breast tissue indicating that the EGF/EGFR pathway is involved in normal breast growth and

differentiation [Bates, *et al*, 1990]. The pattern of EGF receptor staining in normal tissue is variable - immunohistochemical staining detects receptor in lobular, ductal, myoepithelial and stromal cells [Chrysogelos, *et al*, 1994]. EGF stimulates both normal and malignant mammary growth in rats and is necessary for lobulo-alveolar development of mouse mammary glands in organ culture [Turkington, 1969; Tonelli, *et al*, 1980]. EGF may also play a physiological role in development of the mouse mammary gland during pregnancy [Okamoto, *et al*, 1984]. EGF enhances growth of normal human mammary epithelial cells in short term culture [Taylor-Papadimitriou, *et al*, 1977; Gabelman, *et al*, 1992] and stimulates proliferation of breast cancer cell lines [Osborne, *et al*, 1980]. EGF may have role in the promotion of mammary carcinoma. In a strain of mice with a high incidence of mammary tumours, removal of the submaxillary gland which is the major site of EGF synthesis reduced the occurrence of tumours. The high incidence of mammary tumours is restored if EGF is given [Kurachi, *et al*, 1985].

2. 3. Epidermal Growth Factor Receptor (EGFR)

Specific, saturable receptors for EGF were first demonstrated by binding iodinated growth factor to human fibroblasts [Carpenter, *et al*, 1976]. EGF binds to the receptor with a high affinity -K_D values are in the range 10^{-9} to 10^{-10}M. Studies in fibroblasts indicate that a normal cell has between 10,000 and 100,000 binding sites for EGF [Carpenter, *et al*, 1979]. Exceptions to this include the A431 cell line and several epithelial carcinoma cell lines which express in the region of 2×10^6 receptors/cell [Carpenter, 1987]. Most cells with the exception of haemopoetic cells, express receptors for EGF [Carpenter, 1987]. EGFR have been detected in the undifferentiated basal cells of stratified epithelia and other proliferating cells of adnexal structures. Levels are also detectable on non-proliferating cells and the ducts of bronchial glands, bile ducts and breast ducts. It is clear therefore that EGFR is not necessarily a marker for growth in normal tissue and may have other physiological effects in differentiated cells [Gusterson, *et al*, 1984].

The isolation and characterisation of the receptor for EGF was greatly facilitated by the study of the epidermoid carcinoma cell line A431 [Giard, *et al*, 1973]. This cell line vastly overexpresses the receptor by 20-100 fold - EGF receptor comprises 0.15% of the total protein content of this cell line [Stoscheck, *et al*, 1984]. Although many of the physiological responses of the cell line are atypical e.g. EGF produces growth inhibition under most circumstances, the line remains a useful tool for the study of the receptor and early responses to its activation [Gill, *et al*, 1981].

The EGF receptor purified from the A431 cell line by affinity chromatography was characterised as a 170kDa glycoprotein with intrinsic protein tyrosine kinase activity [Cohen, *et al*, 1980; Ushiro, *et al*, 1980; Cohen, *et al*, 1982]. Subsequent cDNA cloning and amino acid sequencing of the receptor revealed more information about the receptor structure which can be divided into 3 domains: extracellular, transmembrane and cytoplasmic (Figure 1) [Ullrich, *et al*, 1984].

The extracellular domain is responsible for ligand binding. This region, which contains the N terminus of the protein, consists of 622 amino acids with 12 possible sites for N-linked glycosylation of which probably 11 are actually glyosylated [Mayes, *et al*, 1984]. The receptor is cotranslationally glycosylated to give an immature 160K glycoprotein. Further processing on the oligosaccharide chains of this precursor yields the mature protein [Soderquist, *et al*, 1986]. No evidence exists that the receptor contains O-linked oligosaccharides [Carpenter, 1987]. The domain has a relatively high cysteine content (9%) found in two Cys rich regions [Ullrich, *et al*, 1984]. Cys-rich extracellular domains are a common feature of other tyrosine kinase receptors including the insulin receptor and are found in EGFR from organisms as diverse as *Drosophila* and humans [Livneh, *et al*, 1985]. The ligand binding domain of the receptor lies between these two Cys rich regions [Lax, *et al*, 1989]. Quantitative binding experiments indicate that each receptor binds one EGF molecule [Weber, *et al*, 1984].

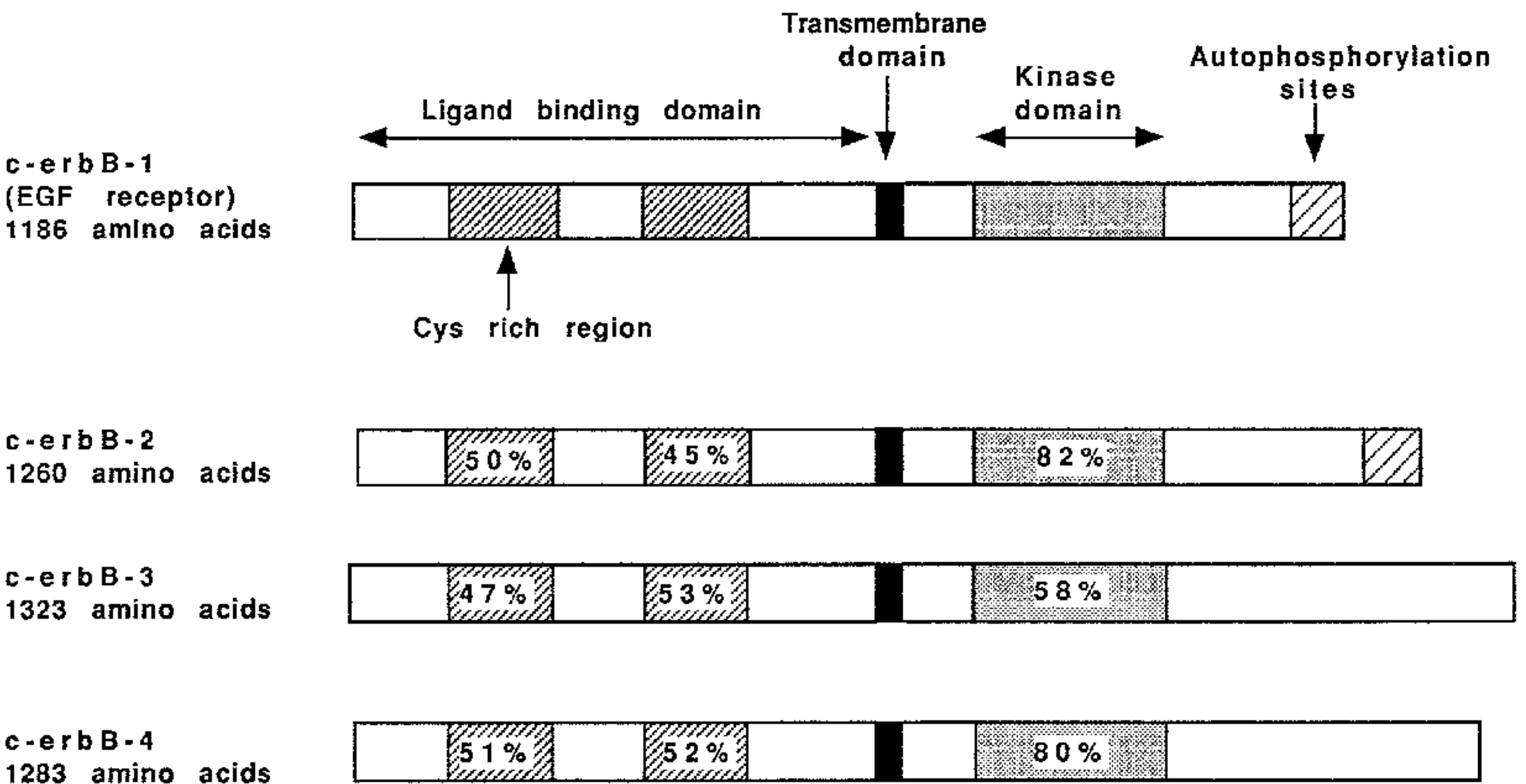


Figure 1. Diagram of the structures of of the class 1 family of protein tyrosine kinase receptors. The percentage homology between the extracellular and the kinase domains of each receptor and the EGFR is given.

The transmembrane domain is a region of 23 mainly hydrophobic amino acids which would be predicted to span the membrane once in an alpha helical structure [Ullrich, *et al*, 1984]. This domain may play only a structural role in linking external and internal domains where only length, hydrophobicity and stability in an alpha helical conformation are essential. Mutation of this domain has no effect on signal transduction [Kashles, *et al*, 1988]. However in the related molecules neu and c-erbB-2, the domain has a role in stabilising dimeric forms of the receptor since a point mutation within this region (Val to Glu) results in dimerisation and activation of the receptor.[Segatto, *et al*, 1988] (Section 2.3.2.).

The cytoplasmic domain consists of 542 amino acid residues including a region of approximately 300 residues which has homology to the src family of protein kinases. Sequence data demonstrated that this domain is highly homologous to the product of the v-erbB oncogene from avian erythroblastosis virus (Section 2.3.2.) [Ullrich, *et al*, 1984]. A conserved ATP binding site is found within this domain with an essential Lys residue at 721 [Russo, *et al*, 1985]. Mutation of this Lysine residue abolishes kinase activity [Honegger, *et al*, 1987a & 1987b] and immediate and late signalling events via the receptor such as alterations in intracellular calcium, activation of gene transcription, receptor down-regulation and cell proliferation [Chen, *et al*, 1987]. The C terminal region also contains the three major sites of receptor autophosphorylation, Tyr 1068, 1148 and 1173 - Tyr 1173 is the major autophosphorylation site in intact cells [Downward, *et al*, 1984b]. These C terminal tyrosines appear to define an auto inhibitory region which can be relieved by autophosphorylation or truncation [Walton, *et al*, 1990] and have also been implicated in binding receptor substrates by their SH2 domains (Section 2.3.4.) . The domain also contains a number of other phosphorylation sites on Ser and Thr residues - these are probably targets for phosphorylation by PLC. One well characterised site is Thr-654 which is located 10 residues beneath the transmembrane region of EGFR. Phosphorylation at this site by PKC abolishes the

high affinity receptor state and reduces its kinase activity [Downward, *et al*, 1985]. Since PKC is activated by EGFR this may represent a negative feedback mechanism to control signalling via the receptor.

2. 3. 1. Other ligands for EGFR

A number of other ligands for the EGFR exist which bind to the receptor with near identical affinities, and produce the same responses in target cells. These ligands all possess the 6 cysteine residues spaced at defined intervals which have been shown to be essential for the secondary structure and biological activity of EGF [Davies, *et al*, 1996]. Apart from EGF, transforming growth factor alpha, vaccinia virus growth factor, amphiregulin, heparin-binding EGF, β-cellulin , cripto-1 and a number of other virally encoded proteins also bind to the receptor [Normanno, *et al*, 1994].

Transforming Growth Factor α

TGFα was first isolated in 1978 from the medium of retrovirally transformed fibroblasts [DeLarco, *et al*, 1978]. It had been observed that fibroblasts transformed by the Moloney murine sarcoma virus rapidly lost their ability to bind EGF. Peptide growth factors were isolated from the conditioned medium of these cells. These transforming growth factors (TGF) were small, heat stable polypeptides which were also secreted by human tumour cells and were capable of inducing proliferation and transformation of rat fibroblasts [Todaro, *et al*, 1980]. The TGF preparation capable of inducing transformation was subsequently shown to contain two peptides: TGFα and TGFβ [Derynck, 1988]. Both peptides are required to produce anchorage-independent growth of normal fibroblasts but it is TGFα which competes for EGF binding and was responsible for the observed loss of EGF binding in transformed cells [Carpenter, *et al*, 1986]. TGFα has subsequently been identified in a variety of retrovirally transformed cells , human tumour cells and embryonic cells [Ozanne, *et al*, 1980; Todaro, *et al*, 1980; Twardzik, *et al*, 1982].

Mature TGFα is a single polypeptide chain of 50 amino acids - molecular weight 5kd. TGFα is 37% homologous to human EGF including conservation of all 6 cysteine residues involved in formation of intramolecular disulphide bonds [Marquardt, *et al*, 1984]. Like EGF, TGFα is synthesised as part of a larger 160 amino acid transmembrane precursor [Derynck, *et al*, 1984]. Differential proteolytic cleavage and glycosylation of the precursor accounts for the differing molecular weight forms of TGFα which have been isolated from the conditioned medium of TGFα producing cells [Derynck, 1988].

Unlike EGF, TGFα is known to be expressed by a wide range of tissues in both the adult and foetus. It is expressed during normal embryogenesis in the developing kidney, pharynx and otic vesicle while TGF mRNA and/or protein has been found in adult pituitary, brain and ovarian theca cells [Derynck, 1988]. TGFα can also be detected in human milk suggesting it may be secreted by normal mammary epithelial cells [Zweibel, *et al*, 1986]. It may play a role in wound healing since it is expressed in activated macrophages and normal skin keratinocytes [Derynck, 1988].

Elevated TGFα secretion has been demonstrated in several types of human carcinoma cell lines, in primary tumours and in transformed fibroblasts and epithelial cells , and has been linked to cell transformation [Salomon, *et al*, 1990]. The role of TGFα in breast cancer has been well studied. TGFα is a potent mitogen for both normal and malignant breast cells and is produced by chemically transformed rat mammary carcinomas - this expression is enhanced by estrogen treatment [Normanno, *et al*, 1994]. A number of human breast cell lines have also been demonstrated to secrete TGFα and in estrogen responsive cell lines this expression is also stimulated by estradiol. One effect of the anti-cancer drug tamoxifen is to block this elevation [Dickson, *et al*, 1987].

The parameters of TGFα binding to mammalian EGFR is very similar to EGF and TGFα has a similar activity to EGF in stimulating DNA synthesis in various cell lines, inducing premature eyelid opening in newborn mice and in promoting cell transformation [Schreiber, *et al*, 1986; Derynck, 1988]. However in other assay systems the two growth factors exert different responses - TGFα is more potent than EGF in inducing membrane ruffling, neovascularisation, keratinocyte migration and induction of Ca^{2+} release from bone in culture. Thus, while acting via the same receptor, cellular response to the two peptides can differ [Derynck, 1988].

Vaccinia Virus Growth Factor

The existence of a viral homologue of EGF, vaccinia virus growth factor (VVGF), was first detected via a search of protein sequence data bases. This protein is encoded in the inverted terminal repeat of the viral genome and is transcribed early in viral infection. The secreted protein has 77 amino acids including a region of 50 amino acids with 35% similarity to EGF and TGFα and retains the ability to form 3 disulphide bonds similar to EGF. Unlike EGF and TGFα, VVGF is glycosylated. This growth factor is capable of stimulating the EGFR and hence cell proliferation [Stroobant, *et al*, 1985]. Thus a virus has acquired sequences coding for a growth factor which may allow it to subvert EGFR dependent functions and may confer some advantage for viral growth. The exact function of this protein in the virus is unknown since vaccinia virus infection does not involve increased cellular proliferation. However, several members of the related Pox family such as cytomegalovirus do cause abnormal cellular proliferation during infection [Carpenter, *et al*, 1986].

Amphiregulin

Amphiregulin (AR) is a fourth protein which binds to the EGFR. It is a 14k glycoprotein secreted by MCF-7 breast cancer cells which have been treated with a

tumour promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA) [Shoyab, *et al*, 1988]. AR inhibits growth of breast tumour cells but not normal cells [Plowman, *et al*, 1990]. Mature AR exists as both a 78 and 84 amino acid peptide which binds to, and activates the EGFR although it has a lower binding affinity than EGF or TGFα [Normanno, *et al*, 1994]. AR also binds to the related protein c-erbB-3 [Gullick, 1991]. AR is expressed by a range of normal tissues and also a number of other tumour cell types including several breast cell lines [Normanno, *et al*, 1994].

2. 3 . 2. Molecules related to the EGFR

EGFR is only one of a family of similar proteins which share a common structure and, possibly, similar functions (Figure 1). All possess a large, glycosylated extracellular ligand binding domain, a single hydrophobic transmembrane domain, and a cytoplasmic tyrosine kinase domain. The family is called the class 1 receptor tyrosine kinases and in addition to EGFR (c-erbB-1) it includes, c-erbB-2, c-erbB-3 and c-erbB-4. The viral oncogene v-erbB is related in structure [Hynes, *et al*, 1994].

v-erbB

Initial partial sequencing of the EGFR revealed a striking similarity to the v-erbB oncogene of avian erythroblastosis virus [Downward, *et al*, 1984a]. Complete cDNA sequencing of the EGFR confirmed this similarity [Ullrich, *et al*, 1984]. A sequence of approximately 400 residues which includes the kinase domain is 95% identical between the two molecules [Carpenter, *et al*, 1987]. It is probable that this oncogene derives from viral capture of part of the cellular EGFR gene (c-erbB-1). The two molecules differ in that v-erbB lacks most of the extracellular ligand binding domain, plus 34 residues at the C-terminus including the major site of receptor autophosphorylation, Tyr 1173. Removal of these regulatory regions has led to the proposal that this molecule functions as a constitutively active, ligand

independent, protein tyrosine kinase in AEV-transformed cells [Downward, *et al*, 1984a & 1984b] (Section 2.3.4.).

c-erbB-2

The neu oncogene (also called HER2) from chemically-induced rat neuroblastomas was isolated in 1985 by its ability to transform normal NIH3T3 fibroblasts. This was shown to have significant homology to the EGFR [Schechter, *et al*, 1984]. Independently an erbB-related gene, c-erbB-2, was sequenced and cloned from a human breast carcinoma cell line. Analysis of these 2 genes identified the neu oncogene as the activated rat homologue of the proto-oncogene c-erbB-2 [Yamamoto, *et al*, 1986]. The normal protein is a 185K glycoprotein which is widely expressed on epithelial cells, particularly on secretory epithelia [Gullick, 1991]. The function of the receptor in normal tissue is not clear although it may be important in neural and neuromuscular junction development [Dickson, *et al*, 1996a]. Comparison of the sequences of EGFR and human c-erbB-2 reveals many similarities. There is 40% identity between the extracellular domains of these two proteins with conservation of the two Cys rich regions. The catalytic domains are 82% identical and c-erbB-2 has autophosphorylation sites related to those of EGFR [Gullick, 1990].

Oncogenic activation of neu/erbB-2 occurs due to a single mutation of valine to glutamate in the transmembrane domain of the receptor which apparently causes the mutant protein to exist in a permanent dimer with activated tyrosine kinase in the absence of ligand [Weiner, *et al*, 1989]. This may be due to stabilisation of the dimer by intrareceptor hydrogen bonding between the glutamate residues [Sternberg, *et al*, 1989]. Other potential methods of activation exist, including truncation of the extracellular or cytoplasmic domains [Segatto, *et al*, 1988]. The gene can also be activated oncogenically by overexpression and this process has

been implicated in the development of several human cancers [Di Fiore, *et al*, 1987b] (Section 3).

c-erbB-2 does not itself bind EGF or TGFα but heterodimers of EGFR/c-erbB-2 have been demonstrated to bind EGF. These have a increased affinity for EGF and a higher protein tyrosine kinase activity than homodimers [Dougall, *et al*, 1993]. It is possible that co-expression of EGFR and c-erbB-2 may confer an additional growth advantage on a cell at low ligand concentrations. Co-expression commonly occurs in human breast cancers [Gullick, 1990]. The implications of this for tumour growth are discussed in Section 3.3.2.

A potential ligand for c-erbB-2 was identified in the conditioned medium of *ras* transformed rat fibroblasts. This heat stable, 44K glycoprotein (called neu differentiation factor or NDF) was capable of stimulating neu receptor in mammary cells resulting in their differentiation to mature, milk-producing cells [Bacus, *et al*, 1994]. NDF stimulates tyrosine phosphorylation of c-erbB-2 and can be covalently cross linked to the receptor in some human breast cancer cells [Carraway, *et al*, 1994]. The human form of NDF, heregulin, was isolated as a protein secreted by MDA-MB-231 breast cancer cells which stimulated c-erbB-2 kinase activity in MCF-7 cells [Normanno, *et al*, 1994]. NDF is not able to stimulate erbB-2 kinase activity in all cells suggesting that another receptor may exist for this protein. More recent studies demonstrate that activation of c-erbB-2 by heregulin is dependant on coexpression of c-erbB-3 or c-erbB-4 and that heregulin actually binds directly c-erbB-4 and weakly to c-erbB-3 [Lemmon, *et al*, 1994]. Another factor of 15-17K called neu-activating factor (NAF) has been proposed as an erbB-2 specific ligand [Dickson, *et al*, 1996b].

c-erbB-3 and c-erbB-4

c-erbB-3 or HER-3 was isolated due to its sequence homology with v-erbB [Kraus, *et al*, 1989]. It is less related EGFR and c-erbB-2 than they are to each other [Rajkumar, *et al*, 1994]. c-erbB-3 lacks certain residues which are highly conserved in the catalytic domains of other tyrosine and ser/thr kinases which suggests that this protein either lacks or has only a weak kinase activity [Hynes, *et al*, 1994]. c-erbB-3 has been found expressed at moderately high levels on epithelial cells and on cells of neuroectodermal origin. The protein is expressed at low level on normal myoepithelial cells of the breast but may have elevated expression in some breast cancers [Bacus, *et al*, 1994]. Most breast cell lines which overexpress c-erbB-2 also overexpress c-erbB-3 [Rajkumar, *et al*, 1994].

c-erbB-4 is the most recently discovered member of the type 1 growth factor receptor family [Plowman, *et al*, 1993]. This shows a greater homology in its kinase domain to EGFR and c-erbB-2 than to c-erbB-3. However the extracellular domain of c-erbB-4 is more similar to c-erbB-3 (65%) than to the other two (41-46%) [Carraway, *et al*, 1994]. c-erbB-4 is overexpressed by a number of breast cell lines [Rajkumar, *et al*, 1994].

It is not yet clear whether c-erbB-3 and c-erbB-4 are commonly amplified in human cancers but both possess transforming activity in fibroblasts in vitro. Their function in normal development and physiology is even less certain [Dickson, *et al*, 1996a].

2. 3. 3. EGFR activation

The EGFR is a protein tyrosine kinase. This activity is essential to signalling via the receptor since mutations which abolish the tyrosine kinase activity abolish the biological function of the receptor [Cadena, *et al*, 1992]. A key question in the activation of the EGFR is how a signal initiated by ligand binding to the external domain is transmitted to the cytoplasmic kinase domain. Two models have been

proposed: an intramolecular model in which a signal generated in the extracellular domain would be transmitted via the single transmembrane span to activate the intracellular kinase domain, or, an intermolecular model where receptor activation would be achieved by allosteric interactions between receptor subunits. Most of the available evidence supports the intermolecular model of activation [Schlessinger, 1988]. This model also bypasses the need for transmission of a conformational change through the hydrophobic transmembrane region which is inherent in the intramolecular model - recent NMR studies of this domain have indicated that such a conformational change is unlikely to occur [Rigby, *et al*, 1996].

An essential feature of the intermolecular model of activation is interaction between receptors. Early studies demonstrated that EGF and other growth factors induce oligomerisation of their receptors and that this plays an important role in transmembrane signalling. EGFR aggregation in response to ligand binding has been observed in living cells, in membranes and in solubilised membrane preparations [Schlessinger, *et al*, 1978a; Yarden, *et al*, 1987]. The use of non-denaturing gel electrophoresis has demonstrated the irreversible, temperature dependent, formation of high molecular weight forms of the EGFR consistent with receptor dimerisation. This ability to undergo oligomerisation is an intrinsic process and is essential to signalling via the receptor [Yarden, *et al*, 1987]. Chemical cross linking studies have shown that EGF induces dimerisation in whole cells [Cochet, *et al*, 1988]. Antibody studies have demonstrated that oligomerisation of receptors alone may be sufficient for receptor activation. The EGFR can be activated by bivalent or multivalent antibodies but not by Fab fragments which lack the ability to cross link receptor monomers [Schreiber, *et al*, 1983]. Further evidence of the importance of dimerisation in the activation of the receptor comes from mutational studies. Insertion of an extra Cys into the extracellular region of EGFR results in a constitutively active dimeric receptor [Sorokin, *et al*, 1994]. The oncogenic activity of the neu oncogene has also been attributed to an intermolecular interaction

stabilising a dimeric form of the receptor to result in a constitutively active receptor [Weiner, *et al*, 1989].

Intermolecular activation of the EGFR requires the receptor to be mobile on the cell surface in order to allow interactions between monomers. EGFR mobility has been measured by a number of methods and in a number of systems. The lateral diffusion coefficient, D_L for EGFR is in the range of 2.8×10^{-10} to 8.5×10^{-10} cm^2s^{-1} [Schlessinger, *et al* 1978b; Hillman, *et al*, 1982; Rees, *et al*, 1984]. Such rapid diffusion rates make the intermolecular model of receptor activation kinetically feasible [Schlessinger, 1988].

A general oligomerisation model has been proposed for activation of growth factor receptor kinases and has been confirmed for the EGFR . This model holds that inactive receptor monomers exist in a state of equilibrium with active receptor dimers and that ligand binding increases the receptors affinity for its neighbours and stabilises the dimeric form [Schlessinger, 1988]. Receptor dimers possess enhanced binding affinity for EGF and elevated levels of PTK activity. Although it has been assumed that stabilisation of the dimeric form of the receptor is due to a conformational change induced by ligand binding, some evidence now exists that EGF may be a dimer in solution [Lemmon, *et al*, 1994].

Receptor binding studies in a number of cell types have shown that the EGFR exists in two forms with distinct affinities for EGF. 5-10% of the total receptor is a high affinity form with K_D of 1-3 $\times 10^{-10}$M while the remaining receptors have a low affinity with K_D 2-15 $\times 10^{-9}$M. Mitogenicity has been attributed to the high affinity receptors [Shechter, *et al*, 1978; Kawamoto, *et al*, 1983; King, *et al*, 1982] It has been proposed that the high affinity sites represent oligomeric forms of the receptor while the low affinity form represents receptor monomers. Measurements of lateral diffusion suggested that the low affinity receptors are highly mobile while the high

affinity types are essentially immobile which would be consistent with the oligomerisation model [Rees, *et al*, 1984].

This allosteric model of activation can also be applied to heterodimerisation such as the interaction between EGFR and erbB-2 and other members of the family of type 1 growth factor receptors. Although erbB-2 does not bind EGF, EGF stimulation of cells results in rapid tyrosine phosphorylation of erbB-2. This could occur via the formation of heterodimers in which EGFR activates erbB-2. Heterodimers of EGFR and c-erbB-2 which have a higher affinity for EGF than EGFR homodimers have been demonstrated and in cells expressing both molecules, heterodimerisation is preferred [Dougall, *et al*, 1993]. Heterodimerisation of members of the EGFR family may serve to increase the diversity of signalling both through altered ligand binding and interaction with cytoplasmic signalling pathways. Since EGFR and c-erbB-2 have different autophosphorylation sites, each would recruit a different set of downstream proteins (Section 2.3.4.) and this has the potential to increase the diversity of signalling [Lemmon, *et al*, 1994]. The existence of two further members of the family which can form hetero-oligomers, c-erbB-3 and c-erbB-4, further increases this potential [Carraway, *et al*, 1994].

2. 3. 4. Receptor autophosphorylation and signal transduction

Dimerisation and kinase activation of the EGFR leads to receptor auto-phosphorylation and a further conformational change to facilitate binding and phosphorylation of cellular substrates [Dickson, *et al*, 1996a]. Evidence exists that autophosphorylation is an intermolecular process. Cross-phosphorylation of tyrosine kinase negative mutants occurs when they are coexpressed with wild type receptors. These mutant receptor are described as dominant -negative since they form inactive heterodimers with wild-type receptors when expressed in the same cell and inhibit the mitogenic response via the wild-type EGFR [Honegger, *et al*, 1989 & 1990].

Autophosphorylation plays an important regulatory role in kinase activity [Bertics, *et al*, 1985]. Autophosphorylation sites in the EGFR lie at the C terminal of the receptor not within the kinase domain as in other receptors [Carpenter, 1987]. The sites can act as alternative substrates for the EGFR kinase - they can competitively inhibit the receptor but do not affect Vmax. Autophosphorylation acts to remove this competitive inhibition and allow exogenous substrates to be phosphorylated [Bertics, *et al*, 1988]. The molecule v-erbB which lacks this regulatory domain is proposed to function as an upregulated tyrosine kinase in virally transformed cells [Carpenter, 1987].

Autophosphorylation sites also act as specific recruitment sites for proteins which transmit the signal downstream of the receptor. These proteins are characterised by a conserved domain of approximately 100 amino acid residues known as a src-homology-2 (SH2) domain. This region forms a binding pocket for the phosphorylated tyrosine and the immediate surrounding residues on the receptor and thus mediates the interaction between the activated receptor and downstream signalling proteins [Boonstra, *et al*, 1995]. At least 7 SH2 domain containing proteins have been shown to associate with the EGFR including phospholipase $C\gamma_1$ ($PLC\gamma_1$), the ras GTPase activating protein (ras-GAP) and the p85 subunit of phosphoinositol-3-kinase [Margolis, *et al*, 1989; Ellis, *et al*, 1990; Hu, *et al*, 1992]. Further interactions occur indirectly via docking protein such as the adaptor protein ShC [Dickson, 1996a]. The specificity of the interaction of the tyrosine kinase with its substrates is determined by the sequences surrounding the autophosphorylation site and sequences within each SH2 domain [Carpenter, 1992]. The formation of receptor signalling complexes mediate the major effects of ligand-EGFR interaction on ion fluxes, cellular phosphorylation events, gene expression, DNA synthesis and malignant growth [Dickson, 1996a].

One of the first characterised signal transduction cascades activated by the EGFR was PLCγ and turnover of phosphatidylinositol bisphosphate (PIP2) [Margolis, *et al*, 1989; Meisenhelder, *et al*, 1989]. PLCγ catalyses PIP2 to produce two second messengers - inositol trisphosphate (IP3) and diacylglycerol (DG). IP3 acts to release Ca^{2+} from intracellular stores resulting in raised intracellular Ca^{2+} levels and activation of the Ca^{2+}/calmodulin dependent serine/threonine kinase,, while diacylglycerol activates protein kinase c (PKC) [Carpenter, 1992]. Activation of PKC has a regulatory role since the EGFR itself is a substrate for PKC. Phosphorylation by PKC on C terminal residues including Thr 654 leads to decreased EGFR kinase activity, decreases the receptors affinity for its ligand and attenuates the biological response to EGF [Hunter, *et al*, 1984; Downward, 1985; Livneh, *et al*, 1988].

2. 3. 5. Receptor Internalisation and degradation

Electron microscope studies have shown that EGFR on the cell surface are initially diffusely distributed. On ligand binding they rapidly (within milliseconds) form clusters associated with coated pits and internalise - 84% of ferritin labelled EGF is internalised and localised to the lysosome after 30 min at 37°C [Haigler, *et al*, 1979]. This process is highly temperature dependent with a maximal rate at physiological temperature [Wiley, *et al*, 1982].

Ligand binding results in rapid internalisation of the receptor [Carpenter, 1987] and degradation of both ligand and receptor in the lysosome [Dunn, *et al*, 1983; Benguinot, *et al*, 1984]. As a result ligand binding results in a substantial decrease in the number of cell surface receptors: a process called receptor down-regulation. As much as 80% of a cell's surface binding can be lost after exposure to growth factor and growth factor treatment also increases the rate of receptor degradation [Sorkin, *et al*, 1993]. EGF rapidly produces a 10-fold increase in the rate of

receptor degradation (from a half life of 10 hours to 1 hour) but does not influence the biosynthesis of receptor molecules [Carpenter, 1985]. Receptors may also be recycled back to the cell surface after internalisation - this has been described in pancreatic carcinoma cells, 3T3 fibroblasts and hepatocytes [Korc, *et al*, 1985; Wakshull, *et al*, 1985; Gladhaug, *et al*, 1987].

EGFR receptors, unlike LDL receptors, only associate with clathrin coated pits after ligand binding. Ligand binding must therefore induce a conformational change in the receptor which facilitates clathrin binding [Schlessinger, 1986] Internalisation of complexes is essential for signal transduction [Decker, 1989]. C-terminal truncations of the receptor interfere with internalisation suggesting that intracellular sequences in the receptor are essential for endocytosis [Livneh, *et al*, 1986b]. The area of the receptor which appears to be essential for internalisation lies between residues 993 and 1022 at the C-terminus [Sorkin, *et al*, 1993]. These regions may be involved in interactions with clathrin coated pits [Sorkin, *et al*, 1996]. Tyrosine kinase activity is also essential for internalisation: internalisation is blocked by antibodies against phosphotyrosine [Glenney, *et al*, 1988] and studies have shown that a receptor deficient in PTK activity fails to cluster in coated pits or internalise [Lamaze, *et al*, 1995]. PTK activity appears to be essential for receptor degradation since the kinase negative mutant also failed to be targeted to the lysosome and was recycled to the cell surface [Honegger, *et al*, 1987b & 1989]. However, internalisation does not appear to be controlled by autophosphorylation since an EGFR lacking the major sites of autophosphorylation but possessing PTK activity, underwent normal ligand induced endocytosis [Glenney, *et al*, 1988]. The processes involved in normal receptor trafficking may be regulated by tyrosine phosphorylation of cellular substrates.

The role of internalisation is unclear - internalised receptor complexes appear to have a high tyrosine kinase activity towards exogenous substrates [Cohen, *et al*, 1985]. This may partly explain why although the initial process of activation and internalisation is very rapid, continual occupation of receptors for up to eight hours is required to commit Go cells to DNA synthesis - internalised receptor or its degradation products may be involved in this signalling [Kay, *et al*, 1986]. Internalisation also serves to remove complexes from the cell surface and attenuation of the signal by internalisation may play an important role in modulating the cells proliferative response to EGF. Mutant receptors which have active tyrosine kinase but do not undergo internalisation, induce mitogenesis and transformation of normal fibroblasts at much lower ligand concentrations than wild type receptors [Wells, *et al*, 1990].

3. EGFR family and cancer

Abnormalities in growth factor signalling pathways, particularly altered levels of expression of normal receptor proteins and mRNAs, are very common in human malignancies. This frequency of occurrence suggests that overexpression of a normal growth factor or its receptor is a cause and not a consequence of malignant transformation. Such overexpression may confer a selective growth advantage on a cell progressing along the malignant pathway by enhancing the level of stimulation of proliferation [Gullick, 1991]. Since members of the EGFR family and their ligands are frequently overexpressed they have been implicated in breast cancer development

3. 1. EGFR in breast cancer

Although studies have demonstrated oncogenic activation of the EGFR by structural alterations, receptor overexpression is a much more common feature of human cancers [Dickson, *et al*, 1996a]. The first demonstration of elevated levels of EGFR was in frozen sections of lung tumours [Hendler, *et al*, 1984]. Normal breast

epithelial cells express the EGF receptor at a low level while elevated expression is found in many breast cell lines and also in breast tumours [VanAgthoven, *et al*, 1994]. EGFR expression is found in approximately 35-60% of primary breast tumours with no clear differences in positivity observed between the different assay methods used [Clark, 1996]. Many different tumour types have now been examined for elevated levels of the receptor and a significant number of tumours of the brain, lung, bladder, head and neck, and gastric tumours have been shown to overexpress EGFR. Overexpression has also been detected in cell lines derived from many of these tumour types [Gullick, 1990]. In breast cancer, overexpression is only rarely due to amplification of the EGFR gene -only 2% of cases. Differences in expression occur at the transcription and protein levels [Chrysogelos, 1994]. By contrast, in head and neck tumours the most common mechanism of overexpression is gene amplification [Dickson, *et al*, 1996a].

In 1987, the EGFR and its ligands were directly implicated in the process of cancer development. Overexpression of normal EGFR in transfected fibroblasts resulted in cell transformation but only in the presence of the ligand, EGF [Di Fiore, *et al*, 1987a]. Coexpression of EGFR and TGFα in the same cells also resulted in transformation - a process mirrored in actual tumours where the genes for both the receptor and ligand are commonly overexpressed [Derynck, *et al*, *1987*; Di Marco, *et al*, 1989; Castellani, *et al*, 1994]. High TGFα levels and elevated EGFR expression were also found in cell lines derived form breast tumours [Ennis, *et al*, 1989]. TGFα can induce cell transformation in culture but is known to be only weakly transforming alone [DeLarco, *et al*, 1978; Derynck, 1988]. It has been proposed that TGFα functions in malignant transformation via an autocrine loop i.e. the tumour produces TGFα which stimulates its own proliferation via the EGFR. In tumours expressing both components of this autocrine system i.e. TGFα or EGF and the EGFR, growth may proceed at an advanced rate. Autocrine growth may account for the reduced requirement for serum for the growth of transformed cells

in culture since these would have a lesser need for exogenous growth factors [Sporn, *et al*, 1980].

In ER positive cell lines expression of EGF and TGFα has been shown to be influenced by estrogen [Bates, *et al*, 1988]. Both these growth factors have been shown to be produced by breast tumours, although TGFα is perhaps the principal ligand in tumour tissue. Thus it is possible that, in addition to its direct effect on breast tissue, estrogen exerts its mitogenic effect indirectly by stimulating the production and secretion of peptide mitogens such as TGFα which in turn stimulate cell growth [Kon, 1989]. Conversely, the antiestrogen tamoxifen inhibits estrogen stimulated secretion of TGFα and EGF and reduces the amount of TGFα in human breast cancer biopsies [Dickson, *et al*, 1987; Gullick, 1990]. Abnormal or constitutive overexpression of these secondary factors may represent one mechanism by which cells overcome their estrogen dependence. In hormone independent breast cell lines, TGFα is produced constitutively which is consistent with the idea that growth factors are involved in a more malignant, hormone independent phenotype [Dickson, *et al*, 1988].

Evidence exists which indicates an *in vivo* role for the TGFα/EGFR pathway in the development of breast cancer. Transgenic mice which overexpress TGFα in multiple tissues, frequently develop adenocarcinoma of the mammary gland. This suggests TGFα is oncogenic in breast tissue *in vivo* [Sandgren, *et al*, 1990].

3. 2. c-erbB-2 in breast cancer

c-erbB-2 is normally expressed at low levels in epithelial and myoepithelial cells of normal breast tissue [Gullick, 1990]. The c-erbB-2 gene is amplified and/or overexpressed in between 10% and 30% of breast, ovarian and gastric cancers and occasionally, in some other tumour types such as colon, oesophageal, kidney and bladder cancers [Gullick, *et al*, 1989]. The most common mechanism (90% of

cases) resulting in high c-erbB-2 protein expression is gene amplification [Hynes, *et al*, 1994]. c-erbB-2 overexpression has been found in all stages of breast carcinoma, but not benign breast disease suggesting the gene is not amplified before the onset of true malignancy and is an early event in tumorigenesis [Gusterson, *et al*, 1988]. Metastatic lesions continue to express c-erbB-2 suggesting a continuing function for the protein [Hynes, *et al*, 1994]. c-erbB-2 has also been examined for its prognostic significance since an early study demonstrated an association between amplification of c-erbB-2 oncogene and poor prognosis in breast cancer [Slamon, *et al*, 1987] (Section 3.3.2.).

It has been demonstrated that overexpression of the normal c-erbB-2 gene also results in transformation of fibroblasts. This overexpression was suggested to be more potent as a transforming agent than overexpression of EGFR [Di Fiore, *et al*, 1987b]. Analysis of the c-erbB-2 gene in human breast cancer cells has demonstrated amplified levels consistent with this model of transformation [Gullick, 1990]. There is no evidence to suggest that a structural mutation similar to that found in the transmembrane region of neu is involved in human breast cancer [Lemoine, *et al*, 1989].

It remains to be determined if c-erbB-3 and c-erbB-4 are similarly overexpressed in human cancers although expression of both proteins has been detected in human breast cancer cells [Kraus, *et al*, 1989; Plowman, *et al*, 1993].

3. 3. EGFR family and prognosis in breast cancer

3. 3. 1. EGFR

In 1985, a study of 104 breast tumours reported that there was a significant inverse relationship between EGFR and ER and that a greater number of EGFR were found in metastatic tumours compared to primary tumours [Sainsbury, *et al*, 1985]. A further study of 135 primary tumours confirmed the existence of an inverse

relationship with ER status and suggested EGFR as a potential indicator of prognosis. EGFR positivity in primary tumours was higher in node-positive than node-negative tumours and EGFR-positive tumours had a significantly lower relapse free survival (RFS) and overall survival (OS) than EGFR-negative tumours. EGFR was found to subdivide the ER-positive group into good and poor prognosis subgroups [Sainsbury, *et al*, 1987]. Several studies have now reported on the relationship between EGFR status and survival. Of fifteen studies reviewed in 1994, five studies showed a significant relationship between EGFR status and RFS and OS [Klijn, *et al*, 1994]. In one study EGFR status was the most important variable in determining RFS and OS in node negative patients and second only to lymph node status in node positive [Sainsbury, *et al*, 1987]. Prognostic factors are of particular importance in the node-negative subgroup in making decisions about adjuvant therapy. However, although EGFR had prognostic significance at 5 year follow-up, this significance was lost by 10 years [Klijn, *et al*, 1994]. Such a loss of prognostic significance with long term follow-up has also been reported for ER status [Clark, 1996].

The role of EGFR as a predictive factor for hormone responsiveness or resistance is perhaps better defined. Cell culture studies suggest an association between EGFR and hormone resistance. Transfection of EGFR into hormone dependent cancer cells induces progression to a hormone independent state and acquired tamoxifen resistance in cell lines is associated with elevated EGFR [Van Agthoven, *et al*, 1992; Long, *et al*, 1992]. Several studies in breast cancer patients have shown that EGFR positive tumours, even when also ER positive, are more likely to be resistant to endocrine therapy which suggests that coexpression of EGFR tends to overide the beneficial effects of ER status [Nicholson, *et al*, 1989, 1991 & 1993]. Conversely EGFR negative patients, particularly if also ER positive, tend to have high response rates to endocrine therapy [Clark, 1996]. One study reported that the response rate for such patients is 80% with 43% achieving complete or partial

remission [Nicholson, *et al*, 1994]. EGFR may therefore identify a poor prognosis subgroup of patients best treated with initial chemotherapy [Klijn, *et al*, 1993].

Since the first demonstration of a relationship, many groups have also demonstrated a strong negative relationship with ER status [Clark, 1996] Mean EGFR positivity is twice as high in ER- or PR-negative patients than in ER- or PR-positive patients [Klijn, *et al*, 1992]. Such inverse correlation is also found in breast cell lines [Davidson, *et al*, 1987]. It appears that in breast tumours, but not in normal tissue, ER and EGFR expression are mutually exclusive within individual cells although both may be present in the tumour [Van Agthoven, *et al*, 1994]. There is also evidence of a statistically significant association between EGFR and poor tumour grades and increased proliferation indices [Clark, 1996]. A few studies report an association with age, menopausal status, histological type, tumour size and axillary node status but evidence on these associations is generally unclear or conflicting [Klijn, *et al*, 1992]. EGFR may also be correlated with overexpression of mutated p53 or angiogenesis factors [Clark, 1996].

Studies in multi drug resistant cell lines have shown onset of drug resistance is associated with enhanced expression of EGFR and hormone insensitivity [Vickers, *et al*, 1988; Dickstein, *et al*, 1993]. EGF treatment has been shown to protect breast tumour cells from actinomycin D cytotoxicity which suggests a role for EGFR in the drug resistant phenotype. It is possible that EGFR signalling pathways control p-glycoprotein function at transcriptional or protein levels and would be consistent with data which suggests that EGFR positive tumours respond less well to chemotherapy [Davies, *et al*, 1996].

3. 3. 2. c-erbB-2

An initial study of c-erbB-2 in breast cancer showed a strong association between c-erbB-2 and short relapse free interval and survival [Slamon, *et al*, 1987]. c-erbB-2

has been proposed as an independent indicator of poor prognosis in node positive breast cancer patients [Slamon, *et al*, 1989]. Further studies which examined the ability of c-erbB-2 status to predict clinical behaviour, particularly those involving large patient numbers, have also tended to reveal a significant association between elevated levels of c-erbB-2 and poor prognosis in node positive patients [Barnes, 1993]. Conflicting evidence does exist on the prognostic significance of c-erbB-2 in node positive patients and c-erbB-2 is not generally found to be prognostic in the node negative subgroup [Hartmann, *et al*, 1993; Clark, 1996]. An association between c-erbB-2 and poor prognosis has also been demonstrated in ovarian and lung cancers [Slamon, *et al*, 1989; Kern, *et al*, 1990].

Some studies suggest that co-expression of EGFR and c-erbB-2 has an additive adverse effect and this may reflect the high PTK activity of the heterodimers [Harris, *et al*, 1989; Wright, *et al*, 1992a]. Cell culture studies show that coexpression of EGFR and c-erbB-2 at levels which are not on their own transforming induces transformation of mouse fibroblasts [Kokai, *et al*, 1989]. There is however no consensus on a positive relationship between EGFR and c-erbB-2 in breast cancer [Klijn, *et al*, 1992]. Studies suggest that while erbB-2 is an independent prognostic indictor, a combination of c-erbB-2 and EGFR expression has an additive adverse effect [Harris, *et al*, 1989; Nicholson, *et al*, 1990].

ErbB-2 expression has also been studied as a predictive indicator for response to hormonal and chemotherapy. Some evidence suggests that c-erbB-2 overexpression may be associated with a reduced response to endocrine therapy and chemotherapy [Barnes, 1993]. Several studies have shown that erbB-2 expression correlates with a poor response to tamoxifen [Nicholson, *et al*, 1990; Wright, *et al*, 1992a; Hynes, *et al*, 1994]. The association between c-erbB-2 and poor response to tamoxifen is found in both ER-positive and -negative tumours [Nicholson, *et al*, 1990]. In ER positive breast cell lines, tamoxifen increases erbB-2 expression and enhances

signalling via the erbB-2 pathway [Taverna, *et al*, 1994]. This is decreased by estradiol. Tamoxifen also induces the expression of erbB-2 immunoreactivity *in vivo* [Johnston, *et al*, 1993]. In one study patients with elevated c-erbB-2 who had a poor response to tamoxifen, responded to chemotherapy better than patients with low erbB-2 levels [Klijn, *et al*, 1993]. However other studies show that high erbB-2 correlates with a poor response to chemotherapy.[Gusterson, *et al*, 1992; Wright, *et al*, 1992b].

The relationship between c-erbB-2 expression and other indicators of prognosis has been extensively studied but many of these have produced conflicting results. Most studies found no association between c-erbB-2 and nodal status [Slamon, *et al*, 1987; Wright, et *al*, 1989]. The majority of studies relating c-erbB-2 to steroid receptor status demonstrate a moderately strong inverse relationship between expression and ER status and possibly a weaker relationship with PR status [Gullick, 1990]. c-erbB-2 may also be associated with increasing tumour grade and large tumour size [Gullick, 1990].

Definitive information is not yet available about the prognostic, or predictive significance, of c-erbB-3 or c-erbB-4 [Clark, 1996]. Preliminary studies however, suggest that erbB-4 may be an indicator of good prognosis [Bacus, *et al*, 1994].

3. 4. EGFR and c-erbB-2 as therapeutic targets

The association of expression of EGFR and c-erbB-2 and poor prognosis makes these receptors an obvious target for therapeutic intervention [Baselga, *et al*, 1994]. A problem arises in selective targeting to tumours since both receptors are expressed by normal tissue in a non-mutated form. One area of interest is interfering with signal transduction via the receptor. Tyrosine kinase inhibitors such as tyrphostins are being examined for their anticancer potential [Reddy, *et al*, 1992]. Although these compounds target the ATP binding site common to many

kinases, specificity to individual tyrosine kinases has been achieved. One compound PD153035 has a Ki of 5pM for EGFR whereas other tyrosine kinases are inhibited at micromolar concentrations [Fry, *et al*, 1994]. Antibodies raised against the ligand binding site of the EGFR can block cell proliferation and a number of such antibodies have entered clinical trials [Baselga, *et al*, 1994].

4. Membrane fluidity

4. 1. Introduction

Membranes are of fundamental importance to cell structure and function. All biological membranes have a common composition and structure with the major constituents being lipids, such as phospholipids, and proteins. The fluid mosaic model proposed by Singer and Nicholson in 1972 is still the simplest structural description of biological membranes. In this model, membrane lipids are organised into a bilayer which is associated with both integral and peripheral membrane proteins [Singer, *et al*, 1972]. The membrane is not rigid but is essentially a two-dimensional dynamic structure in which both lipids and proteins freely diffuse.

There are three potential forms of movement for membrane components:

(i) Lateral diffusion where molecules move within the plane of the membrane.

(ii) Rotation of membrane components along axes perpendicular to the plane of the membrane.

(iii) Transbilayer movement or 'flip-flop' from one leaflet of the bilayer to another. This would be highly energetically unfavourable for proteins with the need for large hydrophilic peptides to traverse the hydrophobic interior of the bilayer, but enzymes (flippases) do exist which speed the rate of flip-flop of lipids [Menon, 1995].

In many respects, the classic fluid-mosaic model is an oversimplification. Different classes of lipids are not homogeneously distributed throughout the membrane but may exist in distinct domains which have a different structure and physical properties to other parts of the membrane [Klausner, *et al*, 1980; Tocanne, *et al*, 1988]. The distribution and mobility of membrane proteins also varies. Some proteins, such as rhodopsin, may be free to diffuse while others are partly or even completely immobilised. In reconstituted synthetic bilayers proteins may diffuse freely and rapidly, but the majority of membrane proteins *in vivo* either diffuse at a very slow rate or are essentially immobile [Cherry, 1979].

4. 2. Factors which affect lipid and protein fluidity

Membrane lipid fluidity is influenced by a number of factors. The most important parameter in determining membrane fluidity is the cholesterol/ phospholipid ratio - the higher this ratio the lower the membrane fluidity. Cholesterol increases membrane microviscosity which increases the degree of order of the lipid domain [Shinitsky, 1984]. The phospholipid sphingomyelin has a similar effect due to the rigid conformation of its apolar chains at physiological temperatures [Van Blitterswijk, 1984]. The next most important parameter determining membrane fluidity is the degree of unsaturation of the phospholipid acyl chains. Polyunsaturated fatty acids such as linolenic acid and arachidonic acid have a marked fluidising effect on the membrane [Van Blitterswijk, 1988]. Finally, membrane proteins have an ordering effect on the membrane similar to the effect of cholesterol [Van Blitterswijk, 1984].

The lateral diffusion of membrane proteins may be constrained in several ways. This may be the result of viscous resistance of the bilayer or the formation of aggregates [Jacobsen, *et al*, 1987]. Steric inhibitions due to high concentrations of receptor in the membrane can also restrict protein diffusion. Studies in liposomes reveal that the diffusion of bacteriorhodopsin decreases with increasing protein

content [Jacobsen, *et al*, 1987]. Protein lateral diffusion may also be constrained by interactions between the protein and immobile elements in the cell interior. Lateral diffusion of the erythrocyte Band 3 protein is restricted by its interaction with spectrin in the red cell cytoskeleton while deletion of the cytoplasmic domain of major histocompatibility (MHC) class II antigens significantly increases their lateral diffusion [Wade, *et al*, 1989]. However, the EGFR, like MHC Class 1 antigens and VSV-G glycoproteins, appears not to be constrained by cytoskeletal interactions since deletions of large parts of the cytoplasmic domain do not affect its lateral diffusion [Livneh, *et al*, 1986a]. The extracellular matrix may also play a role in constraining protein diffusion - the major determinant of the mobility of VSV-G glycoprotein is located in the extracellular domain [Zhang, *et al*, 1992]. In confluent cultured cells the major constraint to lateral diffusion may be the association between major histocompatibility antigens and the extracellular matrix [Weir, *et al*, 1986]. Lateral diffusion of proteins in reconstituted lipid vesicles which lack a cytoskeleton or extracellular matrix is similar to that of lipids. Diffusion of the same proteins in intact cells is at least 100-fold slower suggesting that interactions with other elements of the cell are indeed responsible for the reduction in rate. The mobile fraction in intact cells is also typically less than the 100% measured in artificial bilayers [Jacobsen, *et al*, 1987].

Protein diffusion may also be affected by the presence of lipid domains which restrict the areas within which proteins are free to diffuse [Tocanne, *et al*, 1988]. For example, sodium channels in cultured neuronal cells have a rapid diffusion over the cell body with almost complete recovery, a reduced rate and extent of diffusion over the hillock region of the cell while at the presynaptic ending the channels are almost immobile [Angelides, *et al*, 1988]. Macrodomains are particularly well defined in epithelial cells where the maintenance of the cells polarity is critical in its function - it is probable that apical and basolateral domains are preserved by cytoskeletal attachments and the physical barrier formed by tight junctions

[Jacobsen, *et al*, 1987]. A large body of evidence suggests that smaller domains (microdomains) which differ in composition and/or physical state of lipids are also present in membranes. These domains could explain the existence of lipid immobile fractions which are a common feature of many cells and may have implications for the function of proteins located within them [Klausner, *et al*, 1980; Tocanne, *et al*, 1988].

4. 3. Measurement of membrane fluidity

Lateral diffusion of membrane proteins was first demonstrated in 1970 by a fusion experiment. A mouse and a human cell line were labelled with rhodamine and fluorescein respectively which allowed the surface antigens from each to be distinguished. After fusion of the two cell types, it was observed that protein diffusion must have occurred since the two labels were both heterogeneously distributed over the surface of the heterokaryon [Frye, *et al*, 1970].

The rate of lateral diffusion of a protein was first quantified in 1974. The lateral diffusion coefficient (D_L) of rhodopsin in the disc membrane of rod outer segments was estimated to be 3.5×10^{-9} cm^2s^{-1}. In this experiment rhodopsin in one half of the membrane was bleached with a pulse of light to create an asymmetric distribution of rhodopsin across the rod. The rate of return to a symmetric distribution was then measured spectrophotometrically [Poo, *et al*, 1974]. This technique formed the basis of fluorescence recovery after photobleaching or FRAP (also known as fluorescence photobleaching recovery (FPR) or fluorescence microphotolysis).

FRAP enables the study of the lateral motion of lipids and proteins within the intact bilayer of single cells. Poo and Cone utilised the intrinsic natural chromophore of rhodopsin, retinal, but in the majority of experiments the molecule of interest must first be labelled . Fluorescent labels allow a high level of sensitivity of detection.

Methods of labelling include the insertion of fluorescent lipid analogues into the bilayer [Cherry, 1979], labelling of proteins with fluorescent antibodies, Fab fragments or lectins [Jacobsen, *et al*, 1984; Jesaitis, *et al*, 1986] or the use of fluorescent ligands to label receptors [Shechter, *et al*, 1978]. The advantage of the FRAP technique is that it is non-invasive and can be applied to a single intact cell or even a small section of the cell surface [Cherry, 1979].

The FRAP technique is discussed in more detail in Chapter 2 but briefly the technique is as follows. A low power argon laser beam is focused on a small area (1.5μm) of membrane. The laser light excites the fluorophores in this area causing them to fluoresce. The power of the laser is then briefly increased which irreversibly bleaches the fluorescence in that area of the membrane and creates a concentration gradient of labelled molecules. By monitoring changes in the level of fluorescence the recovery of fluorescence due to unbleached molecules diffusing into the area can be followed. Figure 2 shows an idealised recovery curve of the type obtained by this method. Information on both the rate of recovery and the fraction of molecules which are free to diffuse can be obtained by analysis of such traces [Axelrod, *et al*, 1976] (Section 6.2). Typically the measured recovery in cell membranes is less than 100% indicating that a proportion of the total protein is immobile within the timescale of the experiment. FRAP experiments have determined that the diffusion coefficients (D_L) of lipids are in the order of 10^{-8} cm^2s^{-1}. The lateral mobility of proteins varies markedly from a highly mobile protein like rhodopsin with a D_L of $4x10^{-9}cm^2s^{-1}$ to a relatively immobile protein like the major surface glycoprotein of fibroblasts with a D_L of $10^{-12}cm^2s^{-1}$ [Cherry, 1979].

A number of other techniques exist to measure fluidity including fluorescence polarisation, nuclear magnetic resonance and electron spin resonance [Van Blitterswijk, 1984]. Fluorescence polarisation is probably the most widely used of

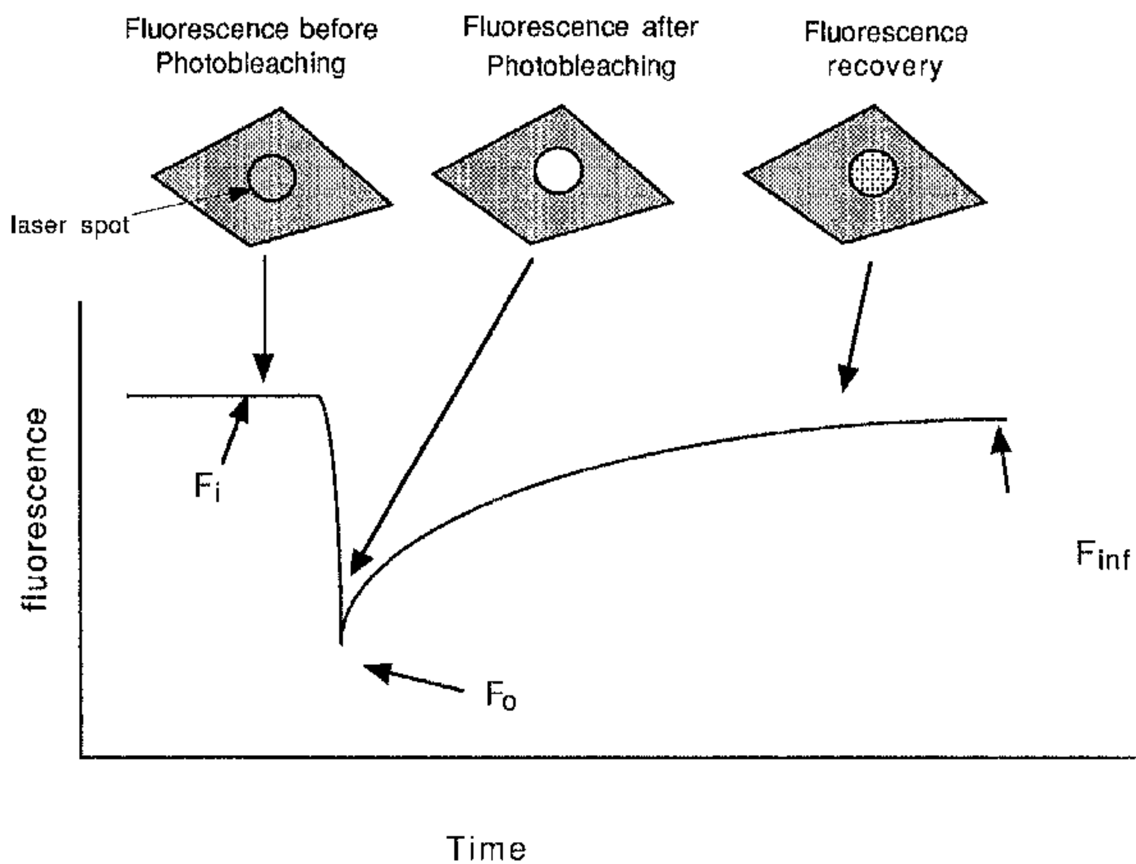


Figure 2. Principle of fluorescence recovery after photobleaching (FRAP) - diagram of an idealised recovery curve obtained by FRAP

these techniques. Fluorescent probes inserted into the membrane are excited with plane-polarised light. The polarisation of the emitted light will vary depending on the extent of molecular motion during the lifetime of the excited state from fully polarised, when the fluorophore is rigidly fixed, to unpolarised if the fluorophore moves rapidly. Comparison of the steady-state fluorescence polarisation of the same probe embedded in different membrane environments gives an assessment of the relative fluidity of these membranes [Van Blitterswijk, 1981]. The most popular probes used for fluorescence polarisation are 1,6-diphenyl-1,3,5,-hexatriene (DPH) and its 4'-trimethylamino derivative (TMP-DPH) [Bashford, 1994]. In whole cells these membrane permeant probes will report an average fluidity weighted according to the distribution of dye among varying membrane compartments including endomembranes and cytoplasmic lipid droplets [Van Blitterswijk, *et al*, 1981]. The probes also partition equally into lipid phase domains and thus the measured fluidity gives no resolution of membrane domains.

4. 4. Criticisms of the FRAP technique

The use of a very intense bleaching pulse of laser light in FRAP measurements has led to a number of criticisms of this technique. It has been suggested that the laser is responsible for the slow rates of protein diffusion and incomplete recoveries. Although intense light may cause protein cross-linking, this does not appear to be a problem with the short time of bleach pulse in experimental FRAP [Sheetz, *et al*, 1979]. Examination of cells after FRAP measurements by scanning electron microscopy showed no evidence of damage to the cell membrane. The cells were also shown to remain viable and thus it was concluded that cellular damage caused by FRAP is not responsible for low recovery rates and slow diffusion coefficients observed in some experiments [Jacobsen, *et al*, 1978]. Values for rhodopsin fluidity obtained by FRAP closely agree with those obtained by the original absorption experiment [Wey, *et al*, 1981], In addition, values obtained by FRAP for H2 antigens from mouse fibroblasts were similar to the heterokaryon experiments

[Edidin, *et al*, 1982]. Recovery kinetics are also not apparently affected by repeated bleaching of the same spot or by ten-fold changes in bleaching intensity [Jacobsen, *et al*, 1978]. Local heating effects on the cell surface could conceivably cause problems with FRAP. However, calculations show that any local heating produced by FRAP is negligible (0.03%) [Axelrod, 1977]. Thus damage to cells induced by the technique of FRAP itself does not appear to be a significant factor influencing the experimental data produced by such studies.

4. 5. Role of membrane fluidity in cellular functions

It is now clear that membrane fluidity strongly influences many cellular processes including activity of membrane associated enzymes, receptors and channels [Shinitsky, 1984]. The enzyme adenylate cyclase is highly dependent on membrane fluidity. Adenylate cyclase activity decreases when membrane fluidity is decreased by increased cholesterol content. This is probably due to decreased efficacy of collision coupling between the enzyme and the occupied receptor [Houslay, 1985]. In the retina, lateral diffusion of bleached rhodopsin molecules is a critical step whereby photons are converted to electrical signals [Axelrod, 1983]. Lateral diffusion allows a single photoexcited rhodopsin molecule to activate the G-protein transducin and triggers a cascade which leads to hyperpolarisation of the outer rod segment and subsequent nerve transmission [Stryer, 1987]. Lateral diffusion may also play a role in cell invasion by viruses. During Semliki Forest virus infection, newly synthesised viral glycoproteins diffuse from random sites of incorporation in the cell membrane to form aggregates at the viral capsid. Cells with greater fluidity are more susceptible to expression of early viral antigens during Epstein Barr virus infection [Pozzi, *et al*, 1996].

Membrane fluidity and lateral diffusion are important in the activation of many cellular receptors. The binding characteristics of cell surface membrane antigens and receptors to their ligands has been shown to be altered by modification of the

membrane lipid composition and fluidity [Axelrod, 1983; Neufeld, 1986]. Clustering of many receptors such as the insulin and EGF receptors is an essential step in their activation (Section 2.3.3.). Multivalent antibodies which can induce receptor clustering, can induce some of the mitogenic effects of EGF and results in internalisation of the receptor. However, monovalent Fab fragments which cannot induce clustering, do not have mitogenic activity although this can be restored by crosslinking the fragments with anti-immunoglobulin antibodies [Schreiber, *et al*, 1983]. Since these receptors are initially diffusely distributed on the membrane surface [Schlessinger, *et al*, 1978a], aggregation presumably involves lateral diffusion of receptor monomers . Both the EGFR and the insulin receptor have been demonstrated to be laterally mobile by FRAP [Schlessinger, *et al*, 1978b]. Clustering is also essential for internalisation of receptors. Thus both the activation and down-regulation of transmembrane tyrosine kinase receptors is dependant on the lateral motion of receptor molecules within the bilayer.

Carrier-mediated transport processes are also affected by membrane fluidity [Axelrod, 1983]. An increase in cholesterol content, which would stabilise lipid fluidity of the membrane, increases the activity of the organic cation transporter in renal brush border membranes [Nabekura, *et al*, 1996]. The activity of p-glycoprotein, the membrane associated ATP dependent pump which is involved in multi-drug resistance is altered by agents which modulate membrane fluidity such as tamoxifen or detergents (Section 4.6.2.). Increased membrane fluidity or rigidity may alter the activity of channels by increasing membrane resistance to protein conformational changes [Wadkins, *et al*, 1993].

4. 6. Membrane fluidity and cancer

Many studies have examined changes in membrane fluidity in the development of cancer. Factors examined include both the chemical composition of membrane such as cholesterol content or the degree of unsaturation and physical techniques for

measuring fluidity such as fluorescence polarisation and FRAP [van Blitterswijk, 1984]. Changes in fluidity parameters have been found in almost all types of tumour cell examined but the physiological significance of such changes remains unclear. Alterations may be commonly found because tumour cells lack the homeostatic mechanisms which maintain correct fluidity in normal cells [Shinitsky, 1984].

Transformed fibroblasts have reduced lipid fluidity relative to non-transformed cells [Kawasaki, *et al*, 1989]. Leukaemia cells have been extensively examined for changes in both membrane composition and fluidity. The consensus of these studies is that leukaemia cells have increased membrane fluidity relative to normal blood cells. This is mainly due to a decrease in the cholesterol/phospholipid ratio [Deliconstantinos, 1987]. The magnitude of the increased membrane fluidity reflects the virulence of the leukaemic cells [Shinitsky, 1984]. The best studied solid tumour type, hepatomas, consistently show an increased cholesterol/ phospholipid ratio and an increased DPH fluorescence polarisation value indicating such tumours have a more rigid plasma membrane compared to normal liver cells [Galeotti, *et al*, 1984; Cheeseman, 1993]. Unfortunately, although membrane changes are relatively common in cancers, little overall consensus about the type of change exists [Van Blitterswijk, 1984].

Studies which have examined membrane fluidity in relation to metastasis have generally shown that increased metastatic potential is associate with increased membrane fluidity [Deliconstantinos, 1987; Taraboletti, *et al*, 1989]. This may be due to alterations in the mobility and cell surface exposure of antigens, receptors and adhesive molecules which may affect the metastatic process. Adhesion of tumour cells to endothelial cells is enhanced by increased membrane fluidity as a result of decreased cholesterol synthesis [Ramachandran, *et al*, 1986].

Changes in membrane fluidity may be important in regulating the growth and metastasis of tumour cells by altering the activity of membrane bound receptors, enzymes and pumps (Section 4.5). Membrane modifying agents may therefore be effective against tumour growth by altering the activity of these molecules [Grunicke, 1991]. Changes already present in tumour membrane composition and fluidity may result in the cancer cell having a disadvantage compared with normal cells when treated such agents. Membrane active agents are being studied in an attempt to overcome drug resistance (Section 4.7). Alterations in membrane fluidity may also have an impact on immunotherapy for tumours by exposing or masking antigens and receptors on the cell surface. Increased rigidity of tumour cell membranes has been used to enhance their immunogenicity by exposing cryptic antigens [Skornik, *et al*, 1984].

4. 7. The cell membrane in drug resistance

4. 7. 1. Changes in the plasma membrane in drug resistant cells

A number of studies have also looked at fluidity of drug resistant cells relative to their drug sensitive parent cells. Doxorubicin resistant P388 cells have a higher degree of lipid structural order and decreased fluidity relative to the parent cell line [Ramu, *et al*, 1983]. In chinese hamster ovary cells a decrease in membrane fluidity is consistently found both in cells which are selected for drug resistance and cells which are transfected with the mdr gene [Callaghan, *et al*, 1992]. However these observed differences in fluidity are not consistent between different resistant cell lines - other studies report increases in membrane fluidity [Wheeler, *et al*, 1982; Rintoul, *et al*, 1984]. Changes in fluidity may be associated with alterations in membrane lipid composition. Although studies of drug resistant cells have shown no differences in gross lipid composition, significant differences may exist in minor lipid components [Callaghan, *et al*, 1992]. For example drug resistant P388 cells have an increased triacylglycerol content and phosphatidylethanolamine: sphingomyelin ratio relative to their drug sensitive parent [Ramu, *et al*, 1984a].

The drug resistant line also has a higher cholesterol and lower phosphatidylserine content. This change in lipid composition has been suggested to contribute to the drug resistant phenotype [Escriba, *et al*, 1990]. Alterations in membrane fluidity may also result from changes in plasma membrane protein content such as overexpression of p-glycoprotein or EGFR [Meyers, *et al*, 1986; Vickers, *et al*, 1988; Dickstein, *et al*, 1993]. Multidrug resistant cell lines have increased sensitivity to membrane active agents such as detergents and to disruption by physical shear. This could be due to disruption of normal membrane structure by lipid alterations or the high levels of membrane protein [Gottesman, 1993].

4. 7. 2. Agents which reverse drug resistance

Drug resistance is the major obstacle to successful treatment of cancer and the ability to overcome it is obviously a major goal in cancer chemotherapy. With the identification of p-glycoprotein as a mechanism for multidrug resistance, a target for anticancer therapy was identified. A number of agents which are capable of reversing p-glycoprotein mediated multi-drug resistance - chemosensitisers - have been identified both in vitro and in animal models [Lehnert, 1993]. It is believed that these compounds function by blocking p-glycoprotein mediated efflux of cytotoxic drugs thereby increasing the intracellular accumulation of the drug by 2- to 5- fold and restoring its cytotoxicity [Gottesman, *et al*, 1993].

An early observation was that the calcium channel blocker, verapamil, was capable of circumventing multidrug resistance [Tsuruo, *et al*, 1981]. Subsequently, a wide variety of drugs with a similar effect have been identified. Just as drugs transported by p-glycoprotein vary greatly, there is significant heterogeneity in drugs capable of reversing the phenotype. In addition to calcium channel blockers such as verapamil and nifedipine, calcium antagonists, steroids such as progesterone and tamoxifen and the immunosuppressants cyclosporin and FK506 have all been reported to circumvent multi-drug resistance [Gottesman, *et al*, 1993]. Tamoxifen's anti-mdr

action may be related to the drugs ability to reduce membrane fluidity [Kayyali, *et al*, 1994]. Many chemosensitisers are hydrophobic compounds which would be expected to interact with membrane lipids. P-glycoprotein function is affected by compounds which increase and decrease membrane fluidity. [Woodcock, *et al*, 1992; Sinicrope, *et al*, 1992; Callaghan, *et al*, 1993]. A number of compounds which reverse multidrug resistance, including tamoxifen, have entered clinical trials [Bosch, *et al*, 1996].

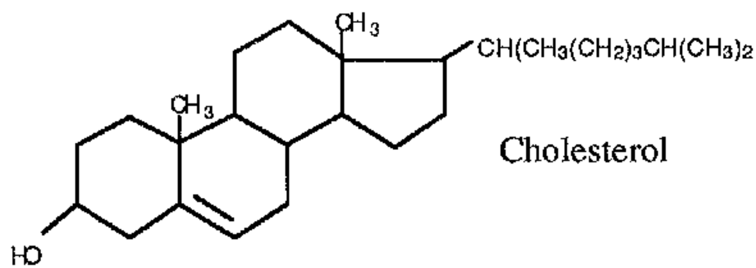
5. Tamoxifen

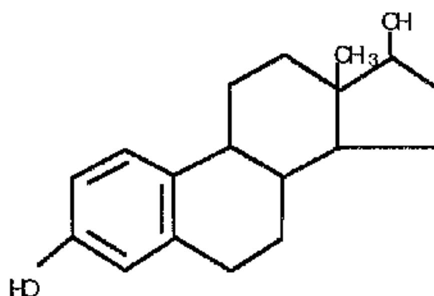
5. 1. Introduction

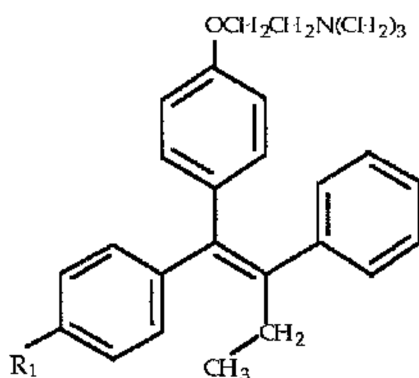
Tamoxifen, (1- (p- dimethylaminoethoxyphenyl)- 1,2- trans-diethyl-but-1-ene, ICI 46,474) (Figure 3) is a synthetic triphenylethylene compound synthesised during the 1950's and marketed by ICI under the brand name Nolvadex. It was initially developed as an anti-estrogen oral contraceptive but was found to be capable of inducing ovulation in subfertile women [Wolf, *et al*, 1995]. Subsequent research led to its use in endocrine therapy of breast cancer where the benefits of tamoxifen treatment were first observed in metastatic breast cancer: 22% of post-menopausal patients responded to treatment with tumour regression [Cole, *et al*, 1971]. Adjuvant use of tamoxifen produces survival advantages and prevents the appearance of a second primary breast cancer [Early Breast Cancer Trialists' Collaborative Group, 1992; Plowman, 1993]. Currently, tamoxifen is the first line endocrine treatment of choice in both pre- and post-menopausal women with all stages of breast cancer [Wolf, *et al*, 1995]. Trials are also underway to evaluate the efficacy of tamoxifen as a preventative treatment in women at high risk of developing breast cancer [Nayfield, *et al*, 1991].

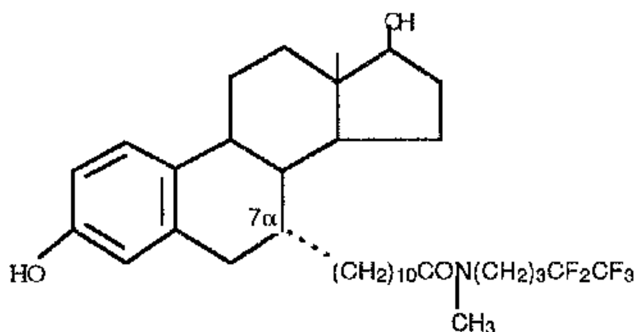
5. 2. Mechanism of action

Originally, the anti tumour action of tamoxifen was thought to be solely due to its ability to compete with estrogen for binding to the estrogen receptor which prevents









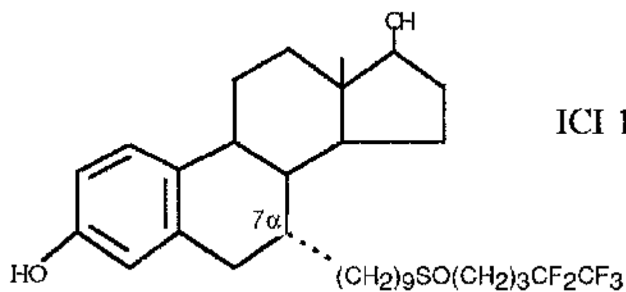


Figure 3. Diagram of the structures of cholesterol, estradiol, tamoxifen and ICI164 and ICI182,780

estrogen stimulation of growth [Wakeling, *et al*, 1984]. Molecular modelling has shown that the compound is indeed capable of adopting a structural conformation which closely resembles the steroid nucleus [Wiseman, *et al*, 1992]. Tamoxifen also exhibits partial estrogen agonist action which again may be related to its similarity in conformation to estrogen itself (see below).

The active metabolite of tamoxifen, 4-hydroxytamoxifen, even more closely resembles this structure due to the possession of an OH group at an analogous position to the steroid [Wiseman, *et al*, 1992]. 4-hydroxytamoxifen which is produced in humans by the metabolism of tamoxifen by cytochrome p450 has a greater biological activity than tamoxifen. 4-hydroxytamoxifen binds to the ER with high affinity which is almost equivalent to estradiol and, like estradiol, has a slow dissociation rate from the receptor and can translocate ER to the nucleus. [Wiseman, 1994b].

The action of tamoxifen is influenced by the biological context in which it acts. Depending on the system under investigation, tamoxifen may act as an estrogen agonist, partial agonist or an antagonist. The agonist action of tamoxifen is also probably related to its conformational similarity to estrogen. The action may vary between animal species, cell type and even vary in the same tissue between *in vivo* and *in vitro* conditions [Wakeling, 1987a]. The agonist action of tamoxifen may account for one of the drug's serious side effects - an increased risk of endometrial cancer. The endometrium is strongly stimulated by estrogens and tamoxifen action in this tissue is predominantly estrogenic [Wolf, *et al*, 1995]. The standard dose of tamoxifen of 10mg twice a day is associated with a doubling of the risk of endometrial cancer [Fisher, *et al*, 1994]. Tamoxifen also has beneficial side effects which have been ascribed to its estrogenic action. Tamoxifen has an estrogenic action in preserving bone density - of particular importance to post-menopausal women in preventing osteoporosis [Wolf, *et al*, 1995; Grainger, 1996]. Tamoxifen

also has an estrogenic effect on serum cholesterol levels acting to reduce levels of the serum lipids cholesterol, LDL and apolipoprotein B which are all risk factors for heart disease[Wiseman, 1994b].

The assumption that the tamoxifen acts simply by competitive inhibition of the estrogen receptor is an oversimplification. Both estrogen receptor positive and negative cancer cells have been shown to respond to the drug [Taylor, *et al*, 1984; Chouvet, *et al*, 1988; Langdon, *et al*, 1990]. Inhibition of cell growth by micromolar concentrations of tamoxifen is not overcome by estradiol suggesting that the drug is not solely acting via the ER [Darbre, *et al* , 1984; Reddel, *et al*, 1985]. Tamoxifen resistance, which often occurs after an initial period of responsiveness, is not associated with a loss of estrogen receptor and this is also the case in variants of MCF-7 cells which have developed tamoxifen resistance - these have identical receptor levels to the wild type cells [Kon, 1989].

Tamoxifen is known to have a number of effects unrelated to its ability to act via the estrogen receptor. It is known to inhibit both protein kinase C and Ca^{2+}-calmodulin-dependent cAMP phosphodiesterase [Lam, 1984; O'Brian, *et al*, 1986]. It may be that tamoxifen's ability to inhibit cancer growth is due in part to its anti-PKC activity since agents which block PKC are often effective at inhibiting the tumour promoting activity of phorbol esters [Verma, 1988]. In hormone-dependent breast cell lines, tamoxifen can induce secretion of the negative growth regulator, transforming growth factor β (TGFβ) [Knabbe, *et al*, 1987]. Induction of TGFβ by tamoxifen in tumours has been reported and this occurs in both ER-positive and -negative tumours [Butta, *et al*, 1992] TGFβ has a negative effect on growth stimulation via the EGFR and this may partly account for the effects of tamoxifen on estrogen receptor negative cells [Arteaga, *et al*, 1988]. Tamoxifen also inhibits estrogen stimulated secretion of TGFα and EGF and long term tamoxifen treatment decreases EGFR expression [Dickson, *et al*, 1987; Berthois, *et al*, 1989].

Triphenylethylene antiestrogens such as tamoxifen may also exert their effects via interactions with a group of proteins called antiestrogen binding sites (AEBS) [Wolf, *et al*, 1995]. AEBS are membrane-associated microsomal proteins which have been found in cell types examined including breast cancer cells [Watts, *et al*, 1987].. Non-steroidal anti-estrogens bind to these sites with high affinity - the binding affinity to AEBS may be greater than to the ER. However, the actual cellular function of these proteins remains hypothetical [Wolf, *et al*, 1995].

Tamoxifen has been shown to affect the functioning of p-glycoprotein and thus potentiates the toxicity of chemotherapeutic drugs which are substrates for this protein. In cultured P388 leukaemia cells, tamoxifen can reverse doxorubicin resistance [Ramu, *et al*, 1984b]. Modulation of the action of p-glycoprotein has also been observed in other cell lines including breast cancer cells at clinically achievable concentrations of tamoxifen [Berman, *et al*, 1991; Leonessa, *et al*, 1994; De Vincenzo, *et al*, 1996]. This use for tamoxifen is now being investigated in clinical trials [Trump, *et al*, 1992; Millward, *et al*, 1992]. Although tamoxifen modulates the activity of pgp it is probably not a substrate for pgp [Wiseman, *et al*, 1992]. This action is unlikely to be mediated by the estrogen receptor since it can be demonstrated in ER negative cells [De Vincenzo, *et al*, 1996]. Tamoxifen is highly lipophilic and can incorporate into membranes, decreasing membrane fluidity by a mechanism similar to cholesterol [Custodio, *et al*, 1993a]. Its ability to inhibit pgp may arise from this ability to decrease membrane fluidity sufficiently to sterically inhibit pgp function [Kayyali, *et al*, 1994]. Tamoxifen has also been shown to inhibit HIV virus production by cultured infected cells [Laurence, *et al*, 1990]. This may be again due to its ability to decrease membrane fluidity which would inhibit lysis of viral infected cells [Wiseman, 1994b].

The use of tamoxifen is limited because some estrogen receptor positive tumours fail to respond to the drug initially while others develop resistance during tamoxifen

treatment [Maass, *et al*, 1980; Osborne, *et al*, 1992]. Cells treated with tamoxifen in the laboratory which are initially inhibited by anti-estrogens will also eventually develop drug resistance. However, patients who develop resistance to tamoxifen continue to respond to second and third line endocrine therapies indicating that resistance is a specific phenomenon and not a general loss of endocrine responsiveness [Wolf, *et al*, 1995].

5. 3. Pure Antiestrogens and other antiestrogens

Although the mixed agonist-antagonist action of non-steroidal antiestrogens such as tamoxifen are responsible for many of their beneficial side effects, many undesirable effects also occur e.g. endometrial stimulation, development of resistance and possibly, hepatic effects [Wolf, *et al*, 1995]. These problems led to the development of so called pure antiestrogens such as ICI164,384 and ICI182,780 (Figure 3).

These new compounds are steroidal in base and should in theory exert only estrogen antagonist effects. The compounds are derived from 17β-estradiol by addition of an alkyl chain substituted at the 7α position [Wakeling, 1987b; Wakeling, *et al*, 1991]. However these compounds do have effects in the absence of estrogens [Coradini, *et al*, 1994]

ICI164,384 binds to the estrogen receptor with a ten-fold higher affinity than tamoxifen and is more effective than tamoxifen in inhibiting the growth of estrogen-responsive breast cancer cells [Wakeling, *et al*, 1988]. ICI 164,384 has no estrogenic effects on the uterus and competitively inhibits the trophic actions of tamoxifen on the uterus and mammary gland and on tamoxifen-resistant breast tumours [Wakeling, *et al*, 1989; Nicholson, *et al*, 1988; Gottardis, 1989]. However, ICI 164, 384 has poor oral bioavailability which led to the development of ICI 182,780. This compound binds to the ER with the same affinity as estradiol and is

more effective at inhibiting mammary tumour cell lines in vitro and transplants into nude mice in vivo [Wakeling, *et al*, 1991 & 1992]. ICI182,780 is also active against tamoxifen resistant tumours in vitro and in vivo and lacks a uterotrophic effect [Howell, *et al*, 1996]. Both ICI164,384 and ICI182,780 have been reported to modulate the mdr phenotype in vitro [Kirk, *et al*, 1994; De Vincenzo, *et al*, 1996].

A number of new non-steroidal antiestrogens have also been developed. Toremifene is similar in effectiveness to tamoxifen but is less estrogenic. It has been studied as a potential modulator of multidrug resistance [Bajetta, *et al*, 1994; Howell, *et al*, 1996]. The tamoxifen derivative droloxifene (3-hydroxytamoxifen) has several advantages over tamoxifen including a shorter terminal elimination half life, lower accumulation, improved drug tolerability, decreased occurrence of resistance and decreased risk of endometrial cancer [Wiseman, 1994b]. It is on clinical trial in women with advanced breast cancer [Bruning, 1992].

5. 4. Antioxidant action of tamoxifen

Antioxidants play an important role in protecting DNA, proteins and membrane lipids against oxidative damage. In the cell membrane, free radicals can cause lipid peroxidation, or oxidative breakdown, which leads to the formation of lipid hydroperoxides and to secondary products such as biologically active aldehydes. Lipid hydroperoxides themselves break down to generate radicals which further propagate lipid peroxidation in an autocatalytic process [Halliwell, 1989]. The process can be inhibited by antioxidants: preventative antioxidants which act to prevent lipid hydroperoxide breakdown and chain-breaking antioxidants which scavenge the free radicals that propagate the process [Cheeseman, 1993]. Many antioxidants are found in the diet including vitamins A, C and E [Hunter, 1996]. Damage due to lipid peroxidation is important in two respects; direct damage to the membrane structures and indirect damage caused by the release of active products. In cancer this is important since DNA damage as a result of the products of

membrane lipid peroxidation is likely to be important in malignant progression [Vaca, *et al*, 1988]. The products of lipid peroxidation have been demonstrated to be capable of tumour initiation [Chung, *et al*, 1993].

Tamoxifen and its derivatives and 17β estradiol have been shown to have an antioxidant action inhibiting lipid peroxidation in a number of model and native systems[Wiseman, *et al*, 1990a & 1990b]. This action is probably due to the ability of tamoxifen to stabilise the membrane in a mechanism similar to cholesterol [Wiseman, *et al*, 1990a & 1993a]. Cholesterol (and its derivatives) is thought to stabilise membranes against lipid peroxidation via an interaction between the hydrophobic rings of cholesterol and fatty acid side chains present in membrane phospholipids which reduces the fluidity of the membrane bilayer [Wiseman, 1994a]. Tamoxifen's preventative effect against nuclear membrane peroxidation may help prevent DNA damage and subsequent malignant progression [Wiseman, 1994b].

This role in the protection of DNA against oxidative damage may explain why tamoxifen has a number of other potential clinical uses. The role of tamoxifen as an antioxidant may partly explain its cardioprotective action since oxidative damage to low density lipoproteins (LDL) is well documented as being a cause of atherosclerosis [Wiseman, 1993b &1994b]. 17β-estradiol which similarly decreases membrane fluidity also protects against oxidative LDL damage[Wiseman, 1994b]. ICI 164,384 has also been shown to have an antioxidant action. This steroidal antiestrogen may have a greater effect against lipid peroxidation than tamoxifen [Wiseman, 1994c].

6. Aims of this thesis

The cell membrane is the site of action of many proteins involved in cell signalling and tumour growth and development. Alterations in the membrane fluidity may affect the activity and expression of these proteins. The epidermal growth factor receptor is a transmembrane protein tyrosine kinase which has been implicated in the growth and development of breast cancer. Activation of the EGFR depends on the lateral diffusion and dimerisation of receptor monomers and alterations in membrane fluidity may affect this process and hence signalling via this receptor. Membrane fluidity may be altered by drugs such as the antiestrogen tamoxifen which has previously been reported to decrease membrane fluidity in both liposomes and breast cancer cells.

The aims of this thesis were to examine whether tamoxifen and related compounds did induce changes in membrane fluidity and how such changes in membrane fluidity might influence both EGFR function and overall breast cancer cell growth.

Chapter 2

Materials & Methods

1. Suppliers

Fine Chemicals

All chemicals were of AR grade or equivalent and were obtained from Fisher Scientific (Fisons) with the following exceptions which were obtained from Sigma: Bovine serum albumin (fraction V), dextran, dithiothreitol, PMSF, sodium orthovanadate and TEMED.

Cell culture materials

DMEM, fetal calf serum (FCS), phenol red free RPMI, phenol red free DMEM and phosphate free medium were obtained from Sigma.

Fetal calf serum, RPMI-1640 and penicillin/streptomycin were obtained from Gibco.

Cell culture plasticware was obtained from Nunc and Costar.

Human recombinant EGF and TGFα were obtained from Sigma and R&D Systems.

Adriamycin was obtained from the Beatson Oncology Centre, Western Infirmary, Glasgow.

Radiochemicals

γ^{32}P-ATP, Biotrak EGF-receptor tyrosine kinase enzyme assay kit and carrier-free ^{32}P orthophosphate were obtained from Amersham.

^{125}I-EGF was obtained from Vienna Labs.

Miscellaneous Materials

Anti-EGFR antibody R1 and Hyperfilm x-ray film were obtained from Amersham.

coverslips and microscope slides were obtained from BDH.

Acrylamide:bisacrylamide, Bradford dye reagents and prestained high molecular weight SDS-PAGE markers were obtained from BioRad.

Staphylococcus aureus cells (Pansorbin) was obtained from Calbiochem.

AF18, BODIPY-phosphatidylcholine, BODIPY-sphingomyelin and FITC-EGF were obtained from Molecular Probes, Inc.

Ultima Flo scintillation fluid was obtained from Packard.

Sephadex G10 and G25 were obtained from Pharmacia.

Goat anti-mouse antibody and serums were obtained from Scottish Antibody Production Unit.

Aprotinin, calf thymus DNA, 17β-estradiol, fluorescein isothiocyanate isomer-1, FITC-BSA, Hoechst N°33258, leupeptin, MTT, ribonuclease A, soyabean trypsin inhibitor, tamoxifen and trypan blue (0.4% solution) were obtained from Sigma.

Addresses:

Amersham International plc, Little Chalfont, Bucks., UK

Amicon Ltd, Stonehouse, Gloucestershire, UK

BDH, Merck Ltd., Lutterworth, Leics., UK

BioRad Laboratories Ltd, Hemel Hempstead, Herts., UK

Boehringer Mannheim UK Ltd., Lewes, East Sussex, UK

Calbiochem-Novabiochem, Beeston, Nottingham, UK

Fisher Scientific UK, Loughborough, Leics., UK

Gibco BRL, Life Technologies, Inchinnan Business Park, Paisley, UK

Molecular Probes, Cambridge Bioscience, Newmarket Rd., Cambridge, UK

Packard, Pangbourne, Berks., UK

Pharmacia, St Albans, Herts. UK

R&D Systems Europe, Abingdon, Oxon., UK

Scottish Antibody Production Unit, Law Hospital, Carluke, UK

Sigma-Aldrich Co. Ltd, Poole, Dorset, UK

Vienna Laboratories, Vienna, Austria

2. Buffers and Solutions

All solutions were prepared using deionised H_2O, with the exception of cell culture solutions which were prepared with high-purity tissue culture grade H_2O.

2. 1. Buffers

2.1.1 Phosphate buffered saline

137mM NaCl; 2.7mM KCl; 8mM Na_2HPO_4; 1.8mM KH_2PO_4,

adjusted to pH 7.2 with 1N HCl

2.1.2 ETN buffer

10mM EDTA; 10mM Tris base; 100mM NaCl

adjusted to pH 7.0 with 1N HCl

2.1.3 HE buffer

20mM HEPES; 1.5mM EDTA; 10% (v/v) glycerol

adjusted to pH 7.4 with 1N HCl

2.1.4 Hank's modified buffer

1.3mM $CaCl_2$; 5.4mM KCl; 0.5mM $MgCl_2$; 0.5mM $MgSO_4$; 137mM NaCl; 4mM $NaHCO_3$; 0.4mM $NaH_2PO_4.2H_2O$.

adjusted to pH 7.2 with 1N HCl

2.1.5 Glycine buffer

0.1M glycine; 0.1M NaCl

adjusted to pH 10.5 with 1N NaOH

2. 2. Cell Culture Solutions

2.2.1 Routine sub-culture medium (MCF-7, MDA-MB-231 & A431 cells)

RPMI-1640 medium with L-glutamine and 25mM HEPES; 10% (v/v) fetal calf serum.

This was further supplemented when necessary with 100 units/ml penicillin and 50μg/ml streptomycin.

2.2.2 Dulbeccos Modified Eagles Medium (MCF-7AdrR cells)

DMEM with L-glutamate and 4500mg/l D-glucose, without sodium bicarbonate. This was prepared from powdered stock with the addition of 44 mM sodium bicarbonate and 25mM HEPES and supplemented with 10% (v/v) FCS.

This was further supplemented when necessary with 100 units/ml penicillin and 50μg/ml streptomycin.

2.2.3 Versene

125mM NaCl; 2.7mM KCl; 6.3mM Na_2HPO_4; 3.2mM KH_2HPO_4; 0.5mM EDTA; 0.0015% (w/v) phenol red

2.2.4 Cell Freezing Medium

90% (v/v) FCS; 10% (v/v) dimethyl sulphoxide

2.2.5 Dextran Coated Charcoal (DCC)

0.5% (w/v) sieved, prewashed charcoal; 0.005% (w/v) dextran; suspended in HE buffer, stirred continuously for 30 minutes at room temperature

2.2.6 Dialysed Heat-Inactivated Dextran-Coated Charcoal-Stripped Fetal Calf Serum (DHIDCCFCS)

100ml of FCS was dialysed against four 1 litre changes of Hank's modified buffer over 48 hours at 4°C. The serum was then heat inactivated by incubating at 56°C for 45 minutes. A pellet of dextran coated charcoal (derived from 12.5ml of DCC solution) was added and this mixture was stirred for 30 minutes at 4°C before centrifuging at 10,000g to pellet the charcoal. The supernatant was filter sterilised through a 0.2 micron filter.

2.2.7 Experimental Medium (MCF-7, MDA-MB-231 and A431 cells)

RPMI-1640 with L-glutamate, without phenol red and sodium bicarbonate. This was prepared from powdered stock with the addition of 23.8mM sodium bicarbonate and 25mM HEPES

2.2.8 Experimental Medium (MCF-7AdrR cells)

DMEM with L-glutamate and 4500mg/l D-glucose, without phenol red and sodium bicarbonate. This was prepared from powdered stock with the addition of 48mM sodium bicarbonate and 25mM HEPES.

2. 3. SDS PAGE Solutions

2.3.1 30% Acrylamide/bisacrylamide stock

29.2% (w/v) acrylamide; 0.8% (w/v) bisacrylamide

2.3.2 7.5% Separating gel

0.375M Tris-HCl pH8.8; 0.1% (w/v) SDS; 7.5% (v/v) acrylamide/bisacrylamide stock; 0.05% (w/v) ammonium persulphate (APS); 0.05% (v/v) TEMED

All the reagents except APS and TEMED were combined and the monomer solution degassed under vacuum for 5 minutes followed by addition of the APS and TEMED to initiate polymerisation.

2.3.3 4.0% Stacking gel

0.125M Tris-HCl pH 6.8; 0.1% (w/v) SDS; 4.0% (v/v) acrylamide/bisacrylamide stock; 0.05% (w/v) ammonium persulphate; 0.1% (v/v) TEMED

All the reagents except APS and TEMED were combined and the monomer solution degassed under vacuum for 5 minutes followed by addition of the APS and TEMED to initiate polymerisation.

2.3.4 Sample buffer

2% (w/v) SDS; 10% glycerol; 60mM Tris-HCl pH6.8; 100mM dithiothreitol; 0.05% (w/v) bromophenol blue

2.3.5 Electrode buffer, pH 8.3

25mM Tris; 192mM glycine; 0.1% (w/v) SDS

2.3.6 Coomassie Blue stain

0.25% (w/v) Coomassie Brilliant Blue R-250; 40% (v/v) methanol; 10% (v/v) acetic acid

2.3.7 Coomassie Blue destain

40% (v/v) methanol; 10% (v/v) acetic acid

2.4. Miscellaneous solutions

2.4.1 Hypotonic buffer pH7.2

20mM HEPES; 3mM KCl; 3mM $MgCl_2$; 100μg/ml PMSF; 2μg/ml aprotinin; 5μg/ml leupeptin; 50μg/ml soyabean trypsin inhibitor

2.4.2 Membrane resuspension buffer pH 7.2

50mM HEPES; 0.1mM EDTA; 0.05% (w/v) BSA; 10% (v/v) glycerol; 100μg/ml PMSF; 2μg/ml aprotinin; 5μg/ml leupeptin; 50μg/ml soyabean trypsin inhibitor

2.4.3 Lysis Buffer pH8

150mM NaCl; 20mM Tris; 10mM NaH_2PO_4; 5mM EDTA; 10% (v/v) glycerol; 1% (v/v) Nonidet P40; 0.5% (w/v) deoxycholate; 0.1% (w/v) SDS; 1mM PMSF; 100μM sodium orthovanadate; 2μg/ml aprotinin; 5μg/ml leupeptin; 50μg/ml soyabean trypsin inhibitor

3. Cell Culture Methods

3.1. Cell Lines

Four cell lines expressing the EGF receptor were used in this study, the breast cell lines MCF-7 and its adriamycin-resistant subclone MCF-7AdrR and MDA-MB-231, and the squamous epithelial cell line A431. MCF-7 and MDA-MB-231 were purchased from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, UK. The A431 and MCF-7AdrR lines were a gift from Dr Ian Freshney, Department of Medical Oncology, University of Glasgow.

MCF-7 and MCF-7AdrR

The MCF-7 cell line was derived from a pleural effusion from a patient suffering from adenocarcinoma of the breast who had received radiotherapy and endocrine therapy [Soule, *et al*, 1973]. This cell line is the most widely used and best characterised of all human breast cell lines and exhibits some of the characteristics of differentiated mammary epithelium . In addition to the expression of estrogen receptors, the cells express estradiol-induced progesterone receptors and receptors for androgens, luteinizing hormone releasing hormone, glucocorticoids, insulin, retinoic acid and prolactin [Clarke, *et al*, 1996]. Since the cell line is estrogen dependent, is sensitive to antiestrogens and has a low metastatic potential, it is considered to be representative of an early stage of disease progression.

The adriamycin resistant subform, MCF-7AdrR, was generated by continuous subculture in the presence of a gradually increasing non-lethal concentration of adriamycin. This cell line is approximately 200 fold more resistant to adriamycin than the parent cell line and is also resistant to a number of other cytotoxic drugs including actinomycin D and vinblastine [Fairchild, *et al*, 1987]. The cell line has an elevated expression of p-glycoprotein and this has been reported to be associated

with an increased expression of the EGF receptor and loss of hormone responsiveness [Vickers, *et al*, 1988].

MDA-MB-231

MDA-MB-231 was also generated from a pleural effusion in an adenocarcinoma patient who had received endocrine therapy and chemotherapy [Caillieau, *et al*, 1973]. The line is not responsive to steroid hormones and has an elevated expression of the EGF receptor relative to the MCF-7 line [Davidson, *et al*, 1987]. MDA-MB-231 cells are highly tumorigenic and can produce lung metastases from mammary fat pad tumours in nude mice [Clarke, *et al*, 1996].

A431

A431 was derived from an epidermal carcinoma of the vulva [Giard, *et al*, 1973]. This cell line is characterised by a large number of EGF binding sites (greater than 10^6 sites/cell) [Fabricant, *et al*, 1977] The existence of this cell line facilitated many of the early studies of the EGF receptor although many of its responses to EGF are atypical [Gill, *et al*, 1981]

3. 2. Routine Growth and Subculture of Cell Lines

All cells were routinely grown in $75cm^2$ tissue culture flasks in a 37^oC incubator with atmospheric air enriched with 5% CO_2. Experimental plates and dishes were grown in a humidified atmosphere to prevent evaporation of medium. All routine and experimental media were changed every 48 hours and antibiotics were not routinely added to medium.

3. 2. 1. Subculture technique

Cells were passaged when they occupied 80-90% of the surface of the culture flask. The growth medium was aseptically decanted and the cell monolayer washed twice with a small volume (approximately 5ml) of sterile PBS prewarmed to 37^oC. 4ml trypsin/versene solution (0.05% trypsin (w/v) in versene solution) was added per $75cm^2$ flask and the flasks were then incubated at 37^oC for 2 minutes. After checking that the cells were well rounded, most of the trypsin/versene was removed to leave 0.5-1ml per flask and the flasks returned to the incubator to allow the cells to detach fully. Trypsinisation was stopped by adding an excess (at least 5ml) of fresh growth medium followed by rapid pipetting to ensure an even cell suspension. The suspension was then dispensed into new culture flasks containing fresh growth medium (approximately 10-15ml). For routine subculture, the cells were split in a ratio of 1:3.

For experimental plates the technique was slightly different. After trypsinisation and resuspension, the cell suspension was counted using a haemocytometer and diluted to the required number of cells per ml. This cell suspension was then plated down on experimental plates; 200μl per well for 96 well plates, 1ml per well for 24 well plates, 2ml per well for 6 well plates or 35mm dishes, 5ml for 60mm dishes, 10ml for 100mm dishes. After 24 hours, the routine medium was removed, the cells were washed twice with PBS and the appropriate experimental medium was added.

3. 2. 2. Cryopreservation of cell lines

All cells used in experiments were of a similar passage number. To ensure this, frozen stocks were prepared and stored at -170°C and new stocks were grown from frozen every few months.

To prepare frozen stocks, cells were grown to 80% confluence in 175cm^2 flasks. Cells were trypsinised and resuspended as described in section 2.2.1. The cell suspension was counted using a haemocytometer, transferred to a sterile universal container and centrifuged for 5 min at 500g. The cell pellet was resuspended in freshly prepared cell freezing medium at a cell density of approximately 5 x 10^7 cells/ml and 1ml aliquots were transferred to sterile Cryo tubes. These were frozen to -70°C in a polystyrene container lined with cotton wool to ensure a slow rate of freezing. Once frozen the vials were immediately transferred to liquid nitrogen vats.

To grow cells from frozen stocks, the vial was thawed rapidly in a water bath at 37°C (approximately 1 minute). The vials were removed while a few ice crystals still remained and the cell suspension was transferred to a universal container to which 10ml cold routine medium had been added. This was centrifuged at 500g for 5 minutes, the supernatant was discarded and the pellet was slowly resuspended in 10ml of prewarmed medium. A small aliquot was removed and mixed with an equal volume of 0.4% trypan blue. This dye will only enter damaged cells and thus allows non-viable cells to be excluded during counting with a haemocytometer. The cell suspension was then diluted to a density of 5 x10^5 viable cells/ml, transferred to 25cm^2 flasks and incubated at 37°C. After 24 hours the medium was replaced with fresh routine medium.

3. 2. 3. Mycoplasma testing of cell lines

Mycoplasma is a serious intracellular contamination which is widely found in cultured cells [Russel, *et al*, 1975]. The infection cannot be detected with the naked eye but can cause alterations in cell metabolism, growth and viability leading to variability in experimental results [Hay, 1994]. All cell lines used were checked every two months for this contamination.

Cells were plated down onto 13mm diameter sterile glass coverslips in 60mm dishes in antibiotic free medium. The cells were allowed to reach approximately 50% confluence before fixing and staining. 5ml freshly prepared Carnitovs fixative (75% (v/v) methanol; 25% (v/v) glacial acetic acid) was added to each dish without decanting the medium. After 2 minutes the fixative/medium was aspirated, a fresh 5ml of fixative was added and this was left for 5 minutes. This step was repeated with a further 5ml of fixative for 5 minutes before all fixative was removed and the dishes allowed to air dry. The dishes were then flooded with 0.5μg/ml Hoechst 33258 in distilled water, left for 30 minutes then washed 5 times in deionised water. Finally the slips were inverted onto a drop of mounting fluid (90% glycerol; 10% PBS) and viewed under a fluorescent microscope fitted with an appropriate filter. Mycoplasma was indicated by the presence of uniformly shaped bodies of extranuclear DNA which stained with the Hoechst dye. Any contaminated cell line was destroyed and new stocks, frozen before the last negative test date, were retrieved from frozen.

4. Hoechst DNA Assay

The assay depends on the intercalation of Hoechst 33258 dye with solubilised DNA to yield a complex which fluoresces with a maximum emission at 450nm [Leake & Habib 1987].

A 1mg/ml stock solution of calf thymus DNA dissolved in ETN buffer was used to prepare DNA standards. The concentrations used were 0, 10, 20, 30, 40 , 50 and 60 μg/ml DNA. These standards were prepared in ETN buffer and stored at -20°C for up to 1 month.

Cell monolayers were harvested by trypsinisation. The cells were pelleted and the DNA was solubilised in 0.2% SDS in ETN buffer by incubation at 37°C for 30 minutes.

100μl aliquots of each standard and sample in duplicate were placed in plastic test tubes and 3ml of ETN buffer containing 100ng/ml Hoechst and 5μg/ml RNase was added. After thorough mixing, the tubes were incubated for 30 minutes in the dark at room temperature. The fluorescence enhancement at 450nm was measured with an excitation wavelength of 360nm using a Hitachi Perkin-Elmer MPF-2A fluorescent spectrophotometer.

The fluorescence enhancement of the standards was used to construct a standard curve from which values for the concentration of DNA in the samples could be extrapolated.

5. MTT assay

This assay is used to determine viable cell numbers. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow, water soluble, tetrazolium salt which is reduced by dehydrogenase enzymes in the mitochondria to an purple formazan product. This product is insoluble in aqueous solutions but can be dissolved in dimethyl sulphoxide and the absorbance read at 570nm [Mossman, 1983].

Cells were trypsinised and resuspended at a concentration of between 1 and $5x10^4$ cells/ml depending on the assay and the cell line used. The cell suspension was plated down onto a flat bottomed 96 well plate (200µl per well). Growth medium only was added to the first column. Plates were incubated in a humidified atmosphere for 24 hours at 37°C.

After 24 hours the medium was removed from the wells with a hypodermic needle attached to a suction line and replaced with experimental medium containing the appropriate growth factors and drugs. The medium was replaced with fresh medium after 48 hours.

After the appropriate incubation time, 50µl of MTT (5mg/ml in PBS) was added to each well, the plates were wrapped in aluminium foil and returned to the humidified incubator. After a minimum of 4 hours incubation with MTT, the medium was removed from the wells and 200µl per well DMSO added to dissolve the MTT-formazan crystals. Finally 25µl per well glycine buffer was added and the absorbance was read at 540nm immediately using a plate reader. The wells in column 1 were used to blank the plate reader

6. Bradford Protein Assay

The Bradford protein assay is based on the principle that there is a shift in the absorbance maximum of an acidic solution of Coomassie Brilliant Blue G-250 from 465 to 595nm when binding to protein occurs [Bradford, 1976].

A series of protein standards in the range of 1-20µg/ml was created using bovine serum albumen (BSA). A 1mg/ml stock of BSA was diluted on the day of assay in the same buffer as the samples to be measured to create standards of 1,2,5,10,15 and 20µg/ml protein.

800μl of standards and appropriately diluted samples were placed in 75x12mm glass test tubes in duplicate. 200μl Biorad dye reagent was added per tube and mixed gently. After a 15 minute incubation at room temperature, the absorbance at 595nm was read using a spectrophotometer. A standard curve was then constructed of absorbance of the protein standards versus protein concentration. This was used to calculate the protein concentration of the unknown samples.

7. Fluorescence Recovery After Photobleaching (FRAP)

7. 1. Apparatus

Figure 4 is a schematic representation of the apparatus used in FRAP experiments [Johnston, 1982]. The apparatus consists of a 1W water-cooled continuous argon laser (Lexel model 85) set at a wavelength of 514.5nm. Light from the laser passes through a spatial filter (Oriel Scientific) which removes higher spatial frequencies from the beam profile and improves the contrast ratio of the modulated beam by 25 fold [Garland, 1980]. Light then passes through a collimating lens to focus the beam into the microscope apparatus (Leitz). In the fluorescent microscope, the beam is diverted through an epi-illuminator, reflected via dichroic mirrors and focused via a 40X objective lens onto the sample. Fluorescence from the sample is detected via a photomultiplier tube which feeds data into an oscilloscope (Gould). This is connected to a Hewlett-Packard model 86A computer. The control electronics consist of a laser control pack which regulates the power of the laser and a digital-acousto-optic modulator (Model 304D, Coherent Associates) which controls the on/off state of the beam and allows attenuation of the intensity of the beam. This system has the advantage that measuring and bleaching beams differ only in duty cycle time and are perfectly spatially aligned [Garland, 1980].

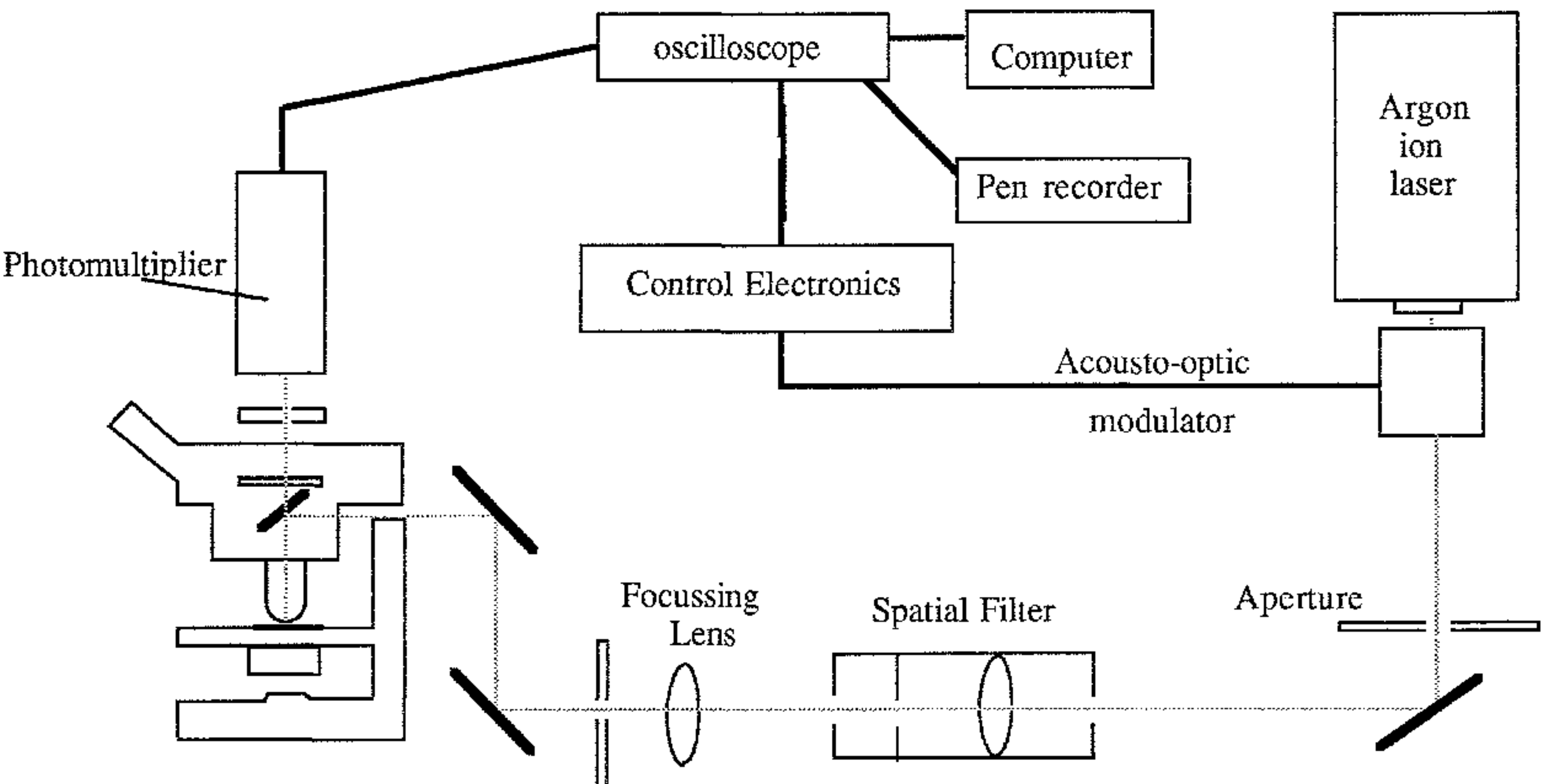


Figure 4. Diagram of equipment used for FRAP

7. 2. FRAP technique

A highly attenuated beam is directed into the back of the microscope and focused by a 40X objective lens onto a small spot (1.5μm radius) on the surface of the specimen. This excites the fluorophores within this area and the resulting fluorescence is detected by the photomultiplier tube. The level of signal is monitored and recorded via the oscilloscope. Once a steady reading of fluorescence signal has been established, the laser control unit is used to briefly (70-100 ms) increase the power of the laser by 10,000 fold. This high intensity pulse irreversibly bleaches the fluorophores in that spot. The time course of recovery of fluorescence levels was then followed via the oscilloscope. Recovery will be due to diffusion of unbleached molecules from the surrounding area into the spot (Figure 2). Traces were then transferred to a computer and the data was analysed using a curve fitting programme. The values obtained for half life of recovery i.e. the time taken for recovery to proceed to half the final recovery, are used to calculate values for the lateral diffusion coefficient. All FRAP measurements were taken at a constant temperature of 22°C.

The equation used to derive values for the lateral diffusion coefficient was derived by Axelrod [Axelrod, *et al*, 1976]. This assumes 2 dimensional diffusion and a laser beam with a Gaussian profile.

$$D_L = (w^2/4t_{1/2})\gamma$$

where D_L is the lateral diffusion coefficient

w is the radius of the spot at $1/e^2$ intensity

$t_{1/2}$ is the half life of recovery

γ is a constant that depends on the beam profile and the bleaching parameter, K which in turn depends on the % bleach. Factors which influence the percentage bleach are laser intensity, duration of bleach pulse, quantum efficiency of bleaching and the fluorescence extinction coefficient.

The fraction of molecules which are free to diffuse within the membrane is derived from the percentage recovery relative to the initial level of fluorescence. The fluorescence recovery (R) is given in percentage terms:

$$R = 100 \times (F_\infty - F_0) / (F_i - F_0)$$

F_∞ is the fluorescence signal at an infinite time after bleaching

F_0 is the fluorescence signal immediately after bleaching and before recovery

F_i is the fluorescence signal before bleaching

In an idealised lipid bilayer D_L would be expected to be approximately $10^{-7}cm^2/s$ and recovery of fluorescence would ultimately be 100% [Cherry, 1979].

7. 3. Estimation of the spot size

The size of the spot was maintained at a constant radius of 1.5μm. This was checked before each set of experiments by performing photobleaching on a thin layer of FITC labelled bovine serum albumin in 95% glycerol. This method also ensures that incomplete recovery is not simply an artefact of the system [Foley, *et al*, 1986]. The laser is focused onto a thin film of this solution on a microscope slide, the fluorescence is bleached and the recovery is recorded and analysed by computer. The kinetics of recovery derived by the computer are used to derive a value for the spot size from the equation given in Section 7.2. Using this system FITC-BSA in 95% glycerol repeatedly demonstrated 100% recovery rates.

7. 4. Fluorescent probes

Lipid probes were dissolved as a concentrated stock in ethanol (2mg/ml) and stored at -20°C.

FITC conjugated antibodies and EGF were prepared as follows: Antibody or EGF was dissolved in 0.25M sodium carbonate pH 9.0 containing 0.1M sodium chloride

at a final protein concentration of between 10 and 20 mg/ml. 0.05mg of fluorescein isothiocyanate isomer 1 was added per mg of protein and the mixture was rotated overnight at 4°C.

Free FITC was separated from the conjugate by passing down a Sephadex G25 column (or G10 for EGF). The column was equilibrated with PBS for 1 hour before use. The sample was then applied to the column and eluted with PBS. The first coloured fraction which represents the conjugate was collected and concentrated by centrifugation in an Centricon concentrator (Amicon). The fluorescein/protein ratio was determined by measuring the absorbance of the conjugate at 280 and 490nm:

$$\text{molar ratio} = \frac{2.87 \times A_{490}}{A_{280} - 0.35 \times A_{490}}$$

This ratio is usually between 1 and 4 for antibodies

7. 5. Sample preparation

Cells were plated down in 35mm tissue culture dishes at a density of approximately 2.5×10^5 cells/ml. and allowed to grow to no more than 50% confluence. To label cells with lipid probes, the medium was removed and the cells were washed twice in serum- free routine medium . 1ml of serum-free medium containing 5µl probe in ethanol carrier (10µg) was then added per dish and the cells were incubated at room temperature for 15 to 60 minutes depending on the experiment. After this incubation, the labelling medium was removed and the cells were washed four times in serum-free medium. Finally 2ml serum-free medium was added per dish and the dishes were mounted under the FRAP microscope to allow measurements.

The location of the probes can be checked using trypan blue quenching. 0.25% trypan blue will quench the fluorescence of all probes located in the outer monolayer by resonance energy transfer but will not enter living, intact cells.

Quenching will therefore result in a loss of fluorescence from probes located at the cell surface and any fluorescence remaining above background levels indicates internalisation of the probe [Foley, *et al*, 1986].

8. Whole cell EGFR receptor binding assay

Cells were grown to approximately 80% confluence in 24 well plates in experimental medium containing DHIDCCFCS. The medium was removed from the wells and the cell monolayers were washed twice with PBS. 200µl of serum-free medium containing 0.5nM ^{125}I-EGF was added to 4 wells, 200µl of serum-free medium containing 0.5nM ^{125}I-EGF and 50nM unlabelled EGF was added to a further 4 wells. The ^{125}I-EGF used in this assay was labelled using the lactoperoxidase method. The remaining wells were used to estimate the DNA content by the Hoechst assay (Section 4.). The plates were incubated at 37°C for 30 minutes before binding was terminated by the addition of 1ml ice-cold PBS. The wells were then washed twice with 0.5ml PBS, 100µl 5N NaOH was added to each well and the plates incubated at 37°C for 15 minutes. The extract was then transferred to gamma tubes and counted using a gamma counter.

9. EGF receptor tyrosine kinase assays

These assays were performed using an Amersham kit which is designed to be specific for the EGF receptor tyrosine kinase. The principle of the assay is that the enzyme present in membrane preparation of cells catalyses the transfer of the γ-phosphate of adenosine-5'-triphosphate to the tyrosine group of a synthetic peptide substrate. This peptide (poly tyr-glu) has been designed to be specific for the EGF receptor tyrosine kinase. EGF is used to activate the enzyme and the difference between enzyme activity in the presence and absence of added EGF can be used to determine the EGF dependant tyrosine kinase activity. The phosphorylated peptide is separated by binding papers and the extent of phosphorylation can be determined by scintillation counting

9. 1. Preparation of cell homogenates

Cells were grown to confluence in 100mm tissue culture dishes in routine medium. 24 hours before preparation of membranes, the cell monolayers were washed twice with PBS and serum-free medium was added. Cells were removed by scrapping with a rubber policeman and pelleted by a 5 minute spin at 500g. The supernatant was removed and the pellet resuspended in 2ml ice cold hypotonic buffer and left on ice for 10 minutes. The cell suspension was then homogenised using a glass-glass homogeniser and the resulting cell preparation was centrifuged at 40,000g (4°C) for 30 minutes. The pellet was resuspended by gentle homogenisation in a small volume of membrane solubilisation buffer.

9. 2. Assay Procedure

5µl EGF or water as appropriate, was added to 10µl of cell membrane homogenate in an Eppendorf tube. 10µl of substrate buffer was added to each tube and the reaction was started by the addition of 5µl magnesium ATP buffer containing 40mCi/ml γ^{32}P-ATP. The final concentration of EGF was 5 $\times 10^{-7}$M. The tubes were vortexed briefly, microfuged for 20 seconds to wash all reagent to the base of the tube, and incubated at 30°C for 30 minutes. The reaction was stopped by the addition of 10µl stop reagent and the tubes were again vortexed and microfuged.

The phosphopeptide was then separated from the reaction mix. 30µl of the terminated reaction mix was spotted onto the centre of a binding disc in a wash tray. The discs were allowed to dry briefly and then washed twice in 1% (v/v) acetic acid for 2 minutes with gentle shaking, followed by three washes in distilled water. After the final wash the disc was placed in a scintillation vial, 10ml scintillant was added and the vials were counted for 3 minutes using a ^{32}P programme on a scintillation counter.

9. 3. Calculation of results

A number of controls were included in this assay to allow calculation of the EGF dependant tyrosine kinase activity. In the presence of sample and EGF, the ^{32}P counted on the papers represents non-specific ^{32}P-ATP binding, specific binding of the phosphopeptide and binding of phosphorylated proteins in the cell extract. By performing the assay in the presence of sample but not EGF, the counts due to non specific ^{32}P-ATP binding and non EGF dependant tyrosine kinase phosphorylation of both the peptide and cellular proteins can be determined and subtracted from the total counts in the presence of EGF.

10. Determination of EGF receptor tyrosine kinase activity in whole cells

10. 1. Labelling intact cells with ^{32}P

1 $\times 10^6$ cells were plated down in 60mm tissue culture dishes and grown to confluence. To label the intracellular pool with ^{32}P, the medium was removed, monolayers washed twice with PBS and 1ml phosphate-free medium containing 0.5mCi/ml ^{32}P was added per dish. The dishes were incubated for 16 hours in a humidified incubator at 37°C. Cell viability in this medium over this time period had previously been confirmed using trypan blue exclusion to detect non-viable cells (Section 2.2.2.). Over 90% of cells remained viable over this period. The ^{32}P medium was then aspirated and fresh phosphate-free medium added, containing 10^{-9}M EGF as appropriate. The cells were incubated for a further 15 minutes at 37°C in the presence of ligand before lysis. Lysis was achieved as follows: the medium was removed, the dishes placed on ice and the monolayers washed once in ice cold lysis buffer before addition of 1ml lysis buffer per dish. The cells were incubated on ice for 30 minutes with occasional rocking. The cell lysate was then scraped to the side of the dish with a rubber policeman and transferred to an Eppendorf tube. The lysates were cleared by centrifugation at 100,000g, 4°C, for 30 minutes.

10. 2. Preparation of preloaded fixed *S. aureus* Cowan I (SAC)

The lysate was first precleared with normal rabbit serum bound to protein A cells, prepared as follows. 9ml fixed *S. aureus* Cowan I (SAC) was thawed and centrifuged at 5000g for 5 min. The pellet was resuspended in lysis buffer and the volume adjusted to equal the original volume. 1ml normal rabbit serum was added and incubated on ice for 30 minutes. The preloaded SAC was then washed three times in lysis buffer by centrifugation and resuspension and finally resuspended in half the original buffer volume before storing at -70°C.

10. 3. Immunoprecipitation of EGF receptors

Cell lysates were incubated with 10% (v/v) preloaded SAC for 2 hours at 4°C. The preloaded SAC was removed by centrifugation at 10,000g for 5 minutes and the supernatant was carefully removed and transferred to a fresh Eppendorf tube containing anti-receptor antibody (100μl R1 clone tissue culture hybridoma medium per 1 ml lysate) and rotated at 4°C for 1 hour. 50μl 10% SAC (washed twice by centrifugation and resuspension in lysis buffer) was added per 1ml lysate and rotated for 30 minutes at 4°C. The SAC was pelleted by centrifugation at 10,000g, 4°C for 1 minute and the pellet was washed three times by resuspension in 1ml lysis buffer followed by centrifugation . After the final centrifugation all the lysis buffer was removed and the pellet was resuspended in 50μl sample buffer, heated to 85°C for 10 minutes, microfuged, and the supernatant run on SDS-PAGE.

11. SDS PAGE

11.1 Preparation of discontinuous SDS-PAGE gels

The methodology for discontinuous SDS-PAGE is based on that of Laemmli in which the use of an upper stacking gel and a lower resolving gel results in a better band resolution than a continuous gel with no stacking section [Laemmli, 1970].

The gel apparatus used was a BioRad Mini-Protean II dual slab cell and the gels were assembled according to the manufacturers instructions. 0.75mm combs and spacers were used to assemble the gels. Resolving gel monomer solution was prepared as for section 1.3.2. omitting APS and TEMED. The monomer solution was degassed under vacuum for 5 minutes and APS and TEMED were then added and the resolving gel was poured into the assembled gel apparatus to a predetermined level. This was obtained by inserting the comb before pouring the gel and marking a position 1cm below the bottom of the teeth. After the resolving gel was poured it was immediately overlaid with isobutanol to ensure an even surface on the gel and allowed to polymerise for 45 minutes. After polymerisation, the isobutanol was rinsed off completely with deionised water. The surface was dried with filter paper before addition of the stacking gel mixture. The stacking gel was poured to the top of the glass plates, the comb was inserted immediately and the gel allowed to polymerise. After polymerisation, the comb was removed and the wells were rinsed thoroughly with deionised water. Finally the gel apparatus was assembled according to the manufacturers instructions - the upper buffer chamber was filled with electrophoresis tank buffer and the lower chamber was filled to a level that covered the bottom 1cm of the gel. Any air bubbles were removed from the bottom of the gel by swirling the buffer in the lower chamber to achieve good electrical contact.

11. 2. Loading and electrophoresis of samples and standard proteins

Samples and standards were loaded carefully into the wells with a Gilson micropipette. Care was taken to prevent overspill between wells. The electrophoresis cell was then attached to a BioRad constant voltage power supply (Model 200/2.0) and a constant voltage of 200v was applied to the gel. Electrophoresis was continued until the bromophenol blue tracker dye was at the bottom of the gel. The gel was then removed from the apparatus and stained for total protein content by the Coomassie blue method.

To determine the molecular weight of observed proteins, one lane in each gel contained a set of electrophoresis standards (BioRad high molecular weight standards diluted 1:20 in sample buffer and heated to 85°C for 10 minutes before use). The standard mix used contained rabbit skeletal muscle myosin (Mr 200,000), *E. coli* β-galactosidase (Mr 116,000), rabbit muscle phosphorylase b (Mr 97,400), bovine serum albumin Mr 66,000) and hen egg white ovalbumin (Mr 45,000). A linear calibration curve can be obtained by plotting the logarithm of the molecular weight of the standard against the distance that protein has migrated in the gel.

11. 3. Coomassie Blue staining of SDS-PAGE gels

Gels were soaked in Coomassie Blue stain solution for 1 hour at room temperature. Destaining was achieved by soaking in 4 changes of destain solution with gentle agitation for between 4 hours to overnight.

11. 4 . Autoradiography

Stained gels were dried before autoradiography as follows. The gel was placed onto a piece of Whatman 3MM paper cut to slightly larger than the size of the gel. The upper surface was protected with a layer of Saran wrap (Dow). The gels were then dried for 1 hour at 85°C under constant vacuum. Dried gels were exposed to x-ray film in an autoradiography cassette. The period of exposure varied with the degree of labelling from 24 to 72 hours. X-ray film was developed using a Kodak X-Omat Film Processor Model ME3.

12. Determination of internalisation of ^{125}I-EGF

This assay is a modification of one used to measure FcγR1 receptor internalisation [Harrison, *et al*, 1994].

Cells were grown to confluence in 100mm dishes . The medium was removed and the cell monolayers washed twice with 2mM EDTA in PBS before overlaying the

monolayer with 5ml EDTA/PBS. The dishes were then left at room temperature until all the cells had detached to give a single cell suspension. This suspension was then counted with a haemocytometer, centrifuged at 500g for 5 minutes to pellet the cells and the cells were resuspended in routine medium at a density of 10^7 cells/ml. ^{125}I-EGF was added at a concentration of 10^{-9}M and the cells were incubated on ice for 30 minutes. This allows cell surface occupation of the EGFR in the absence of internalisation. After the labelling period the cells were washed in a 10 fold excess of cold medium to remove unbound ligand, the cells were pelleted by centrifugation at 1000g for 5 minutes and resuspended in fresh medium at a density of 2×10^6 cells/ml.

Non-specific binding which was determined in the presence of a 100 fold excess of unlabelled EGF accounted for less than 5% of the total cell associated counts

The cell suspension was then divided into 500μl aliquots, rapidly warmed to 37°C and assayed at various time points. One 500μl aliquot was retained on ice throughout for determination of internalisation at time zero. At each time point, duplicate 100μl aliquots were diluted 20 fold in ice-cold PBS at pH7.2 and at pH2.5. After a 5 minute incubation on ice in the PBS wash, cells were pelleted by centrifugation at 2000g for 5 minutes. The supernatant was removed and cell-associated counts were determined in a gamma counter.

The pH7.2 wash gives the total cell associated counts both internal and external, while the acid wash typically removes 80-85% of the cell surface associated counts [Haigler, *et al*, 1980]. Thus for each time point the percentage of the total cell associated counts can be determined and from this an idea of rate of internalisation can be gained.

13. Computer Software

This thesis was created on an Apple Macintosh® computer using Microsoft Word® Version 5.1. Statistical analysis of data was performed using the Instat™ statistical programme version 2.01 (Graphpad Software) and graphs were created using CA-Cricket Graph III® version 1.0 (Computer Associates Ltd.). Diagrams were created using Clarisworks™ version 1.0Bv3 (Claris Corporation).

13. Statistics

The Instat™ statistical programme version 2.01 was used to calculate means and standard deviations and perform statistical tests of significance. Unless otherwise stated control and experimental means were compared using a two-tailed unpaired Student t test. The t test assumes a Gaussian distribution of data with the standard deviations of the two populations being equal. Significance of results is reported by the p value with p values below 0.05 considered significant.

Chapter 3

Results

3. 1. DNA content and MTT reduction as a measure of cell number

In this thesis two methods were used to assess cell growth and survival; measurement of DNA content and reduction of MTT. In each case it is important to ensure that the level of DNA and MTT reduction do accurately reflect cell number. Two initial experiments were performed to show the correlation between each method and cell number.

3. 1. 1. Correlation of cell number and DNA content.

Method

80% confluent $75cm^2$ flasks of cells were trypsinised, counted and resuspended at a density of $5x10^4$ cells/ml as described in Methods section 2.2.1 The cells were then allowed to plate down in two 6-well plates. After 24 hours, the cells were removed from 2 wells by trypsinisation and resuspension in routine medium to a final volume of 1ml. A small sample (100μl) was removed and the number of cells counted on a haemocytometer. The remaining cells were lysed and a DNA assay performed as in Methods section 3. Every 24 hours both cell count and DNA assay were repeated with a further 2 wells.

Results

Figure 5 shows the linear correlation obtained when cell number is plotted against DNA content. A good correlation exists between the amount of DNA in the sample as measured by the Hoechst assay and the number of cells in the same sample.

3. 1. 2. Correlation of cell number and MTT reduction.

Method

80% confluent $75cm^2$ flasks of cells were trypsinised, counted and resuspended at a density of $5x10^4$ cells/ml as described in Methods section 2.2.1. The cells were then allowed to plate down in 6 well plates. After 24 hours, the cells were removed

from 2 wells by trypsinisation and resuspension in routine medium to a final volume of 1ml. A small sample was removed and the number of cells counted on a haemocytometer. 400µl aliquots were placed in Eppendorf tubes and the MTT assay was performed as in Methods section 4 with a modification for suspension cells. Essentially, after 4 hours incubation with 100µl MTT, the cells were pelleted by centrifugation then resuspended in 400µl DMSO. 50µl glycine buffer was added and the absorbance was measured at 540nm in a spectrophotometer. Every 24 hours both cell count and MTT assay were repeated with a further 2 wells.

Results

Figure 6 shows the linear correlation obtained when cell number is plotted against absorbance at 540nm. A good correlation exists between the amount of MTT reduction by a sample and the number of cells in the same sample.

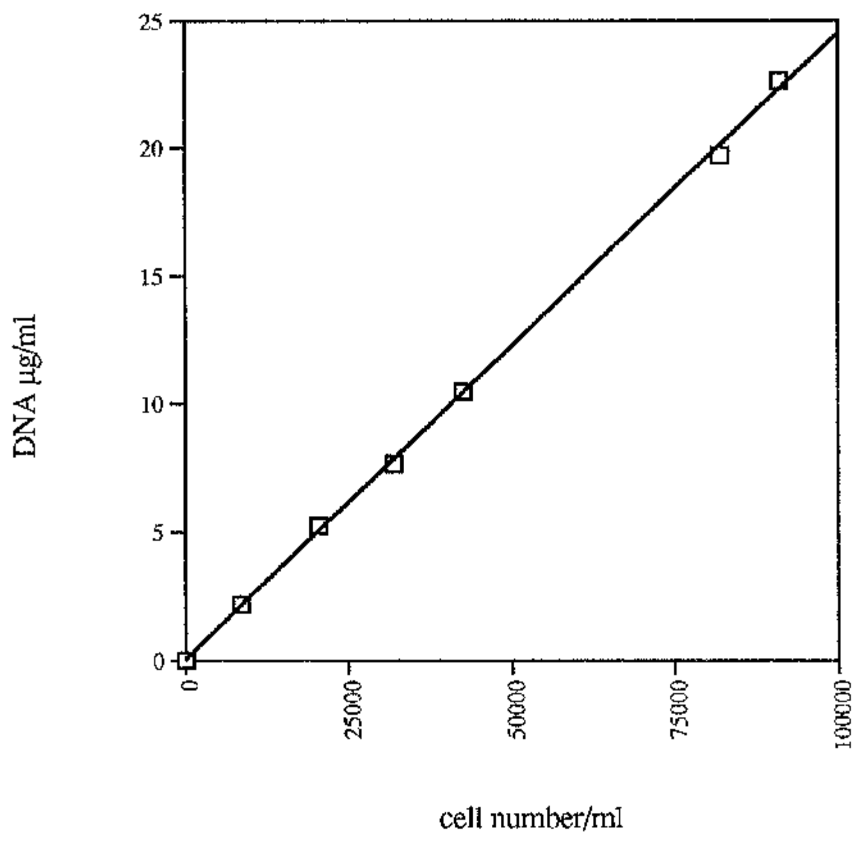
Conclusion

Both DNA concentration and MTT reduction are linearly correlated with cell number and so it is valid to use either as a measure of cell number or proliferation.

Figure 5. Correlation of cell number with DNA content.

This graph shows an example of the correlation found between cell number and DNA concentration. Each data point represents a sample of cells which has been counted using a haemocytometer to obtain the number of cells/ml and assayed for DNA content by the Hoechst method (Methods section 4.).

Cell number versus DNA concentration

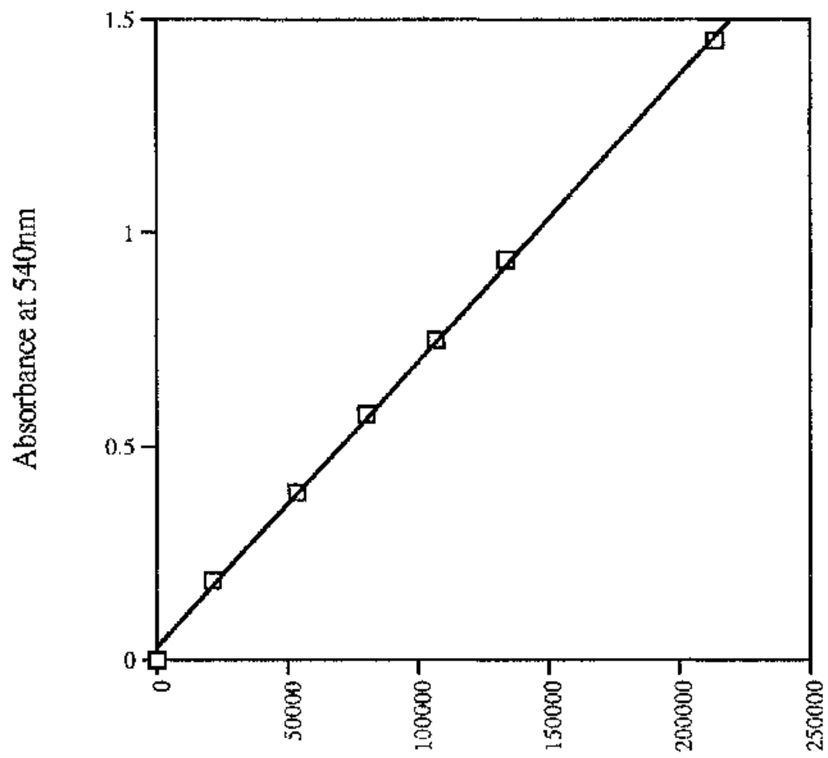


$y = 0.00024x + 0.046 \quad r = 1.000$

Figure 6. Correlation of cell number with MTT reduction

This graph shows an example of the correlation found between cell number and MTT reduction as measured by absorbance at 540nm. Each data point represents a sample of cells which has been counted using a haemocytometer to obtain the number of cells/ml and assayed by a modification of the MTT method (Methods section 5.).

Cell number versus MTT reduction



Cell number/ml

y = 0.00006x + 0.027   r = 1.000

3. 2. Effect of EGF and TGFα on breast cancer cell growth

EGF has been implicated in both normal breast cell growth and in the development and growth of breast tumours [Bates, *et al*, 1988 & 1990]. It was important to characterise the dose-response to EGF of each of the cell lines used, under normal conditions, since cells with different levels of EGFR have differing responses to EGF [Davidson, *et al*, 1987; Godden, *et al*, 1992]. The effect of TGFα on MCF-7 cell growth was also examined in the presence and absence of EGF, to confirm that this ligand is acting through the same receptor as EGF.

Method

80% confluent flasks of cells were trypsinised, counted and resuspended at a density of $5x10^4$ cells/ml as described in Methods Section 2.2.1. The cell suspension was plated down onto 6 well plates and allowed to settle for 24 hours. The routine medium was then removed and the monolayers were washed twice with sterile PBS. Different concentrations of EGF (or TGFα or EGF and TGFα in the case of MCF-7 cells) in phenol red free medium containing 2% DHIDCCFCS were then added in duplicate to wells in the 6 well plates. Phenol red free medium was used since a contaminant in phenol red is known to be estrogenic and this could affect growth [Berthois, *et al*, 1986]. DHIDCCFCS has had all detectable steroids and growth factors removed.

After 72 hours at 37^oC, the medium was removed and the monolayers washed twice in PBS. The cells were harvested by trypsinisation, resuspension and centrifugation. The cell pellets were lysed by incubation with 0.2% SDS for 30 minutes at 37^oC. The DNA content was assayed by the Hoechst method as described in Methods section 3.

Results

Figure 7 shows dose-response curves for the three breast cancer cell lines and the A431 cell line in response to EGF. MCF-7 cell growth was stimulated by EGF with maximal stimulation at 100ng/ml EGF. In contrast MDA-MB-231 and MCF-7AdrR cells were not growth stimulated by EGF and growth was inhibited at higher concentrations of EGF. This supports previous reports that of these three cell lines, only MCF-7 cells are stimulated by EGF [Osborne, *et al*, 1980; Godden, *et al*, 1992]. The A431 cell line was inhibited by all concentrations of EGF used in this study which agrees with previous findings [Gill, *et al*, 1981; Davidson, *et al*, 1987]. Growth stimulatory effects of EGF are confined to the cell lines with relatively low numbers of EGFR.

Figure 8 shows the effect of TGFα in addition to EGF on the growth of the MCF-7 cell line. Growth stimulation due to TGFα alone was very similar to that of EGF with maximal stimulation for both growth factors at 100ng/ml. Addition of EGF and TGFα together had a similar effect to addition of EGF alone. The lack of an additive effect of these two growth factors is additional evidence that both are acting via the same cell surface receptor and are competing for binding to this receptor. Ligand binding studies have previously shown that EGF and TGFα compete for binding to the EGFR although purified TGFα has only around half the binding activity of EGF [Winkler, *et al*, 1986]. The activities of EGF and TGFα have been compared in a number of biological assays and in some of these the two growth factors have similar activity. The two growth factors are equivalent in their ability to stimulate DNA synthesis in a number of cell lines, to induce anchorage independent growth of transformed fibroblasts and to induce eyelid opening in newborn mice [Schreiber, *et al*, 1986; Derynck, 1988].

Figure 7. Dose response curves for EGF in tumour cell lines.

MCF-7, MDA-MB-231, MCF-7AdrR and A431 cells were exposed to a range of concentrations of EGF from 0.1ng/ml to 1000ng/ml for 72 hours. Data is expressed as a percentage of the control of untreated cells and each data point represents the mean of 3 experiments performed in duplicate. The error bars indicate standard deviation.

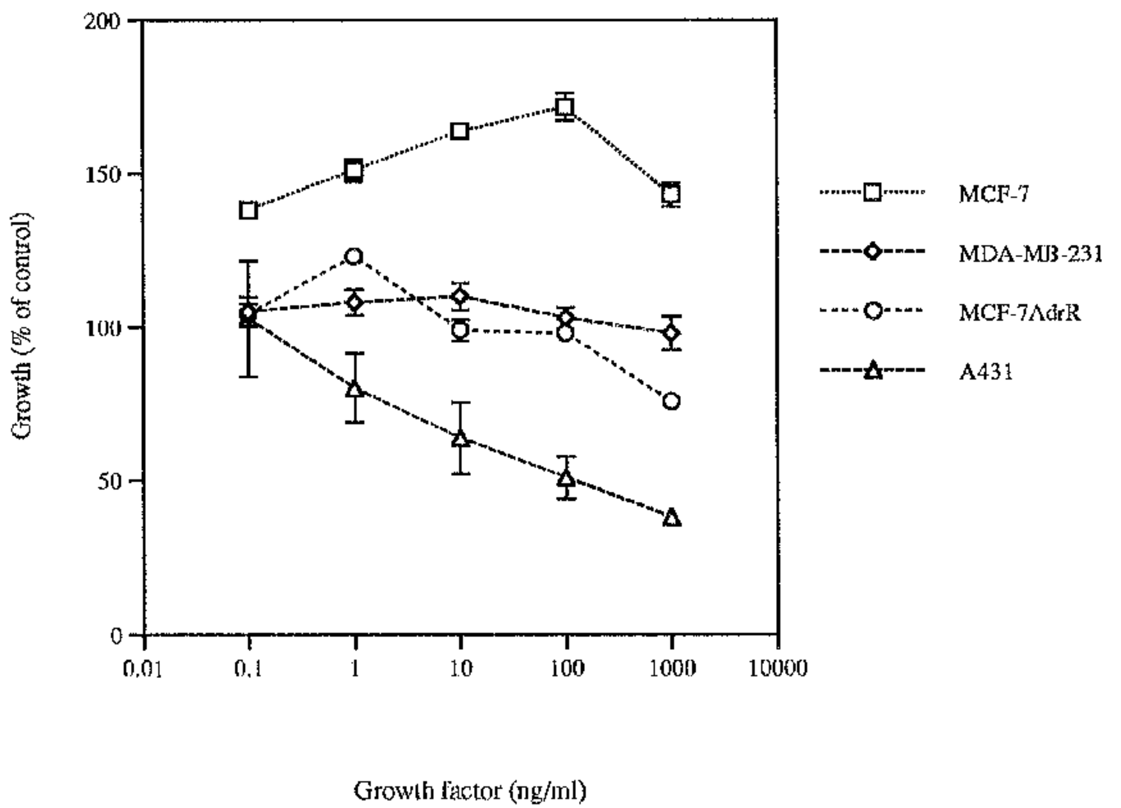
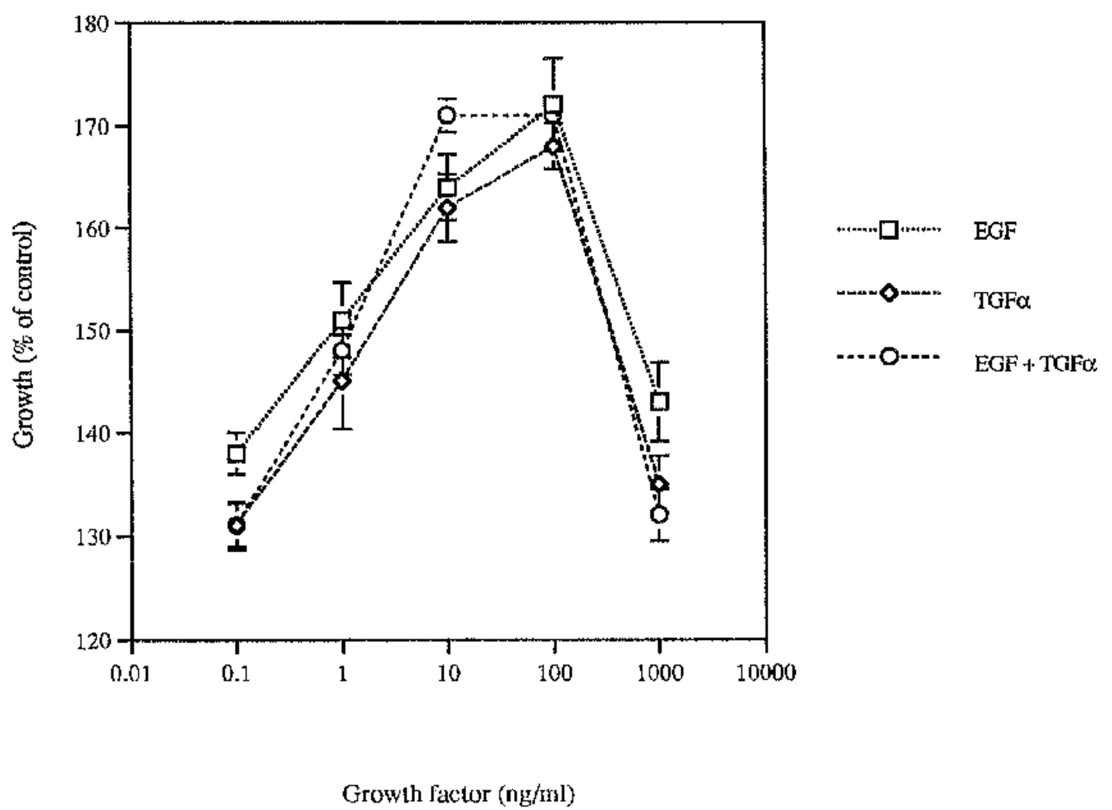

Effect of EGF on growth of cell lines
200
150
100
50
0
Growth (% of control)
0.01
0.1
1
10
100
1000
10000
Growth factor (ng/ml)
MCF-7
MDA-MB-231
MCF-7AdrR
A431

Figure 8. Dose response curves for EGF and TGFα in the MCF-7 breast cancer cell line.

MCF-7 cells were exposed to EGF alone, TGFα alone or both EGF and TGFα. Each growth factor was added in a range of concentrations from 0.1ng/ml to 1000ng/ml for 72 hours.

Data is expressed as a percentage of the control of untreated cells and each data point represents the mean of 3 experiments performed in duplicate. The error bars indicate the standard deviation.

Effect of EGF and TGFα on MCF-7 growth



3. 3. Lateral diffusion of lipids and proteins in breast cancer cells

3. 3. 1 Lipid probes

In this thesis the technique of fluorescence recovery after photobleaching was used to investigate changes in membrane fluidity in breast cancer cells. Initial experiments were performed using this technique to define some fluidity parameters under normal growth conditions for the cell lines under investigation. A number of synthetic lipid probes were utilised for FRAP studies on untreated cells.

Method

80% confluent 75cm^2 flasks were trypsinised, counted and resuspended at a density of 2 x10^{4}cells/ml in routine medium as described in Methods section 2.2.1. The cells were allowed to plate down in plated down in 35mm tissue culture dishes for at least 24 hours. 24 hours before use, the routine medium was aspirated, the monolayers washed twice with sterile PBS and serum-free medium was added. Cells were then stained with lipid probes as described in Methods section 6.4. using a 15 minute incubation at room temperature for AF18 and a 30 minute incubation at room temperature for the BODIPY-PC and BODIPY-SPM probes. The location of the probes was checked by fluorescence quenching with trypan blue - none of the probes had significantly internalised.

Results

Figure 9 shows the general form of the results obtained in FRAP experiments. This trace was obtained from an experiment using FITC-BSA in 95% glycerol. The percentage recovery in this case was 99.6% and the lateral diffusion coefficient was 240.4 x10^{-10} cm^2/s.

Figure 10 shows an example of a typical curve obtained from a FRAP experiment on MCF-7 cells labelled with AF18. The percentage recovery was 37.0% and the lateral diffusion coefficient 27.08 x10^{-10} cm^2/s.

Figures 11, 12, and 13 show results from untreated cells stained with the three lipid probes used in this study. 5-N (octadecanoyl) aminofluorescein (AF18) is a fluorescent lipid analogue with a fluorescein headgroup and a single 18 carbon fatty acid tail which labels most membrane domains. The other two probes used are fluorescent derivatives of the membrane phospholipids sphingomyelin and phosphatidylcholine. Different lipid probes were used since these may associate with different domains in the membrane [Tocanne, *et al*, 1989]. Both sphingomyelin and phosphatidylcholine are enriched in the outer monolayer of the membrane but sphingomyelin is associated with less fluid membrane domains [Van Meer, 1993].

The AF18 probe exhibits similar fluidity in all three breast cell lines. The apparent difference in rate of diffusion observed between the MCF-7 and MCF-7AdrR cell lines is not significant ($p= 0.1504$) and no significant differences are observed between the % recovery values of this probe in the three cell lines (Figure 11).

The rate of lateral diffusion of both BODIPY probes is decreased in the MCF-7 AdrR cell line relative to the MCF 7 parent cell line ($p= 0.0001$ for BODIPY-SPM, $p<0.0001$ for BODIPY-PC). A smaller decrease in D_L is observed in the MDA-MB-231 line relative to MCF-7 ($p <0.0001$ for BODIPY-PC, $p = 0.0081$ for BODIPY-SPM). No significant differences are observed in the % recovery of these two probes in the three cell lines (Figures 12 and 13).

The values obtained for D_L in this study were within the range of values previously reported for lipid diffusion in cells [Tocanne, *et al*, 1989]. The percentage recovery values obtained for all lipid probes was typically less than 50% which indicates that a large fraction of each probe is immobile. In synthetic lipid bilayers and some cell systems, percentage recovery values are usually 100% [Cherry, 1979]. However, previous studies have reported similarly low recovery values for lipid probes in a

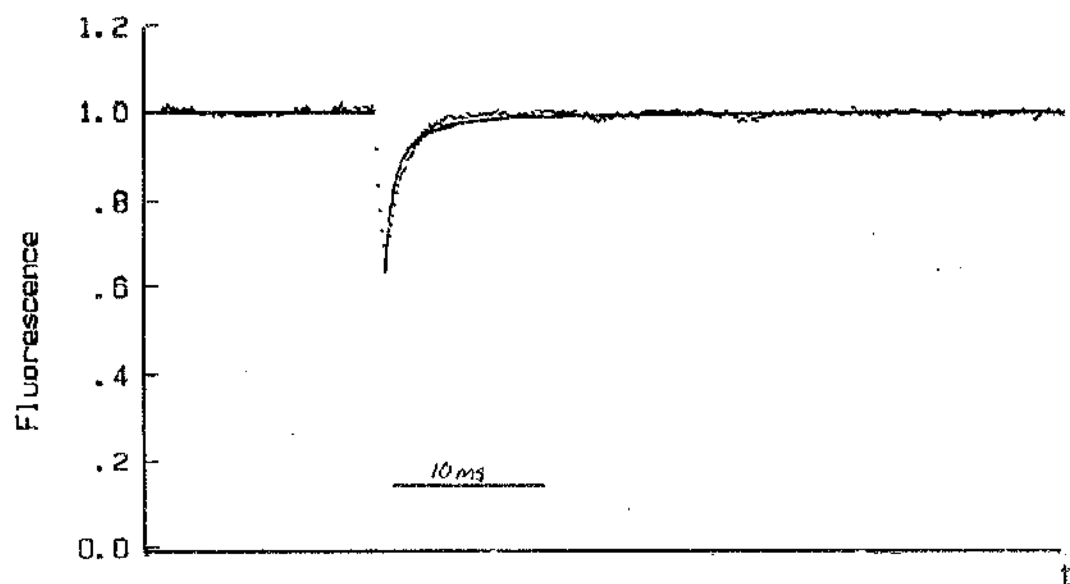
number of cell types [Tocanne, *et al*, 1989]. The presence of lipid immobile fractions suggests that the membrane is composed of lipid microdomains which differ in composition and physical state [Klausner, *et al*, 1980].

It has been suggested that higher growth rates and metastatic potential in tumour cells are associated with greater membrane fluidity [Deliconstantinos, 1987; Taraboletti, *et al*, 1989]. These results contrast with such observations since the highly tumorigenic cell line MDA-MB-231, which would be expected to be more fluid than the MCF-7 cell line, exhibits lower membrane fluidity. This may reflect differences in the techniques used to measure membrane fluidity since studies which show a correlation use the technique of fluorescence polarisation. In this technique the average fluidity in all domains in the membrane is measured and fluorescence polarisation results are not always consistent with results from FRAP experiments [Kleinfeld, *et al*, 1981]. However, the relationship between fluidity and growth rate and metastasis may be dependant on the tumour type since a previous study in breast cancer found no correlation between fluidity and metastasis [Chatterjee, *et al*, 1976].

Comparisons between the MCF-7 and MCF-7AdrR cell lines show that the lateral diffusion coefficients of both BODIPY probes are significantly smaller in the drug resistant cell line. This would indicate that the membranes of the drug resistant cells are less fluid than their drug sensitive counterpart. This supports previous fluorescence polarisation studies which found that drug resistant cells had less fluid membranes [Ramu, *et al*, 1983; Callaghan, *et al*, 1992]. It is possible that this reduction in fluidity is due to the increased membrane protein content of the MCF-7AdrR cell line which could act to stabilise the membrane within microdomains. Both p-glycoprotein and EGFR are overexpressed in this cell line [Vickers, *et al*, 1988].

Figure 9. Example of a single FRAP curve obtained with FITC-BSA in 95% glycerol

This figure shows a curve obtained after a FRAP experiment on a thin film of FITC-BSA in 95% glycerol. Fluorescence (fraction of initial prebleaching level) is plotted against time. The solid line represents the curve fitted by computer program.



1.2
1.0
.8
.6
.4
.2
0.0
Fluorescence
10 ms
t

Figure 10. Example of a single FRAP curve obtained with MCF-7 cells stained with the AF18 probe.

The cells were labelled with 10μg/ml AF18 in serum-free medium for 15 minutes at room temperature. This figure shows a curve obtained after a single bleach. Fluorescence (fraction of initial prebleaching level) is plotted against time. The solid line represents the curve fitted by computer program.

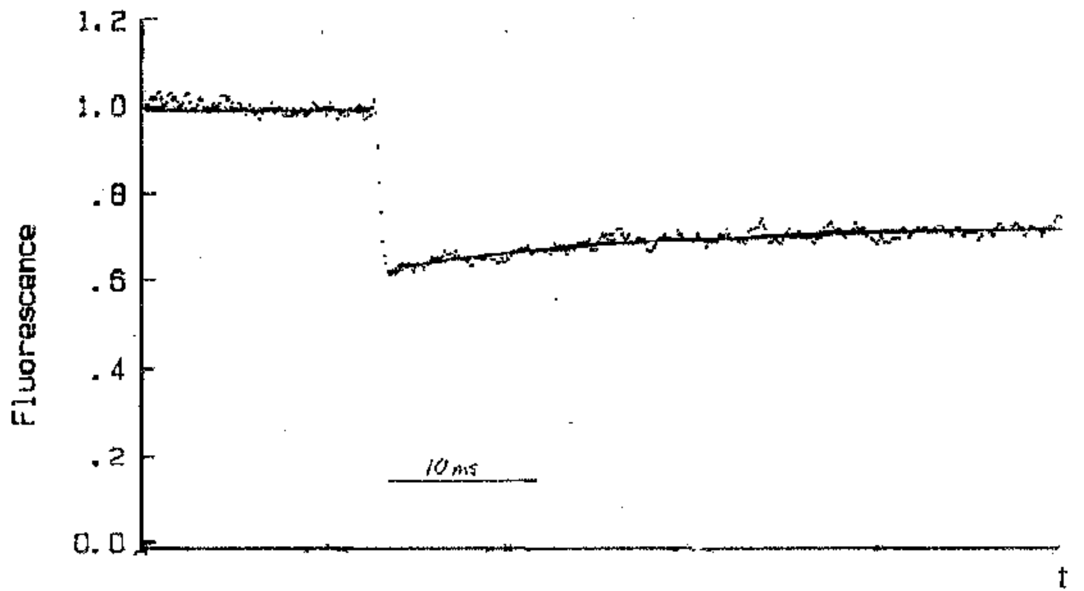
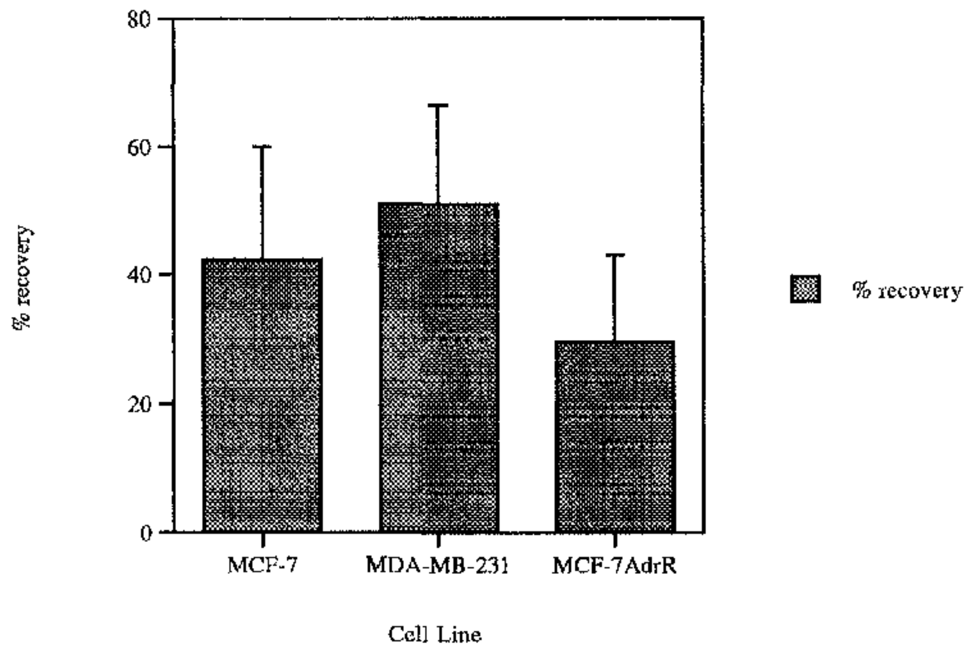

1.2
1.0
.8
.6
.4
.2
0.0
Fluorescence
10 ms
t

Figure 11. Fluidity of the AF18 probe in breast cancer cell lines.

Untreated MCF-7, MDA-MB-231 and MCF-7AdrR cells were stained for 15 minutes at room temperature with AF18.
Each data point represents the mean of 24 measurements taken during 3 separate experiments. The error bars indicate the standard deviation.

No significant difference exists in either % recovery or D_L values between MCF-7 and MDA-MB-231 (p= 0.166 for % recovery, p=0.4603 for D_L), MCF-7 and MCF-7AdrR (p= 0.076 for % recovery, p=0.1504 for D_L) or MDA-MB-231 and MCF-7AdrR (p= 0.054 for % recovery, p=0.577 for D_L)

% recovery of AF18 in breast cancer cells



Lateral diffusion coefficients of AF18 in breast cancer cells

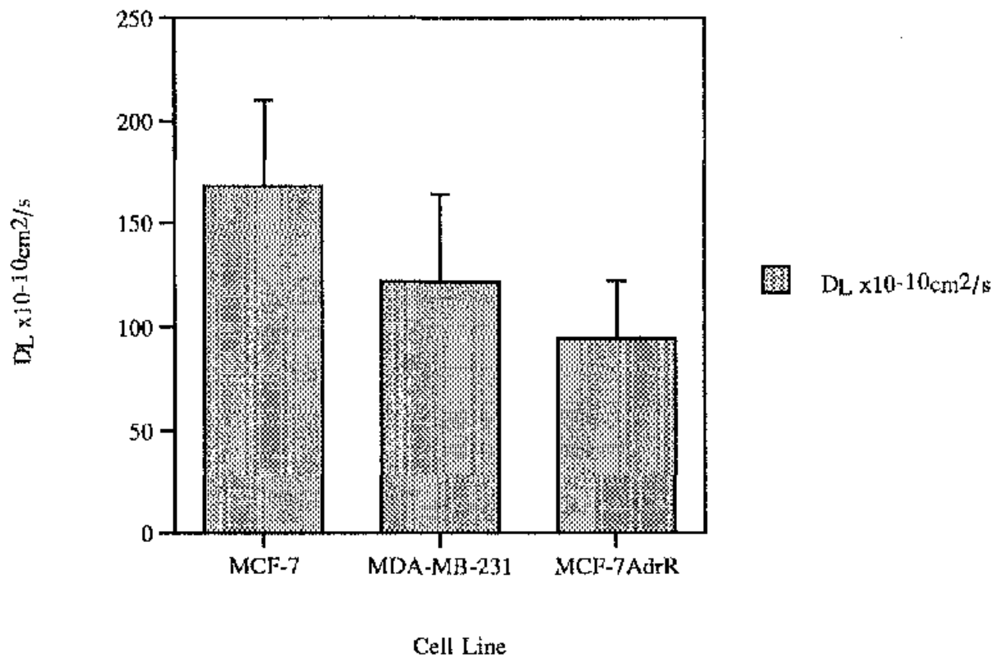
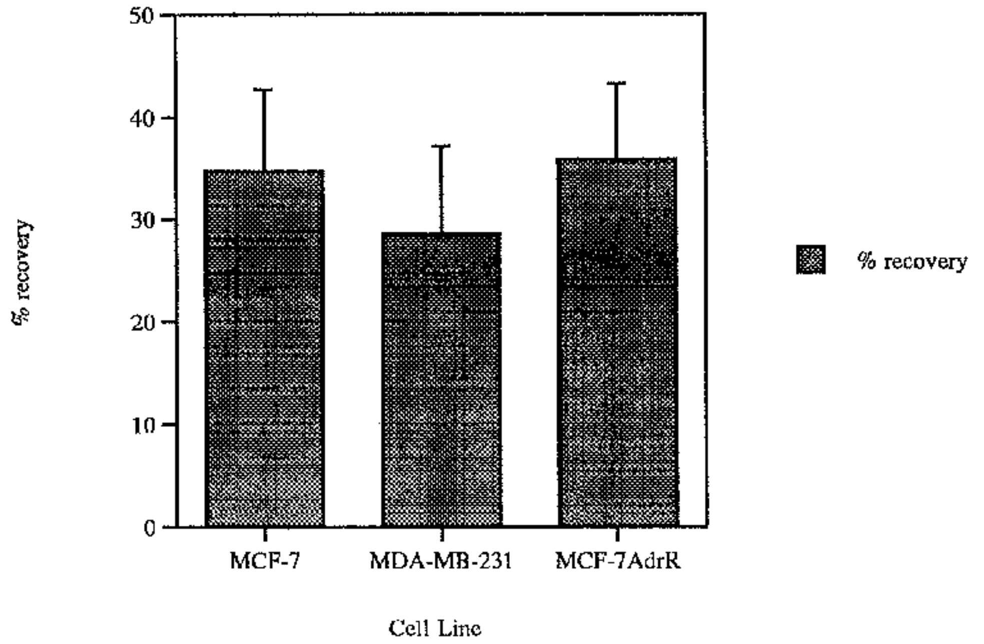


Figure 12 Fluidity of the BODIPY-phosphatidylcholine probe in breast cancer cell lines.

Untreated MCF-7, MDA-MB-231 and MCF-7AdrR cells were stained for 30 minutes at room temperature with BODIPY-phosphatidylcholine.
Each data point represents the mean of 24 measurements taken during 3 separate experiments. The error bars indicate the standard deviation.

No significant differences exist in % recovery values between MCF-7 and MDA-MB-231 ($p= 0.051$), MCF-7 and MCF-7AdrR ($p= 0.5304$) or MDA-MB-231 and MCF-7AdrR ($p= 0.055$).
Lateral diffusion is decreased in both the MDA-MB-231 and MCF-7AdrR cell lines relative to MCF-7 ($p< 0.0001$). The difference between MDA-MB-231 and MCF-7AdrR is not significant ($p= 0.054$).


% recovery of BODIPY-phosphatidylcholine
in breast cancer cells
50
40
30
20
10
0
% recovery
MCF-7
MDA-MB-231
MCF-7AdrR
Cell Line
% recovery

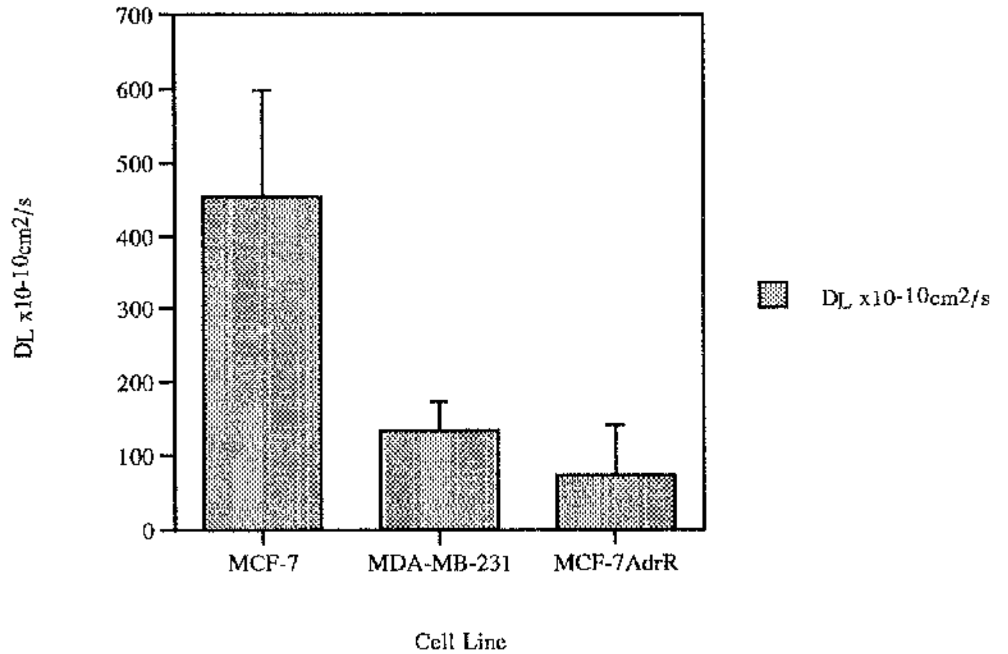
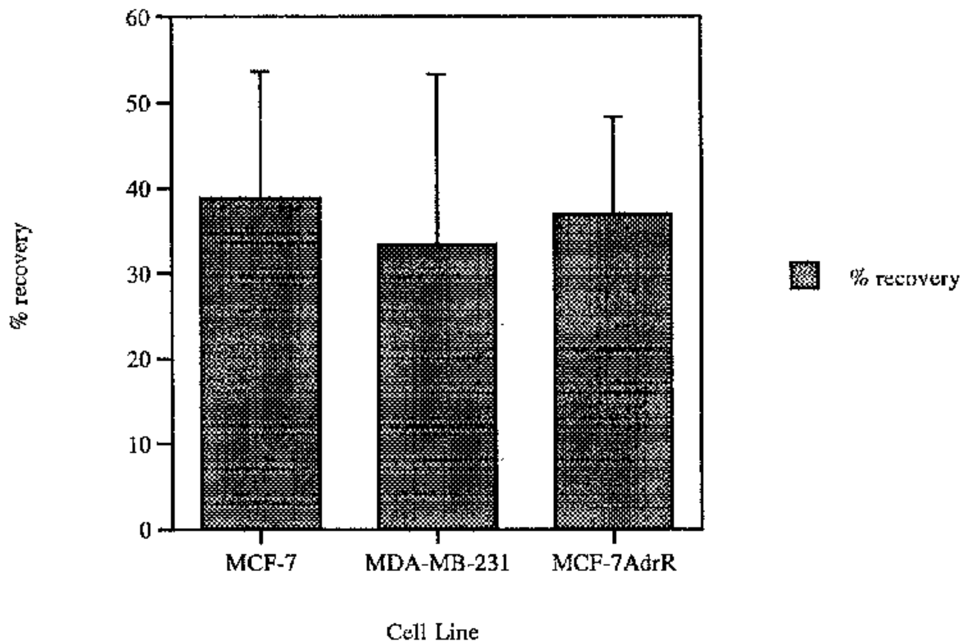

Lateral diffusion coefficients of BODIPY-phosphatidylcholine
in breast cancer cells
700
600
500
400
300
200
100
0
DL x10-10cm2/s
MCF-7
MDA-MB-231
MCF-7AdrR
Cell Line
DL x10-10cm2/s

Figure 13 Fluidity of the BODIPY-sphingomyelin probe in breast cancer cell lines.

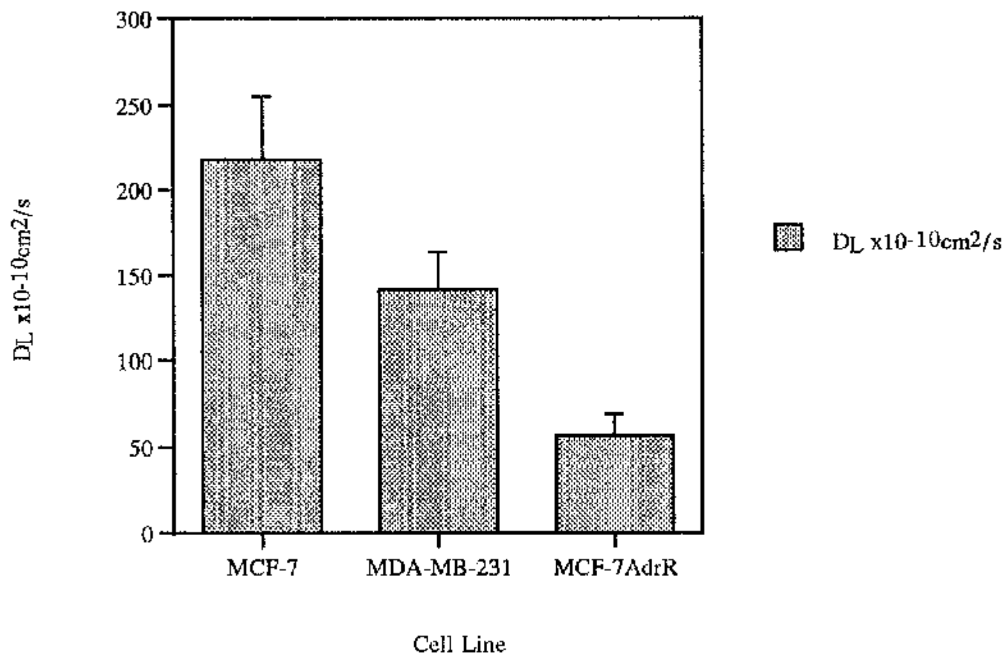
Untreated MCF-7, MDA-MB-231 and MCF-7AdrR cells were stained for 30 minutes at room temperature with BODIPY-sphingomyelin.
Each data point represents the mean of 24 measurements taken during 3 separate experiments. The error bars indicate the standard deviation.

No significant difference exists in % recovery values between MCF-7 and MDA-MB-231 ($p= 0.316$), MCF-7 and MCF-7AdrR ($p= 0.620$) or MDA-MB-231 and MCF-7AdrR ($p= 0.462$).
Lateral diffusion is decreased in both the MDA-MB-231 and MCF-7AdrR cell lines relative to MCF-7 ($p=0.0081$ and $p< 0.0001$ respectively). Lateral diffusion of this probe in MDA-MB-231 cells is decreased relative to MCF-7AdrR ($p= 0.0002$).

% recovery of BODIPY-sphingomyelin in breast cancer cells



Lateral diffusion coefficients of BODIPY-sphingomyelin in breast cancer cells



3. 3. 2. Lateral diffusion of the epidermal growth factor receptor

The most direct method of labelling the EGFR for FRAP is to use labelled epidermal growth factor. A number of studies have used fluorescently labelled EGF to study the EGF receptor in fibroblasts and A431 cells which have high numbers of EGFR [Schlessinger, *et al*, 1978; Hillman, *et al*, 1982; Rees, *et al*, 1984]. In order to study EGFR lateral diffusion in our cells, the binding of commercially available FITC-EGF and our own labelled EGF (Methods section 7.4) to breast cancer cells was studied. As EGF has only a single N-terminal site to allow FITC conjugation whereas antibodies possess multiple sites [Schechter, *et al*, 1978; Benveniste, *et al*, 1988] a labelled monoclonal antibody to EGFR was also used in order to obtain higher levels of fluorescent signalling. The antibody, clone R1, was labelled with fluorescein as described in Methods section 7.4.

Method

80% confluent 75cm^2 flasks were trypsinised, counted and resuspended at a density of 1 x10^4 cells/ml in routine medium as described in Methods section 2.2.1. The cells were allowed to plate down on 13mm diameter sterile glass coverslips in 35mm tissue culture dishes for at least 24 hours. 24 hours before use, the routine medium was aspirated, the monolayers washed twice with sterile PBS and serum-free medium was added. The coverslips were then washed with sterile PBS. To label with EGF, 50µl of 10^{-9}M FITC-EGF was added and incubated for 15 minutes in the dark. Coverslips were incubated with dilutions of antibody for 1 hour. These incubations were performed on ice to prevent internalisation of the label. The slips were then washed with cold PBS and inverted onto a drop of PBS on a slide before examining by fluorescence microscopy and quantitation of the fluorescence levels.

Results

Using FITC-EGF, staining was clearly observed in the A431 and MDA-MB-231 cell lines. However the MCF-7 cells exhibited very little, weak staining (MCF-7AdrR were unavailable at this point and were not included in these experiments). The relative intensity of staining was quantified and varied as expected with intense staining in the A431 cell line and weak staining in the MDA-MB-231 line (data not shown).

FRAP measurements were attempted on the A431 and MDA-MB-231 cell lines - this proved impossible for the MDA-MB-231 cell line since the level of signal was below that required for adequate detection with this apparatus. FRAP measurements were taken with the A431 cell line. The % recovery was 46.7% (+/-12.14) and the lateral diffusion coefficient, D_L was 2.54 $\times 10^{-10}$ (+/-2.06). These results are consistent with previously published results which give values in the range of 2.8 $\times 10^{-10}$ to 8.5 $\times 10^{-10}$ [Schlessinger, *et al*, 1978; Hillman, *et al*, 1982; Rees, *et al*, 1984].

Using the anti-EGFR antibody to label cells, staining was observed in all three cell lines used with the most intense staining in the A431 cell line, intermediate staining in the MDA-MB-231 line and weak staining in the MCF-7 line. However, despite the increased level of signal it was still impossible to take FRAP measurements in the MCF-7 cell line. Measurements with the A431 cell line were readily achieved and these again closely agreed with previously published results taken with labelled EGF [Schlessinger, *et al*, 1978; Hillman, *et al*, 1982; Rees, *et al*, 1984].

From these studies it was concluded that direct measurement of EGFR lateral diffusion in breast cell lines was not feasible using the existing equipment.

3. 4. Effect of tamoxifen on membrane fluidity

Having defined the normal fluidity parameters of the cell lines used, the effect of drugs which may alter this fluidity was examined. The non-steroidal antiestrogen tamoxifen is a highly lipophilic compound and both tamoxifen and its metabolite 4-hydroxytamoxifen have been shown to incorporate into lipid bilayers and induce structural changes in the model and native membranes [Custodio, *et al*, 1991, 1993a & 1993b] Furthermore this interaction has been shown to decrease membrane fluidity in liposomes and breast cell lines by the technique of fluorescence polarisation [Wiseman, *et al*, 1993a; Clarke, *et al*, 1989] Since this decrease in membrane fluidity could have a profound effect on the growth of cells and their response to exogenous factors, experiments were performed to confirm this effect on membrane fluidity using the FRAP technique.

Method

80% confluent $75cm^2$ flasks were trypsinised, counted and resuspended at a density of 1 $x10^4$cells/ml in routine medium as described in Methods section 2.2.1. The cells were allowed to plate down in plated down in 35mm tissue culture dishes for 24 hours. After 24 hours routine medium was aspirated, the monolayers washed twice with sterile PBS and phenol red free medium containing 2% DHIDCCFCS and tamoxifen or ethanol carrier as appropriate was added. 24 hours before FRAP, serum containing medium was withdrawn and replaced with drug dilutions in serum-free medium. Cells were then stained with lipid probes as described in Methods section 6.4 using a 15 minute incubation at room temperature for AF18 and a 30 minute incubation for the other probes.

Results

Figure 14 shows the effect of tamoxifen incubation on AF18 fluidity. The ethanol carrier increased membrane fluidity slightly, relative to untreated cells (see section

3.4.). Ethanol has a well characterised fluidising effect on cell membranes [Yun, *et al*, 1984]. Even the lowest concentration of tamoxifen used (10^{-8}M) reduced membrane fluidity as measured by lateral diffusion coefficient D_L, while higher concentrations reduced lateral diffusion even more ($P < 0.0001$). The % recovery values are not significantly altered by treatment ($P = 0.4937$). The results shown are from a 24 hour incubation with the drug - results from longer incubations are comparable (data not shown).

Figure 15 shows the effect of 10^{-6}M tamoxifen on the fluidity of the other lipid probes. Like AF18, the % recovery of BODIPY-PC and BODIPY-SPM was not significantly altered by tamoxifen ($P= 0.051$, $P= 0.1553$). Lateral diffusion of both probes was significantly decreased by tamoxifen ($P< 0.0001$). Thus the decrease in lateral diffusion is not just observed with the AF18 probe which may reflect a consistency in the effect of tamoxifen between different domains within the membrane.

Figure 16 shows the effect of 10^{-6}M tamoxifen on fluidity of AF18 in the two estrogen receptor negative cell lines, MDA-MB-231 and MCF-7AdrR. In both cell lines, tamoxifen significantly decreased the lateral diffusion of AF18 ($P< 0.0001$). The apparent decrease in the % recovery of the probe after tamoxifen treatment is not significant in either the MDA-MB-231 or MCF-7AdrR cell lines ($p = 0.347$, $p = 0.254$).

Conclusion

Tamoxifen has a general effect in reducing membrane fluidity which is reflected over a number of lipid domains and is not dependent on expression of the estrogen receptor. This effect could have profound implications for cell growth by altering the activity of membrane associated proteins. The membrane action of tamoxifen may partly explain why higher concentrations of tamoxifen have effects which are

not reversed by estradiol [Reddel, *et al*, 1985] and may contribute to tamoxifen's action in estrogen receptor negative cells particularly at high concentrations.

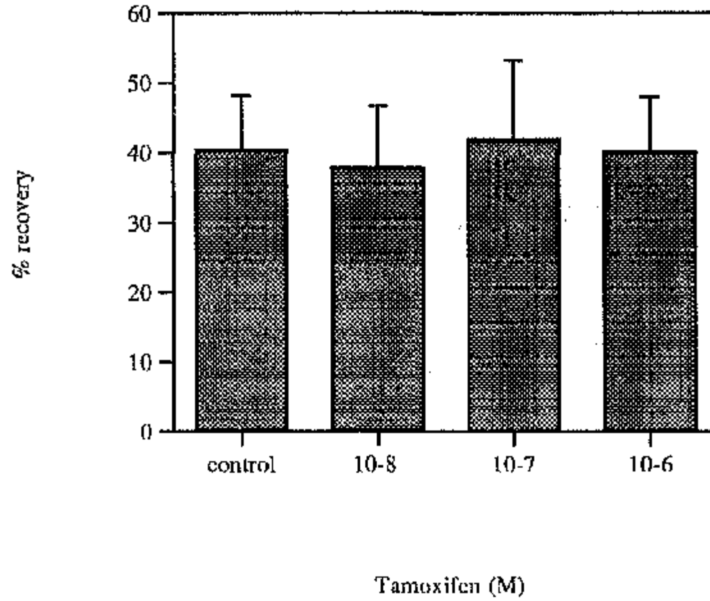
This observation that tamoxifen can decrease fluidity confirms previous observations in liposomes and breast cancer cells [Wiseman, *et al*, 1993a; Clarke, *et al*, 1989]. In these studies, the technique of DPH fluorescence polarisation was used to assess changes in membrane fluidity. Fluorescence polarisation measures the average fluidity of all membranes in the cell and across all domains. Large changes in fluorescence polarisation do not necessarily imply similar changes in lateral diffusion rates of other membrane constituents [Kleinfeld, *et al*, 1981].

Figure 14. Effect of tamoxifen on the fluidity of the AF18 probe in MCF-7 cells.

Cells were exposed to a range of concentrations of tamoxifen (10^{-8} to 10^{-6}M) or ethanol carrier for 24 hours.

Each data point represents the mean of 24 measurements taken during 3 separate experiments. The error bars indicate the standard deviation.

% recovery of AF18 in MCF-7 cells
after 24 hour incubation with tamoxifen



Lateral diffusion coefficient of AF18 in MCF-7 cells
after 24 hour incubation with tamoxifen

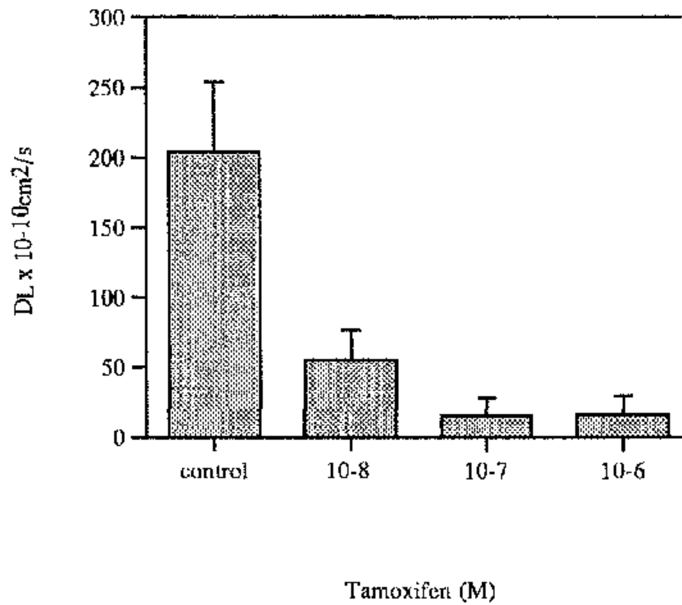
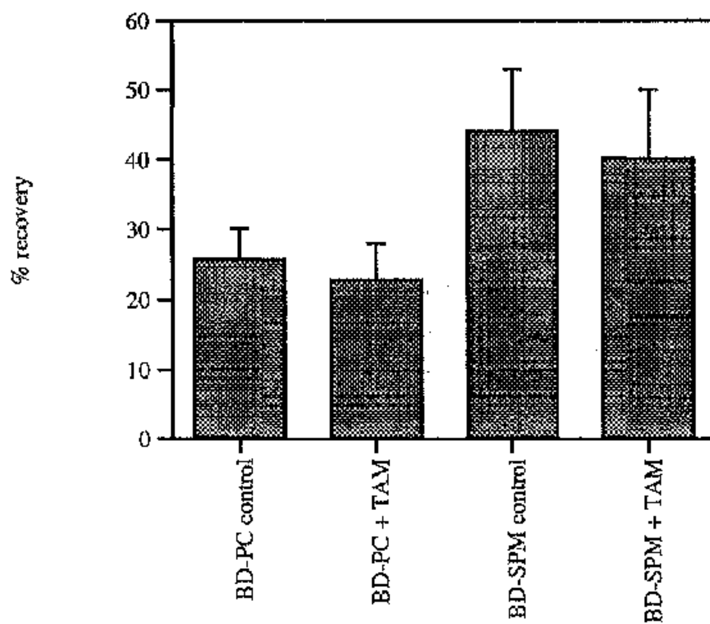


Figure 15. Effect of tamoxifen on the fluidity of the BODIPY-PC and BODIPY-SPM probes in the MCF-7 cell line.

Cells were exposed to ethanol carrier or 10^{-6}M tamoxifen for 24 hours.
Each data point represents the mean of 24 measurements taken during 3 separate experiments. The error bars indicate the standard deviation.


% recovery of BODIPY probes in MCF-7 cells
after 24 hour incubation with tamoxifen
% recovery
60
50
40
30
20
10
0
BD-PC control
BD-PC + TAM
BD-SPM control
BD-SPM + TAM

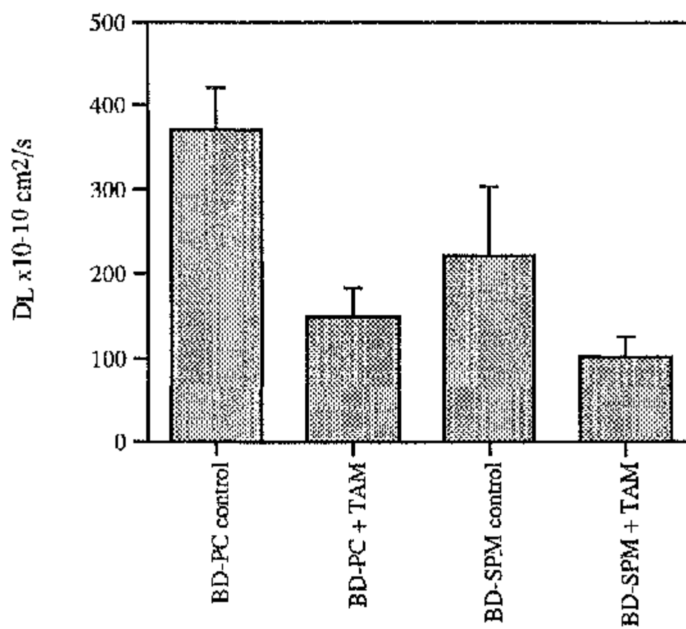
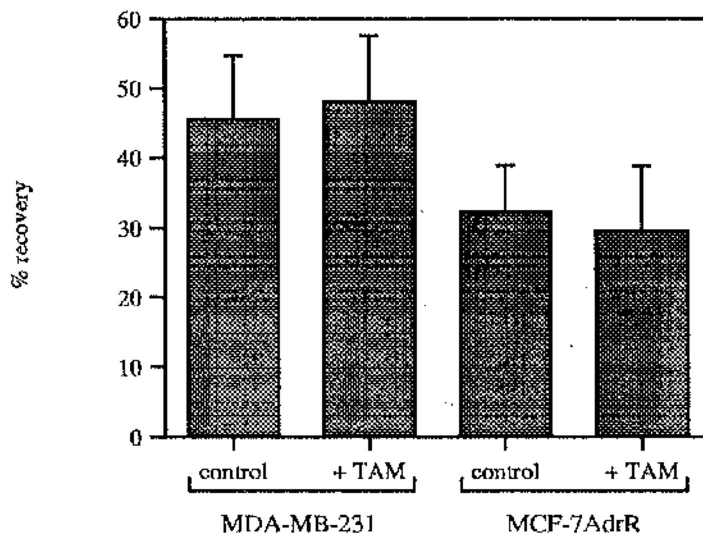

Lateral diffusion of BODIPY probes in MCF-7 cells
after 24 hour incubation with tamoxifen
DL x10-10 cm2/s
500
400
300
200
100
0
BD-PC control
BD-PC + TAM
BD-SPM control
BD-SPM + TAM

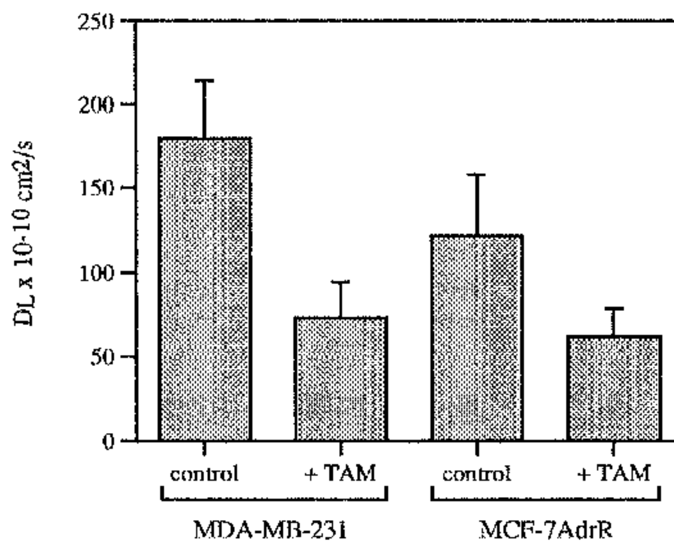
Figure 16. Effect of tamoxifen on the fluidity of the AF18 probe in the MDA-MB-231 and MCF-7AdrR cell lines.

Cells were exposed to ethanol carrier or 10^{-6}M tamoxifen for 24 hours.
Each data point represents the mean of 24 measurements taken during 3 separate experiments. The error bars indicate the standard deviation.

% recovery of AF18 in MDA-MB-231 and MCF-7AdrR cells
after 24 hour incubation with tamoxifen



Lateral diffusion of AF18 in MDA-MB-231 and
MCF-7AdrR cells after 24 hour incubation with tamoxifen



3. 5. Effect of cholesterol, 17β-estradiol and ICI 182,780 on membrane fluidity

A number of other steroidal and related compounds have been suggested to have a similar membrane stabilising effect to tamoxifen and to be effective as membrane antioxidants [Wiseman, *et al*, 1993a]. Tamoxifen's ability to decrease membrane fluidity has been attributed to its ability to adopt a conformation similar to cholesterol [Wiseman, 1992]. Cholesterol has a well defined ability to decrease membrane fluidity in biological membranes: the most important parameter defining membrane fluidity is the cholesterol:phospholipid ratio [Van Blitterswijk, 1984]. The steroidal anti-estrogen ICI164,384 has also been reported to inhibit lipid peroxidation and this may be due to a similar membrane stabilising effect [Wiseman, 1994c]. FRAP was used to examine the effect of cholesterol, 17β-estradiol and ICI 182,780 on membrane fluidity. ICI 182,780 has a structure related to ICI 164,384 and is currently being used in clinical trials [Wakeling, *et al*, 1992; Nicholson, *et al*, 1993b; Howell, *et al*, 1996].

Method

80% confluent 75cm^2 flasks were trypsinised, counted and resuspended at a density of 1 x10^{4}cells/ml in routine medium as described in Methods section 2.2.1. The cells were allowed to plate down in plated down in 35mm tissue culture dishes for 24 hours. After 24 hours routine medium was aspirated, the monolayers washed twice with sterile PBS and phenol red free medium containing 2% DHIDCCFCS and 10^{-6}M tamoxifen, 17β-estradiol, ICI 182,780 or ethanol carrier as appropriate was added. 24 hours before FRAP, serum containing medium was withdrawn and replaced with drug dilutions in serum-free medium. Cells were then stained with AF18 as described in Methods section 6.4 using a 15 minute incubation at room temperature.

Results

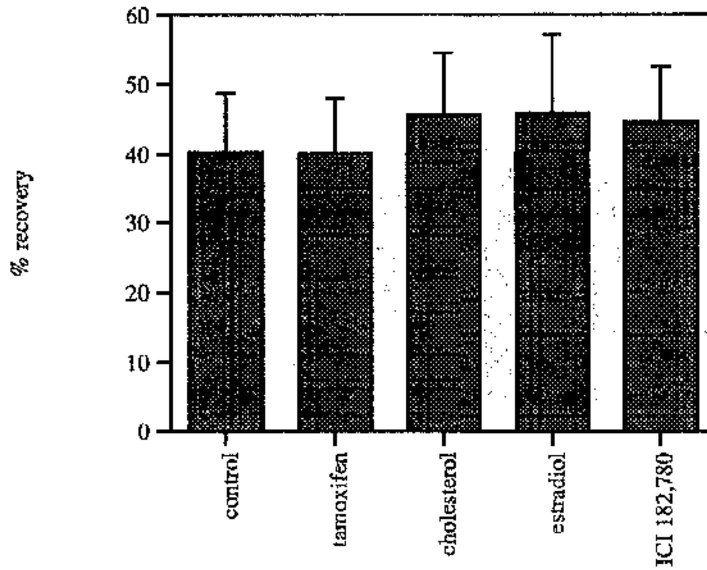
Figure 17 shows the effect of cholesterol, estradiol and ICI 182,780 on the fluidity of MCF-7 cells measured using the AF18 probe. All three compounds significantly decreased AF18 fluidity relative to control treated cells ($P<0.0001$). The decrease in fluidity by these steroids is not significantly different to that produced by tamoxifen ($P= 0.1155$). Although 17β-estradiol has previously been shown to be more effective than tamoxifen in decreasing membrane fluidity [Wiseman, *et al*, 1993a], these results show no significant difference. This may be due to the different techniques used to measure fluidity.

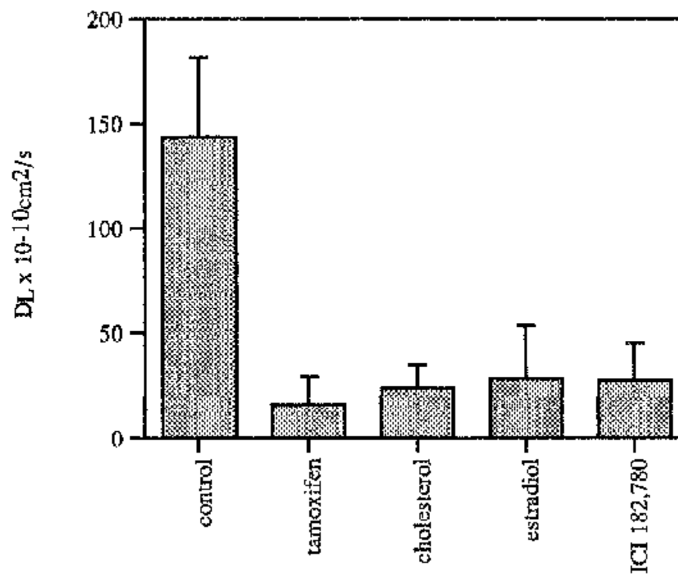
Tamoxifen and related compounds all significantly decrease membrane fluidity in breast cancer cells. Since the membrane is the site of many molecules with important roles in the control of cell growth, this effect could have implications for tumour growth.

Figure 17. Effect of tamoxifen, 17β estradiol and ICI 182,780 on the fluidity of the AF18 probe in the MCF-7 cell line.

Cells were exposed to ethanol carrier or 10^{-6}M tamoxifen, 17β estradiol or ICI 182,780 for 24 hours.

Each data point represents the mean of 30 measurements taken during 3 separate experiments. The error bars indicate the standard deviation.


% recovery of AF18 in MCF-7 cells
% recovery
60
50
40
30
20
10
0
control
tamoxifen
cholesterol
estradiol
ICI 182,780


Lateral diffusion of AF18 in MCF-7 cells
DL x 10-10cm2/s
200
150
100
50
0
control
tamoxifen
cholesterol
estradiol
ICI 182,780

3. 6. Effect of tamoxifen and the steroidal antiestrogen ICI182,780 on breast cancer cell growth

The antiestrogen tamoxifen has been proposed to inhibit breast cancer cell growth by competitively inhibiting the estrogen receptor [Wakeling, *et al*, 1984]. Tamoxifen exhibits a diversity of molecular actions which cannot all be accounted for by its action as an estrogen receptor antagonist. The drug is effective against some estrogen receptor negative cell lines and some tumours and inhibition by high concentrations of tamoxifen is not reversed by estradiol [Langdon, *et al*, 1990; Darbre, *et al*, 1984; Reddel, *et al*, 1985]. Tamoxifen also functions as a partial estrogen receptor agonist which causes a number of side effects such as an increased risk of endometrial cancer. The steroidal anti-estrogens ICI164,384 and ICI182,780 were developed in an attempt to overcome some of the harmful side effects caused by the estrogenic action of tamoxifen and to help overcome resistance to tamoxifen. These drugs have a structure based on 17β-estradiol and should be devoid of estrogenic activity [Wakeling, *et al*, 1988 & 1991]. However studies report that ICI164,384 exhibits anti cancer actions which are not dependent on estrogen [Wiseman 1994c]. Growth assays were performed using the three breast cell lines to determine the dose-response curves of both drugs in the absence of estrogens.

Method

80% confluent flasks of cells were trypsinised, counted and resuspended at a density of $5x10^4$ cells/ml as described in Methods Section 2.2.1. The cell suspension was plated down onto 6 well plates and allowed to settle for 24 hours. The routine medium was then removed and the monolayers were washed twice with sterile PBS. Stock concentrations of tamoxifen and ICI 182,780 were prepared in absolute alcohol and diluted 1:100 in culture medium before addition to cells. The drug dilutions were prepared in phenol red free medium containing 2%

DHIDCCFCS and added in duplicate to wells in the 6 well plates. Control cells were incubated in steroid free medium with the same final concentration of ethanol carrier as the experimental medium. Phenol red is a weak estrogen so in addition to the use of stripped fetal calf serum it is important to use phenol red free medium in these assays [Berthois, *et al*, 1986].

After 72 hours at 37°C, the medium was removed and the monolayers washed twice in PBS. The cells were harvested by trypsinisation, resuspension and centrifugation. The cell pellets were lysed by incubation with 0.2% SDS for 30 minutes at 37°C. The DNA content was assayed by the Hoechst method as described in Methods section 3.

Results

Figure 18 shows the effect of tamoxifen on the growth of the breast cell lines used in this study. Growth of the MCF-7 cell line was slightly stimulated by low concentrations of tamoxifen (10^{-10}M to 10^{-9}M) and only inhibited by concentrations above 10^{-7}M. The two estrogen receptor negative cell lines, MDA-MB-231 and MCF-7AdrR were relatively unaffected by low concentrations but were inhibited by concentrations above 10^{-7}M.

Figure 19 shows the effect of ICI 182,780 on the three breast cell lines. MCF-7 cells were significantly inhibited by ICI182,780 at concentrations of 10^{-10}M and greater. The stimulatory effect observed with 10^{-10} to 10^{-9}M tamoxifen was not seen with ICI 182,780 while the degree of inhibition seen with 10^{-8} to 10^{-6}M was much greater than that observed in these cells with tamoxifen (see Figure 17). The MDA-MB-231 and MCF-7AdrR cell lines were relatively unaffected by ICI182,780 at lower concentrations but inhibited by concentrations greater than 10^{-7}M.

Both tamoxifen and the steroidal anti-estrogen ICI182,780 can inhibit breast cell growth in the absence of exogenous estrogens. This effect was most marked at concentrations of 10^{-7}M and above. At these concentrations tamoxifen's effect has been shown not to be inhibited by estradiol [Darbre, *et al,* 1984; Reddel, *et al,* 1985]. It is probable that the effect at these concentrations which was also found in the estrogen receptor negative cell lines MDA-MB-231 and MCF-7AdrR was due to an effect not mediated by the estrogen receptor.

Figure 18. Dose response curves for tamoxifen in breast cancer cell lines.

MCF-7, MDA-MB-231 and MCF-7AdrR cells were exposed to a range of concentrations of tamoxifen from 10^{-10}M to 10^{-6}M, or ethanol carrier only for 72 hours. Cell growth was assessed by DNA assay.

Data is expressed as a percentage of the control of untreated cells and each data point represents the mean of 3 experiments performed in duplicate. The error bars indicate the standard deviation.

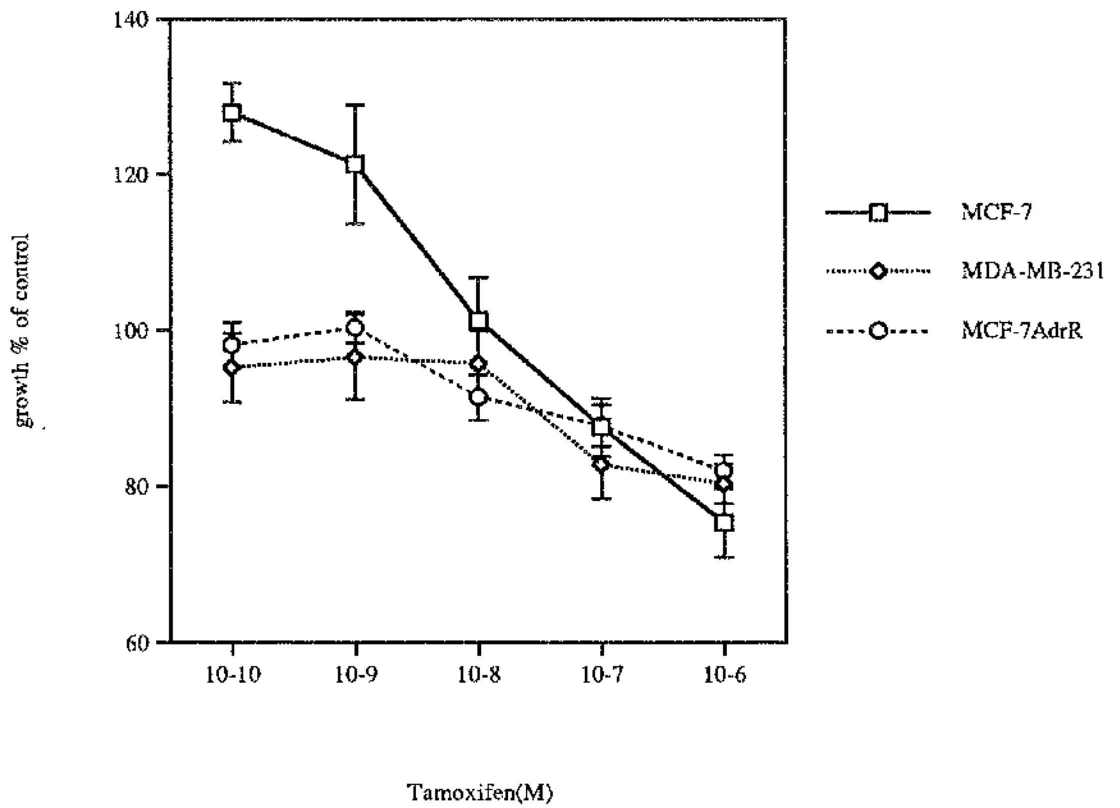
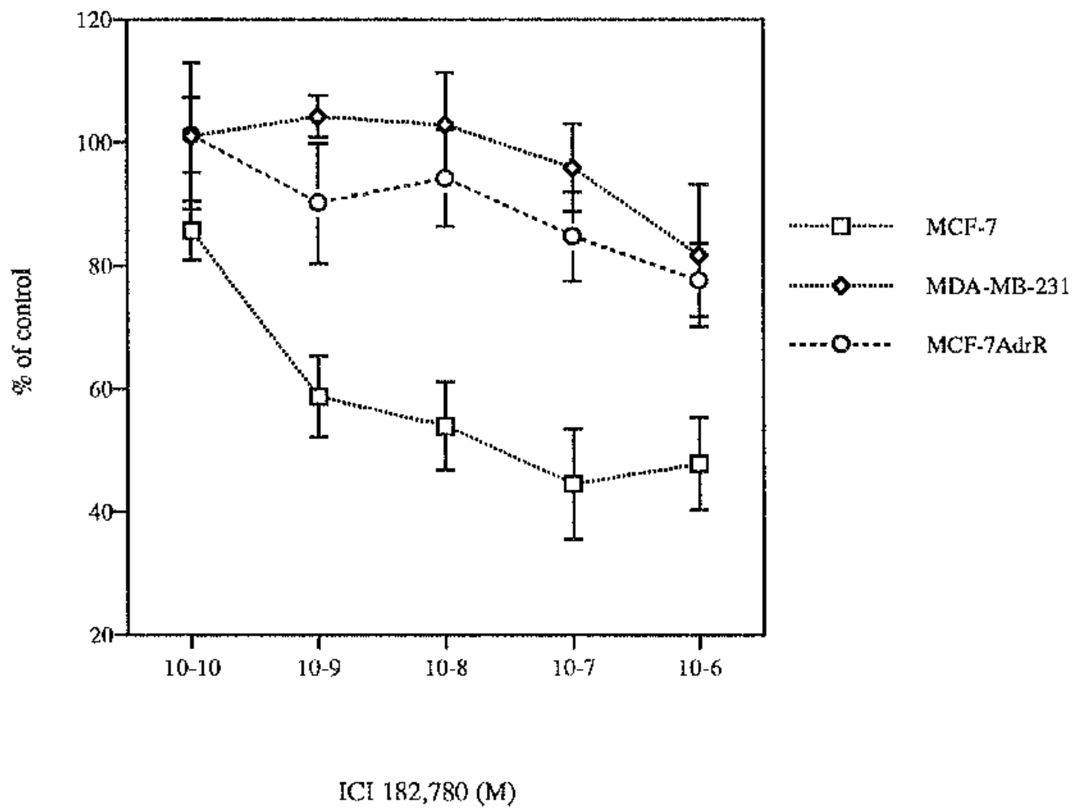

Effect of tamoxifen on growth of breast cancer cells
140
120
100
80
60
growth % of control
10-10
10-9
10-8
10-7
10-6
Tamoxifen(M)
MCF-7
MDA-MB-231
MCF-7AdrR

Figure 19. Dose response curves for ICI 182,780 in breast cancer cell lines.

MCF-7, MDA-MB-231 and MCF-7AdrR cells were exposed to a range of concentrations of ICI 182,780 from 10^{-10}M to 10^{-6}M, or ethanol carrier only for 72 hours. Cell growth was assessed by DNA assay.

Data is expressed as a percentage of the control of untreated cells and each data point represents the mean of 3 experiments performed in duplicate. The error bars indicate the standard deviation.


Effect of ICI 182,780 on breast cancer cell growth
120
100
80
60
40
20
% of control
10-10
10-9
10-8
10-7
10-6
ICI 182,780 (M)
MCF-7
MDA-MB-231
MCF-7AdrR

3. 7. Effect of tamoxifen on response to EGF stimulation

Tamoxifen may have actions other than through the estrogen receptor and may interfere with signalling via the EGFR [Wiseman, 1994b]. Tamoxifen treatment decreases membrane fluidity (section 3.4.) which could interfere with the functioning of membrane associated receptors, enzymes and channels [Van Blitterswijk, 1984]. EGF receptor activation depends on the receptor ability to diffuse laterally. The effect of concentrations of tamoxifen which decrease membrane fluidity on EGF-stimulated growth of MCF-7 cells was examined. MCF-7 cells are maximally stimulated by 100ng/ml EGF (Section 3.2.). The other cell lines in this study do not exhibit a growth response to EGF.

Method

80% confluent flasks of cells were trypsinised, counted and resuspended at a density of $5x10^4$ cells/ml as described in Methods Section 2.2.1. The cell suspension was plated down onto 6 well plates and allowed to settle for 24 hours. The routine medium was then removed and the monolayers were washed twice with sterile PBS. Stock concentrations of tamoxifen were prepared in absolute alcohol and diluted 1:100 in culture medium before addition to cells. The tamoxifen dilutions were prepared in phenol red free medium containing 2% DHIDCCFCS and added in duplicate to wells in the 6 well plates. Tamoxifen negative control cells were incubated in steroid free medium with the same final concentration of ethanol carrier as the experimental medium. Duplicate incubations with various concentrations of tamoxifen were made in the presence and absence of 100ng/ml EGF. The medium was replaced with fresh media containing appropriate additions after 48 hours.

After 72 hours at 37°C, the medium was removed and the monolayers washed twice in PBS. The cells were harvested by trypsinisation, resuspension and centrifugation.

The cell pellets were lysed by incubation with 0.2% SDS for 30 minutes at 37°C. The DNA content was assayed by the Hoechst method as described in Methods section 3.

Results

Figure 20 shows the effect of various concentrations of tamoxifen on EGF stimulation of growth. In the control treated cells (ethanol carrier only), addition of 100ng/ml EGF stimulated growth relative to the control by 174% ($p < 0.0001$). Treatment with 10^{-8}M and 10^{-7}M tamoxifen reduced the level of stimulation by EGF. In cells treated with 10^{-6}M tamoxifen, EGF-stimulated growth was not significantly different from growth in the absence of EGF ($p=0.4732$). Thus EGF stimulation of MCF-7 growth was reduced by tamoxifen treatment. It is possible that this growth inhibition is in part due to the action of TGFβ which is produced by cells in response to tamoxifen [Knabbe, *et al*, 1987]. To exclude this possibility, we repeated the experiment in the presence of TGFβ neutralising antibodies. Elimination of TGFβ from the medium partially reversed the effect but did not completely remove tamoxifen inhibition of EGF-stimulated growth. This suggests that tamoxifen affects the functioning of EGFR by another mechanism.

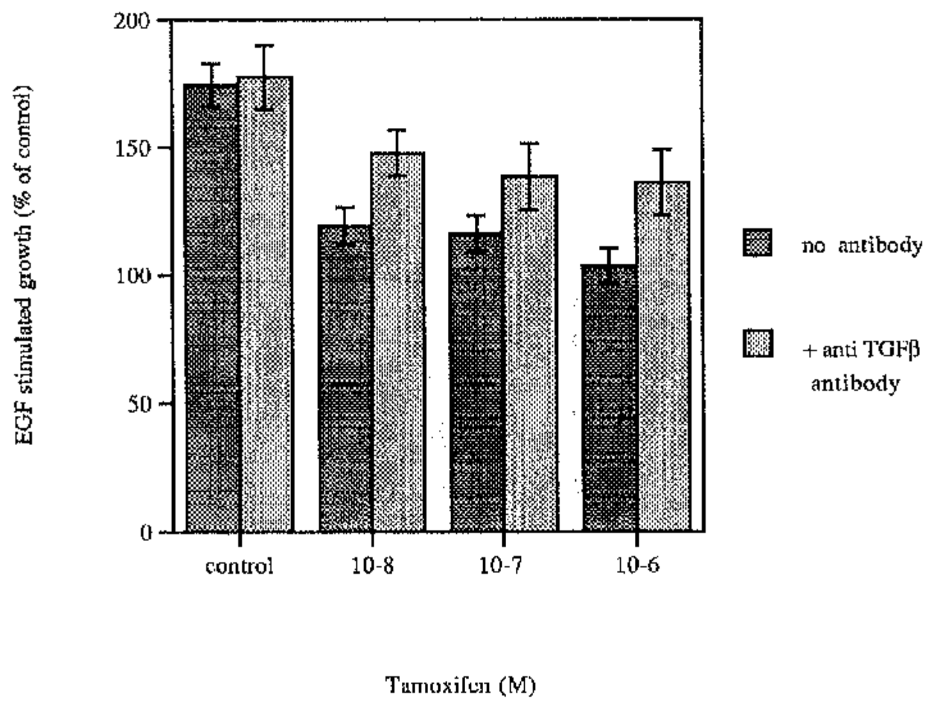
Previous studies have investigated tamoxifen's ability to inhibit growth factor stimulated growth. Tamoxifen and 4-hydroxytamoxifen have been shown to inhibit EGF-stimulated MCF-7 cell growth in some studies [Vignon, *et al*, 1987; Freiss, *et al*, 1990a; Wosikowski, *et al*, 1993] although conflicting reports exist [Cormier *et al*, 1989]. One study suggested the effect was due to a decrease in the level of expression of EGFR induced by tamoxifen treatment which resulted in lower EGF responsiveness [Vignon, *et al*, 1987]. However tamoxifen's effect on EGFR expression is not observed until after 5 days treatment [Berthois, *et al*, 1989]. The effect over this time scale is also unlikely to be due to modulation of the receptor's affinity for EGF by tamoxifen [Freiss, *et al*, 1990b].

Figure 20. Effect of Tamoxifen on EGF-stimulated MCF-7 cell growth.

Cells were exposed to an optimal concentration of 100ng/ml EGF for 72 hours in the presence of a range of concentrations of tamoxifen (10^{-8} to 10^{-6}M) or ethanol carrier only.

Cell growth was assessed by a DNA assay. Each data point represents the mean of 3 separate experiments. The error bars indicate the standard deviation.

Effect of tamoxifen on EGF-stimulated MCF-7 growth



3. 8. Effect of tamoxifen on EGF binding to cells

One possible explanation for tamoxifen's effect on signalling via the EGFR is that tamoxifen alters the level of receptor, or alters the binding of EGF to the receptor. It has been reported that tamoxifen treatment downregulates expression of the epidermal growth factor receptor [Berthois, *et al*, 1989]. Changes in membrane fluidity can potentially alter the cell surface exposure and ligand binding of receptors [Shinitsky, 1984]. Decreasing membrane fluidity increases the exposure and binding capacity of the insulin receptor [Neufeld, 1986]. A whole cell EGF binding assay was performed to examine the effects of tamoxifen treatment on EGF binding and levels of receptor expression.

Method

Cells were plated down onto 24 well plates in routine medium. After 24 hours the medium was removed, the cells washed twice with PBS and experimental medium containing 2% DHIDCCFCS was added. 24 hours before the assay the medium was removed, the cells washed twice with PBS and experimental medium containing tamoxifen or ethanol carrier was added. Whole cell EGF receptor binding was determined as described in Methods section 8.

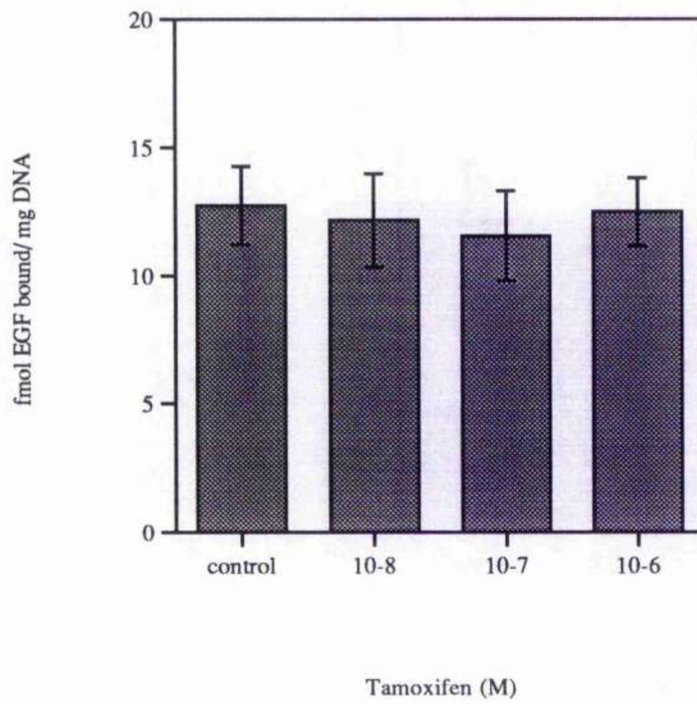
Results

Figure 21 shows the effect of tamoxifen treatment on the EGF binding of MCF-7 cells. No significant difference was observed between treated and control cells. It is therefore unlikely that downregulation of receptor expression contributes to the effect of tamoxifen on EGFR function. These results also suggest that reduction of membrane fluidity does not significantly alter the ligand binding ability of the receptor. This was further confirmed using quantitative fluorescence of stained cells. Cells stained with anti-EGFR antibodies exhibited identical levels of staining both before and after tamoxifen treatment (data not shown).

Figure 21. Effect of tamoxifen on cell surface EGF binding in MCF-7 cells.

Cells were pretreated with a range of concentrations of tamoxifen (10^{-8} to 10^{-6}M) or ethanol carrier only for 24 hours before the EGF binding assay.

Data is expressed as fmol ^{125}I-EGF bound/μg DNA and each data point represents the mean of 3 separate experiments performed in quadruplicate. The error bars indicate the standard deviation.


Effect of tamoxifen on cell surface EGF binding
fmol EGF bound/ mg DNA
20
15
10
5
0
control
10-8
10-7
10-6
Tamoxifen (M)

3. 9. Effect of tamoxifen on the EGF receptor tyrosine kinase activity

3. 9. 1. Assay of tyrosine kinase in isolated membranes

EGFR receptor activity depends on its intrinsic protein tyrosine kinase activity. Tamoxifen has been shown to reduce both membrane fluidity and cell growth response to EGF in MCF-7 cells. The reduction in membrane fluidity caused by tamoxifen could interfere with the ability of receptor monomers to diffuse laterally within the bilayer. This would interfere with the process of intermolecular activation of receptors which would be expected to result in a decrease in the protein tyrosine kinase activity of the receptor and hence signalling processes via the receptor. To assess this effect, cells were pretreated with tamoxifen, membranes were isolated and the EGFR-PTK activity was measured using a commercially available kit.

Method

80% confluent flasks of cells were trypsinised, counted and resuspended at a density of 5×10^4 cells/ml as described in Methods Section 2.2.1. The cell suspension was plated down onto 100mm dishes and allowed to grow to approximately 80% confluence. The routine medium was then removed and the monolayers were washed twice with sterile PBS. Stock concentrations of tamoxifen were prepared in absolute alcohol and diluted 1:100 in culture medium before addition to cells. The tamoxifen dilutions were prepared in phenol red free medium containing 2% DHIDCCFCS and added. Control cells were incubated in steroid free medium with the same final concentration of ethanol carrier as the experimental medium.

After 24 hours, membranes were prepared from the cells and the PTK activity was assayed using the Amersham kit as described in Methods section 8.

Results

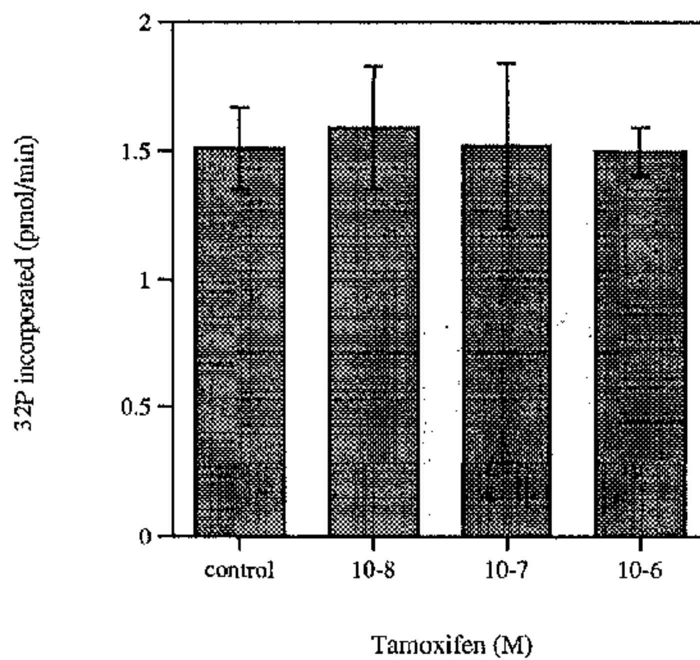
Figure 22 shows EGFR tyrosine kinase activity in membrane treated with various concentrations of tamoxifen. No significant difference in PTK activity was detected between control cells and cells treated with 10^{-8}, 10^{-7} or 10^{-6}M tamoxifen ($p = 0.8161$, $p=0.9972$ and $p=0.9442$). These concentrations of tamoxifen have previously been shown to decrease membrane fluidity and to inhibit EGF stimulated growth. This may indicate that the observed reduction in fluidity caused by tamoxifen has no effect on lateral mobility and hence PTK activity of the EGFR. However this assay is performed using solubilised receptors. This may destroy any constraining effect due to fluidity - receptor dimers may be able to interact and activate each other without the need for lateral diffusion. Thus a whole cell receptor assay is needed to differentiate between these two possibilities.

Figure 22. Effect of preincubation with tamoxifen on EGFR tyrosine kinase activity in membranes isolated from MCF-7 cells.

Cells were exposed to a range of concentrations of tamoxifen (10^{-8}M to 10^{-6}M) or ethanol carrier control before plasma membranes were isolated as described in Methods section 9.1. EGFR tyrosine kinase assay was measured using the Biotrak EGF-receptor protein tyrosine kinase assay system (Amersham).

Each data point represents the mean of 3 separate experiments. The error bars indicate the standard deviation.

Effect of preincubation with tamoxifen on EGF receptor tyrosine kinase activity in MCF-7 cell membranes



3. 9. 2. EGF Receptor Protein Tyrosine kinase assays in whole cells

Autophosphorylation of the EGFR is the next step in the signalling pathway after dimerisation and activation of receptor monomers. EGFR which fail to activate cannot undergo autophosphorylation while constitutive activation of the receptor results in permanent autophosphorylation. Autophosphorylation is necessary for maximal biological activity of the EGFR [Helin, *et al*, 1991]. Autophosphorylation is therefore an expression of receptor's intrinsic tyrosine kinase activity and might be expected to be reduced if dimerisation of receptors is inhibited [Honegger, *et al*, 1987a]. In order to detect receptor autophosphorylation in whole cells, the cells were first preloaded with ^{32}P to label the intracellular pool of ATP. Cells were then stimulated with EGF and the level of autophosphorylation of EGFR determined by autoradiography after separation by SDS-PAGE.

Method

The intracellular pool of ATP was first preloaded with ^{32}P by incubating the cells for 16 hours in the presence of ^{32}P in phosphate free medium (Methods section 9.1). Cell viability over this period was assessed by trypan blue exclusion (Methods section 2.2.2). MCF-7 and MCF-7AdrR cells were used for this assay since greater than 95% of these cells remained viable over the labelling period. The cells were then stimulated with EGF, lysed and the receptor immunoprecipitated as described in Methods section 9. Equal amounts of immunoprecipitate were run on SDS-PAGE as described in Methods section 10.

Results

Figure 23(a) shows the effect of tamoxifen treatment on autophosphorylation of the EGFR in MCF-7 cells. In the control cells, autoradiography demonstrated that EGF stimulation resulted in increased phosphorylation of the 170K EGF receptor (Lanes 1 and 2). After pretreatment of the cells with tamoxifen, EGF-stimulated

incorporation of ^{32}P into the EGFR is significantly reduced (Lanes 3 and 4). This suggests that tamoxifen pretreatment reduces the tyrosine kinase activity of the receptor. A similar effect is found in MCF-7AdrR cells Figure 23(b). Although these cells do not exhibit growth stimulation in response to EGF, ligand binding does induce receptor autophosphorylation (Lanes 1 and 2). Pretreatment of MCF-7AdrR cells with tamoxifen reduces the stimulation of ^{32}P incorporation into the EGFR (Lanes 3 and 4).

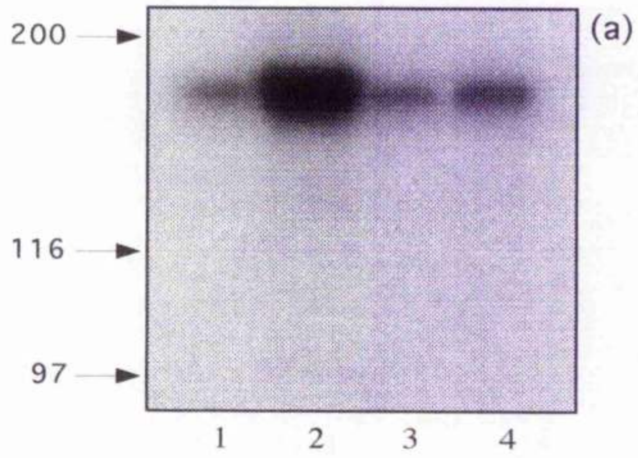
Tamoxifen's effect on EGFR phosphorylation is found in the complete absence of exogenous estrogens and in the MCF-7AdrR cell line which lacks estrogen receptor, it must be unrelated to tamoxifen's estrogen receptor mediated actions. Since tamoxifen can reduce membrane fluidity, such an effect could be due to restricted lateral diffusion of the receptor which would inhibit the ability to form receptor dimers and hence reduce autophosphorylation.

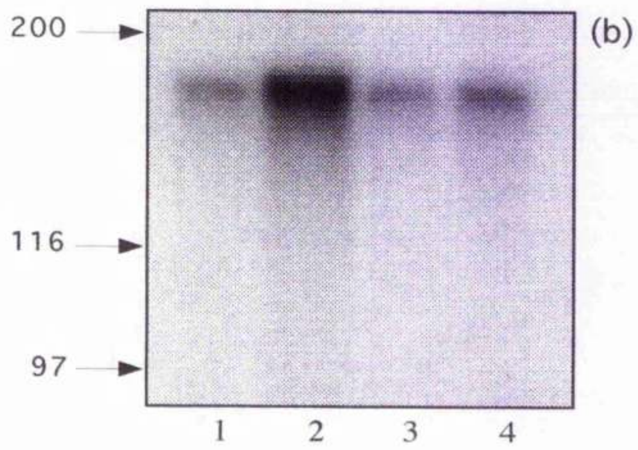
Figure 23. Effect of preincubation with tamoxifen on EGFR tyrosine kinase activity in MCF-7 cells (a) and MCF-7AdrR cells (b). Autoradiographs of immunoprecipitated EGFR.

80% confluent cells were preloaded with ^{32}P by 16 hour incubation at 37°C with 0.5mCi/ml ^{32}P in phosphate free medium. After exposure to EGF for 15 minutes, EGFR were immunoprecipitated as described in Methods section 10 followed by SDS-PAGE. The gel was dried and exposed to X-ray film for 24 hours.

The lanes in each autoradiograph represent the following:

1. unstimulated control treated cells
2. control cells stimulated with 10^{-9}M EGF
3. unstimulated tamoxifen pretreated cells
4. tamoxifen pretreated cells stimulated with 10^{-9}M EGF


200
116
97
(a)
1 2 3 4


200
116
97
(b)
1 2 3 4

3. 10. Effect of tamoxifen on epidermal growth factor receptor lateral diffusion

Although attempts to measure EGFR fluidity directly were restricted by low receptor numbers and consequent low levels of fluorescent signal, it was possible to directly measure EGFR lateral diffusion by FRAP in the A431 cell line. Therefore, this cell line was studied in order to determine if the reduction in membrane fluidity observed with lipid probes were also reflected by changes in EGFR fluidity.

Method

80% confluent $75cm^2$ flasks were trypsinised, counted and resuspended at a density of 1 $x10^4$cells/ml in routine medium as described in Methods section 2.2.1. The cells were allowed to plate down in 35mm tissue culture dishes for 24 hours. After 24 hours routine medium was aspirated, the monolayers washed twice with sterile PBS and phenol red free medium containing 2% DHIDCCFCS and tamoxifen or ethanol carrier as appropriate was added. 24 hours before FRAP, serum containing medium was withdrawn and replaced with drug dilutions in serum-free medium. Cells were then stained with AF18 as described in Methods section 6.5 using a 15 minute incubation at room temperature or, with FITC labelled EGFR1 antibody as described in Section 3.3.2.

Results

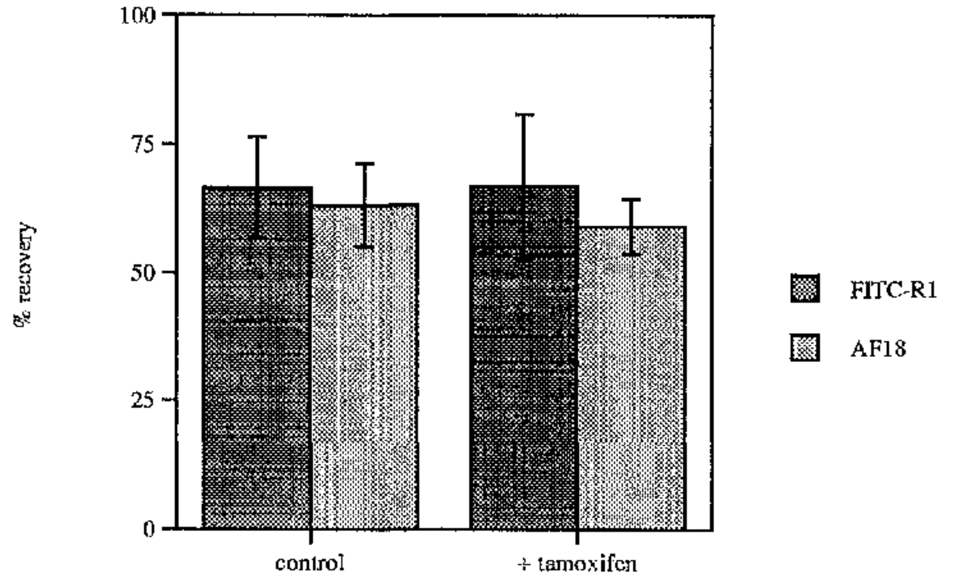
Figure 24 shows the effect of tamoxifen on AF18 and FITC-R1 fluidity in A431 cells. As in the breast cell lines used there has been a significant decrease in the lateral diffusion coefficient of AF18 after treatment with tamoxifen ($P<0.0001$). Lateral diffusion of FITC-R1 is similarly decreased after tamoxifen treatment ($P<0.0001$).

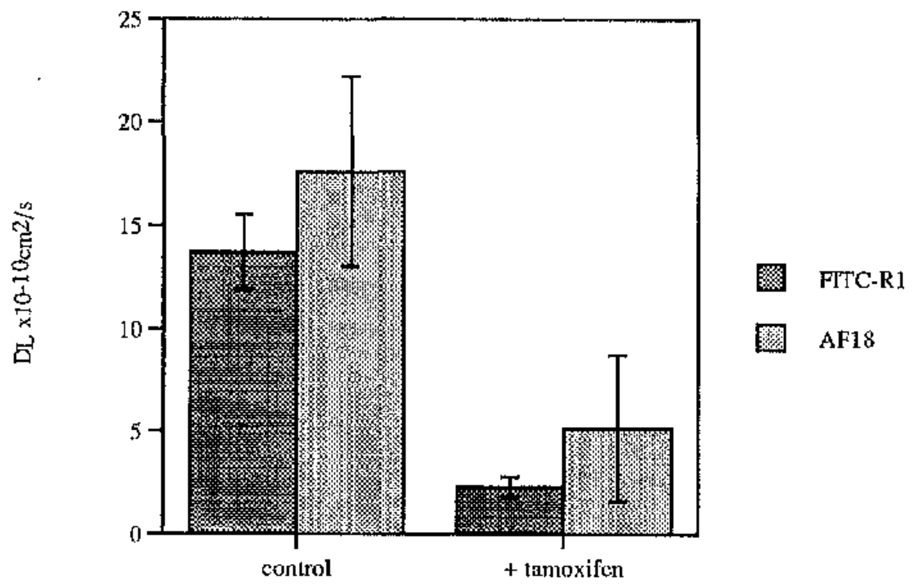
Although A431 has a number of atypical responses to EGF and may have altered receptors [Gill, *et al*, 1981; Weber, *et al*, 1984], the finding that tamoxifen

decreases EGFR mobility in this cell line strongly suggests that this effect would also be found in breast cells.

Figure 24. Effect of tamoxifen on the fluidity of AF18 and the FITC-R1 anti-EGFR antibody probe in the A431 cell line.

Cells were exposed to ethanol carrier or 10^{-6}M tamoxifen for 24 hours and then stained for 15 minutes with AF18 or 1 hour on ice with FITC-R1.
Each data point represents the mean of 24 measurements taken during 3 separate experiments. The error bars indicate the standard deviation.


% recovery of AF18 and FITC-R1 in A431 cells
100
75
50
25
0
% recovery
control
+ tamoxifen
FITC-R1
AF18


Lateral diffusion coefficients of AF18 and FITC-R1 in A431 cells
25
20
15
10
5
0
DL x10-10cm2/s
control
+ tamoxifen
FITC-R1
AF18

3. 11. Effect of membrane active drugs on EGF receptor internalisation

3. 11. 1. EGFR internalisation in normal cells

Ligand-induced internalisation of the EGFR is a specific quantitative indicator of EGFR function. Since the process of internalisation is dependent on the ability of the receptors to diffuse from their initial diffuse distribution to form clusters associated with coated pits, it has been proposed that changes in lateral diffusion may be reflected by changes in the rate of internalisation of the receptor [Jans, *et al*, 1990; Paccaud, *et al*, 1993]. Studies have suggested that the rate of lateral diffusion of EGF, insulin and glucagon receptors is strongly correlated with their rate of internalisation [De Diego, *et al*, 1991]. We looked to investigate the relationship between membrane fluidity and EGFR internalisation by examining the effect of membrane active agents on EGFR internalisation. The first experiment performed defined the normal rates of diffusion of EGFR in the cell lines used.

Method

Cells were grown to confluence in 100mm tissue culture dishes. Serum was withdrawn 24 hours before assay to deprive the cells of endogenous growth factors and enhance radiolabelled EGF binding. A single cell suspension was generated by gentle, non-enzymatic detachment of the cells in 2mM EDTA in PBS followed by centrifugation and resuspension in serum free routine medium at a density of 10^7 cells/ml. Internalisation of ^{125}I-EGF was then determined as for Methods section 7.

Results

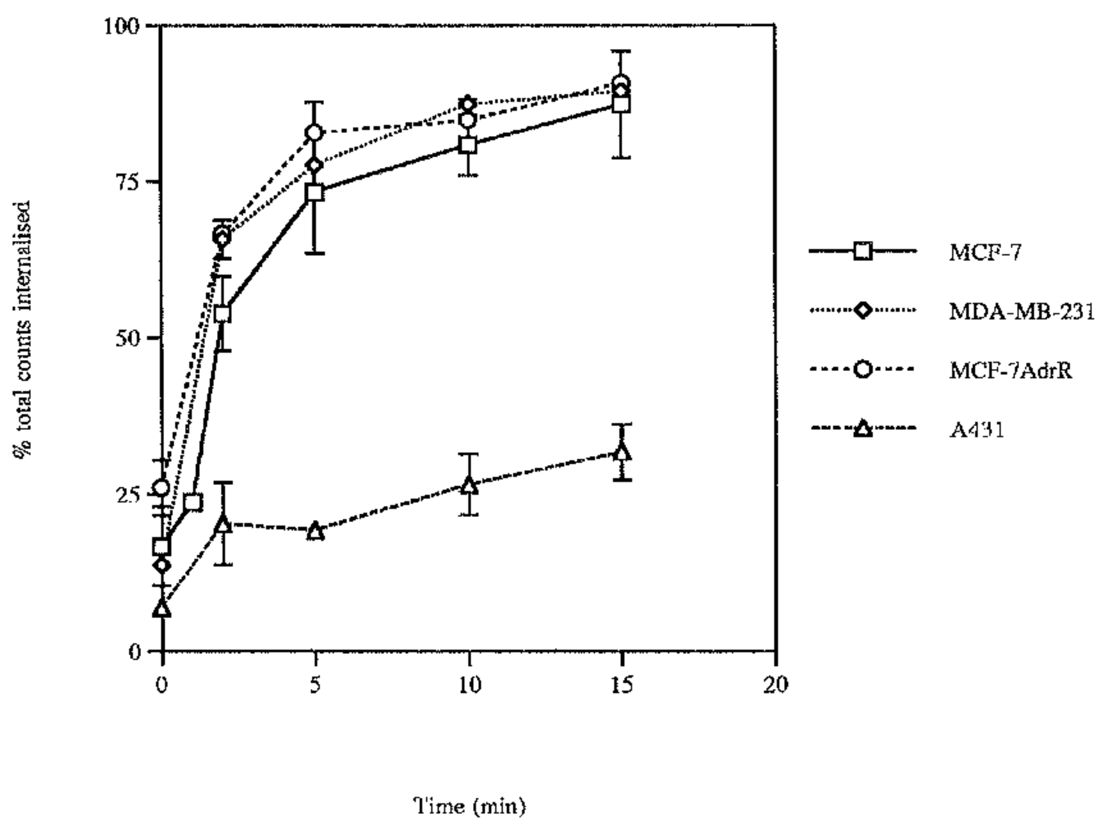
Figure 25 show the rates of internalisation of the three breast cancer cell lines and the A431 cell line under normal conditions of growth. The rate of internalisation in all three breast cell lines is very similar. Internalisation is very rapid with 80-90% of the total receptor internalised after 15 minutes. Thus in spite of the different levels of receptor available, endocytosis is very similar in these breast cell lines. A

previous study using FITC labelled EGF also found no difference in the kinetics of internalisation of EGFR in the MCF-7 and MDA-MB-231 cell lines. In contrast the internalisation of the A431 cell line is incomplete, which may reflect a saturation of the endocytic pathway in this cell due to its vast overexpression of the receptor [Wiley, 1988]. In fibroblasts artificial overexpression of EGFR at levels similar to A431 slows the rate of receptor internalisation and reduces the extent of total internalisation [Benveniste, *et al*, 1988].

Figure 25. Time course of internalisation of ^{125}I-EGF in tumour cell lines.

Data is expressed as a percentage of the total ^{125}I bound and each data point represents the mean of 3 separate experiments performed in duplicate. The error bars indicate the standard deviation. Non-specific binding determined in the presence of a 100 fold excess of unlabelled EGF was less than 5% of the total label bound.

Time course of internalisation of ^{125}I-EGF



3. 11. 2. Effect of drugs which alter membrane fluidity on internalisation

A number of compounds affect the fluidity of lipid probes in the cell lines used. If internalisation is affected by the rate of lateral diffusion of the receptor, changes in membrane fluidity which restrict this lateral diffusion may alter the rate of internalisation. MCF-7 cells were treated with estradiol, tamoxifen and ICI 182,780 at concentrations which reduced membrane fluidity, to examine the effect on internalisation.

Method

Cells were grown to confluence in 100mm tissue culture dishes and treated with the appropriate compound or control for 24 hours before assay. Serum was withdrawn 24 hours before assay to deprive the cells of endogenous growth factors and enhance radiolabelled EGF binding. A single cell suspension was generated by gentle, non-enzymatic, detachment of the cells in 2mM EDTA in PBS followed by centrifugation and resuspension in routine medium at a density of 10^7 cells/ml. Internalisation of ^{125}I-EGF was then determined as for Methods section 7.

Results

Figure 26 shows that none of the compounds which affect cell membrane fluidity significantly alters the rate of internalisation of EGFR. Thus it would appear from these results that membrane fluidity does not significantly affect internalisation of the EGFR.

Conclusion

From these results it would appear that membrane fluidity changes in breast cancer cells do not significantly alter rates of internalisation. This is in contrast to a number of studies which suggest that the rate of internalisation is affected by the rate of receptor lateral diffusion [Jans, *et al*, 1990; De Diego, *et al*, 1991; Paccaud,

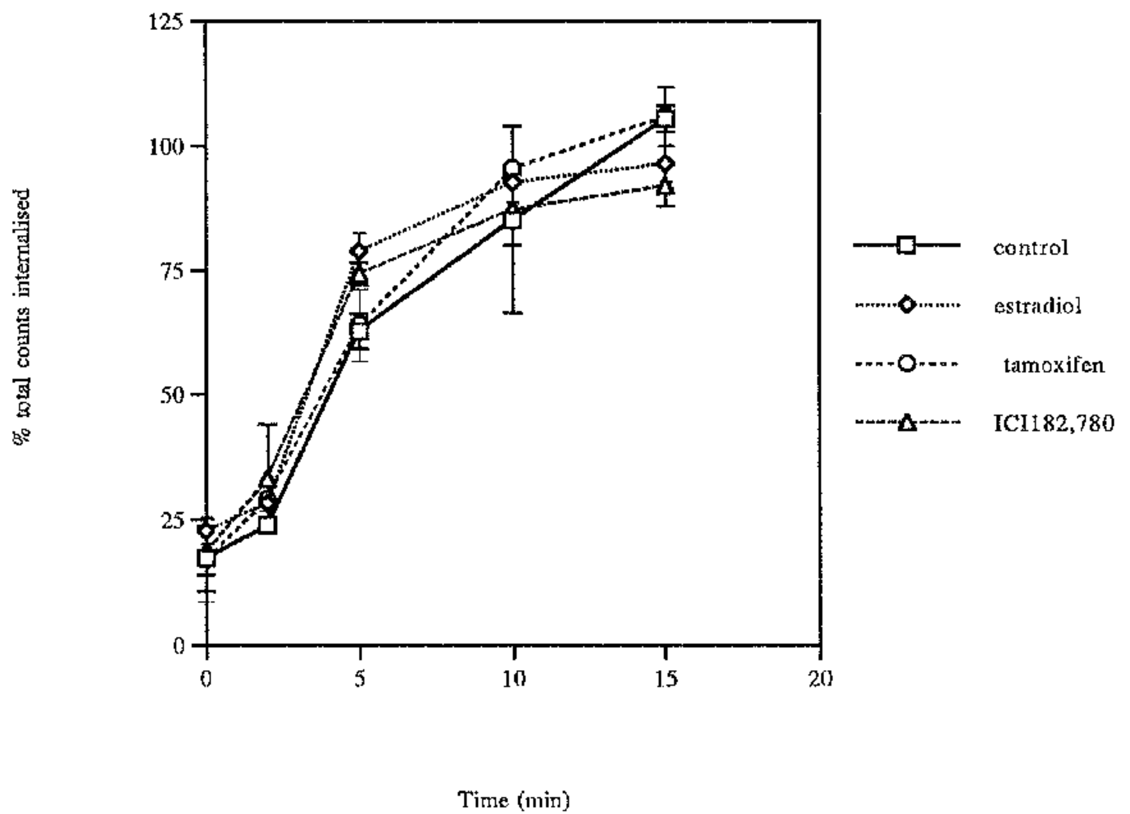
et al, 1993]. However earlier studies suggested that rotational and lateral diffusion of EGFR were not rate determining for receptor internalisation [Schlessinger, 1983]. In one study where reduced lateral diffusion did affect internalisation, the reduced diffusion was also related to alterations in receptor expression which could saturate the available sites for endocytosis [Benveniste, *et al*, 1988]. In our cell lines none of these treatments affect EGFR expression.

One alternative possibility is that the EGFR in these breast cells are present in microdomains which are distinct from those occupied by the lipid probes utilised in this study. However this seems unlikely for several reasons. Firstly, tamoxifen's ability to decrease fluidity has been demonstrated using several probes and is also observed using fluorescence polarisation which measure bulk fluidity in the cell [Clarke, *et al*, 1989]. Microscopic examination of cells labelled with both AF18 and rhodamine-labelled anti-EGFR antibodies shows no difference in the areas stained. Finally in the A431 cell line, reduction in fluidity of AF18, is reflected by a similar reduction in EGFR fluidity (Section 3.10.).

Figure 26. Time course of internalisation of ^{125}I-EGF in MCF-7 breast cancer cells treated with membrane active agents.

Cells were treated with 10^{-6}M estradiol, tamoxifen, ICI182,780 or ethanol carrier only for 24 hours before assaying.

Data is expressed as a percentage of the total ^{125}I bound and each data point represents the mean of 3 separate experiments performed in duplicate. The error bars indicate the standard deviation. Non-specific binding determined in the presence of a 100 fold excess of unlabelled EGF was less than 5% of the total label bound.



Effect of membrane active drugs on internalisation
of 125I-EGF by MCF-7 cells
125
100
75
50
25
0
% total counts internalised
0
5
10
15
20
Time (min)
control
estradiol
tamoxifen
ICI182,780

3. 12. Effect of tamoxifen on adriamycin sensitivity of MCF-7 and MCF-7 AdrR cell lines

Tamoxifen has been shown to reduce membrane fluidity in breast cell lines by FRAP (Section 3.5.) and fluorescence polarisation [Clarke, *et al*, 1989]. This effect may be of particular importance in drug resistance. Compounds which affect membrane fluidity have been demonstrated to alter the functioning of p-glycoprotein and increase drug accumulation in multidrug resistant cell lines [Sinicrope, *et al*, 1992; Callaghan, *et al*, 1993; Drori, *et al*, 1995]. This effect is found both with compounds which decrease membrane fluidity such as cholesterol and membrane fluidising unsaturated fatty acids such as linoleic acid [Callaghan, *et al*, 1993]. Tamoxifen has previously been reported to reverse doxorubicin resistance in leukaemia cells by a mechanism not reversed by estradiol [Ramu, *et al*, 1984b]. It has been suggested that this may be due to tamoxifen's ability to stabilise the cell membrane [Kayyali, *et al*, 1994]. Steroidal anti-estrogens which also alter membrane fluidity have also been shown to affect the functioning of p-glycoprotein [Kirk, *et al*, 1994; De Vincenzo, *et al*, 1996].

Growth assays were performed to determine the effect of tamoxifen on adriamycin sensitivity of the MCF-7 drug sensitive cell line and MCF-7AdrR resistant cell line. The MCF-7AdrR cell line was generated from the MCF-7 parent cell line by incubation in increasing concentrations of the cytotoxic drug adriamycin (doxorubicin). MCF-7AdrR cells have been reported to be 200 fold more resistant to adriamycin [Fairchild, *et al*, 1987]. The MTT assay was used to confirm the adriamycin sensitivity of these two cell lines before examining the effect of tamoxifen on drug sensitivity.

Method

80% confluent flasks of cells were trypsinised, counted and resuspended at a density of $5x10^4$ cells/ml as described in Methods Section 2.2.1. The cell suspension was plated down onto 96 well plates and allowed to settle for 24 hours. The routine medium was then removed and the monolayers were washed with sterile PBS. A range of dilutions of adriamycin, prepared in phenol red free medium containing 2% DHIDCCFCS were added to columns in the plates (8 wells per column). To examine the effect of tamoxifen drug dilutions were prepared in medium containing either 10^{-6}M tamoxifen or ethanol carrier

After 72 hours at 37°C, the viable cell number was measured by the MTT assay as described in Methods section 4.

Results

Figure 27 shows the results of the adriamycin dose-response experiments. As can be seen the parent MCF-7 cell line is sensitive to adriamycin with approximately 50% inhibition at 0.5μg/ml. The MCF-7AdrR cell line is however relatively resistant to growth inhibition by adriamycin at the concentrations used. This result is to be expected since this line was selected by its ability to grow in increasing concentrations of adriamycin. Thus unlike its parent cell line it is resistant to adriamycin, confirming the observation of Fairchild, *et al*, 1987.

Figures 28 and 29 shows the effect of 10^{-6}M tamoxifen on the dose-response of the two cell lines to adriamycin. It can be seen that tamoxifen did not significantly alter the adriamycin sensitivity of either the MCF-7 or the MCF-7AdrR cell line.

It is unclear why our results are not consistent with other studies on the reversal of drug resistance by tamoxifen [Berman, *et al*, 1991; Leonessa, *et al*, 1994; De Vincenzo, *et al*, 1996]. This has been fairly extensively studied and indeed has

entered clinical trials in some cases [Millward, *et al*, 1992]. It remains possible that the MCF-7AdrR cell line used in our study has undergone mutation which is a common problem with the use of cell lines. Alternatively the results may simply highlight the differences which exist between cell lines and the difficulties involved in applying findings from cell culture experiments to an *in vivo* situation. The MCF-7AdrR cell line has been suggested to exhibit further mechanisms of resistance in addition to that mediated by p-glycoprotein and also exhibit antiestrogen resistance [Vickers, *et al*, 1988; Zyad, *et al*, 1994]. This may account for the inability of tamoxifen to reverse doxorubicin resistance in these cells.

Figure 27. Dose response curves for adriamycin in the MCF-7 cell line and its adriamycin resistant subclone, MCF-7AdrR.

The two cell lines were exposed to a range of concentrations of adriamycin (from 0.01μg/ml to 50 μg/ml) for 72 hours. Cell survival was assessed by the MTT assay. Data is expressed as a percentage of the untreated control and each data point represents the mean of 3 separate experiments. The error bars indicate the standard deviation.

Effect of adriamycin on MCF-7 and MCF-7AdrR cells

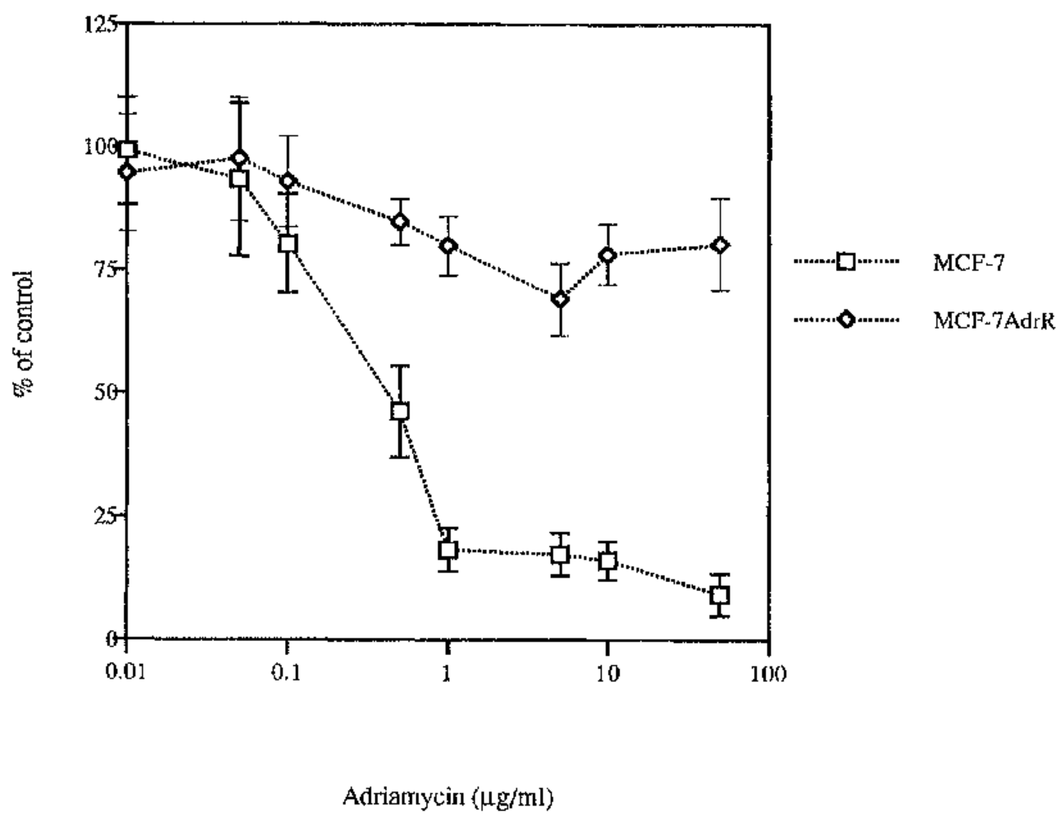


Figure 28. Effect of 10^{-6}M tamoxifen on the adriamycin sensitivity of the MCF-7 cell line.

Cells were exposed to a range of concentrations of adriamycin (from 0.05μg/ml to 10 μg/ml) for 72 hours in the presence of either 10^{-6}M tamoxifen or ethanol carrier only. Cell survival was assessed by the MTT assay.

Data is expressed as a percentage of the untreated control and each data point represents the mean of 3 separate experiments. The error bars indicate the standard deviation.

Effect of tamoxifen on the sensitivity of MCF-7 cells to adriamycin

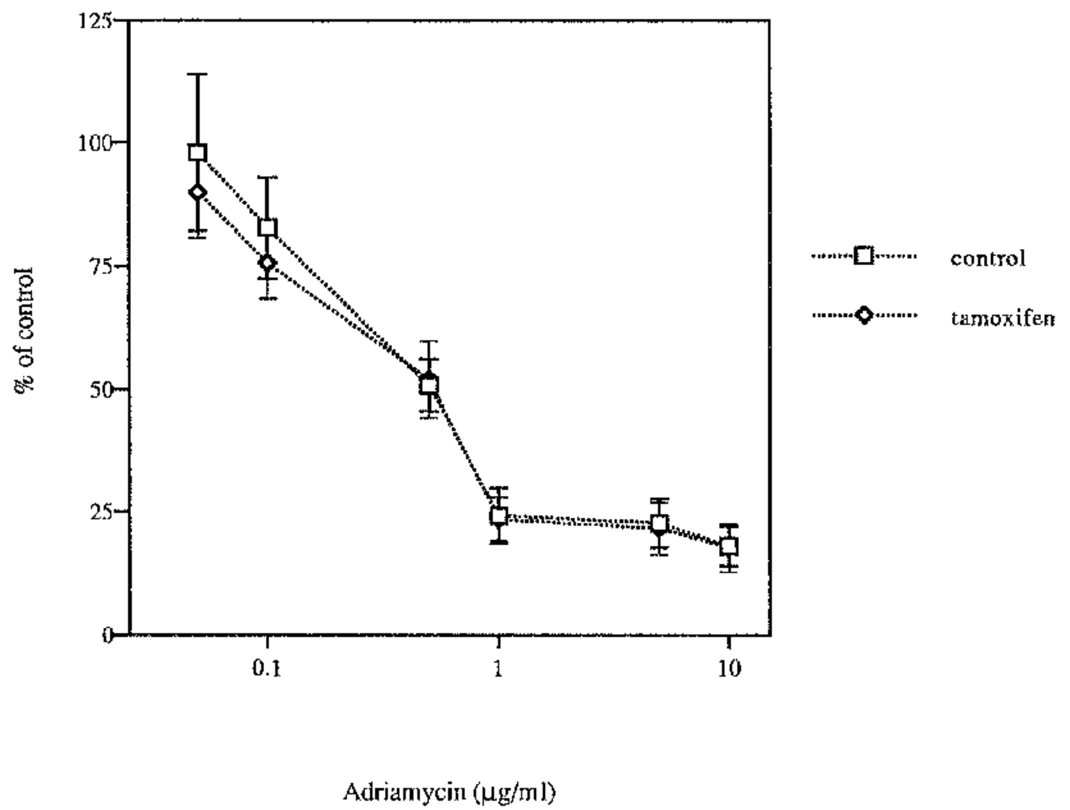
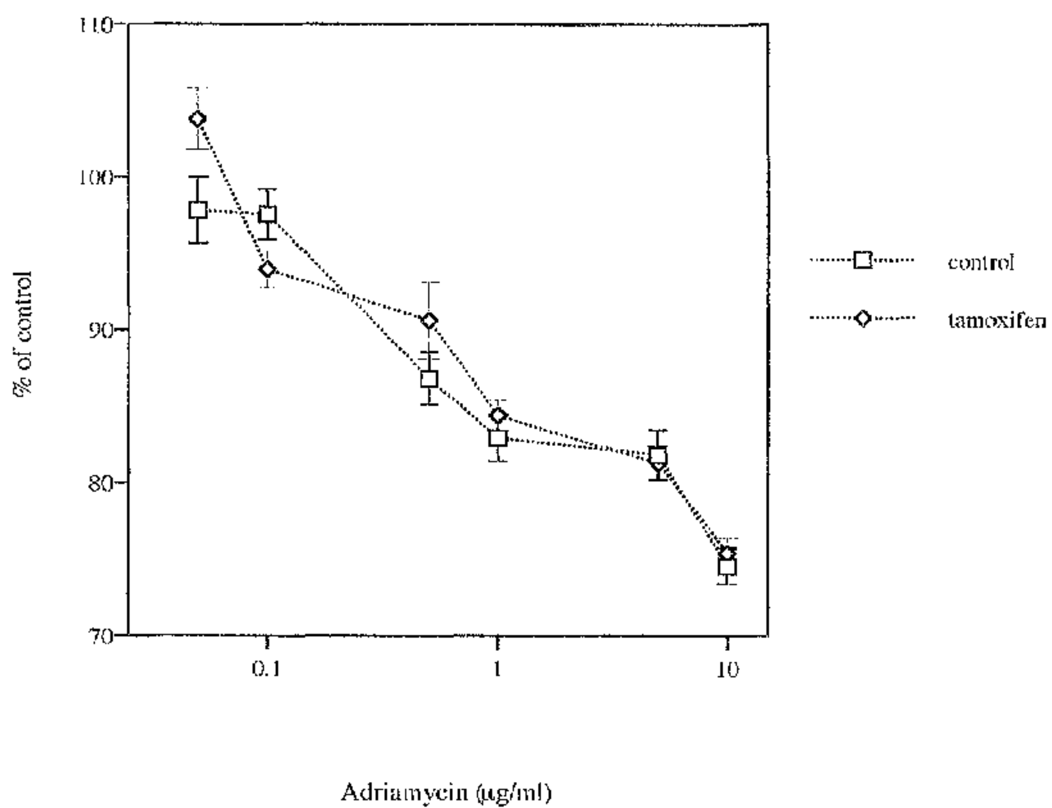


Figure 29. Effect of 10^{-6}M tamoxifen on the adriamycin sensitivity of the MCF-7 AdrR cell line.

Cells were exposed to a range of concentrations of adriamycin (from 0.05μg/ml to 10 μg/ml) for 72 hours in the presence of either 10^{-6}M tamoxifen or ethanol carrier only. Cell survival was assessed by the MTT assay.

Data is expressed as a percentage of the untreated control and each data point represents the mean of 3 separate experiments. The error bars indicate the standard deviation.

Effect of tamoxifen on the sensitivity of MCF-7AdrR cells to adriamycin



Chapter 4

Discussion

The epidermal growth factor receptor (EGFR) has been detected in many human tumours including breast cancer [Gullick, 1991]. In breast cancer, expression of the EGFR is associated with a poor prognosis subgroup of patients [Sainsbury, *et al*, 1987; Klijn, *et al*, 1994]. Overexpression of the receptor in the presence of an activating ligand can induce the transformation of cultured fibroblasts [Di Fiore, *et al*, 1987a]. EGFR-mediated signalling may therefore play an important role in the growth and development of human breast cancer. The EGFR has consequently been identified as a potential target for anti-tumour therapies [Davies, *et al*, 1996].

The EGFR is a membrane-associated protein tyrosine kinase. The cell membrane is the site of action of many proteins involved in cell signalling and tumour growth and development. The activity of membrane-associated receptors and enzymes may be modulated by the physical state of the membrane [Epand, 1991]. Alterations in membrane fluidity can also affect the expression and exposure of membrane associated proteins [Shinitsky, 1984]. The activation of the EGFR tyrosine kinase depends on the lateral diffusion and dimerisation of receptor monomers and alterations in membrane fluidity may affect this process and hence signalling via the EGFR.

It was important to perform initial experiments to define the growth response to EGF of the different cell lines used in this study. MCF-7 cells were growth stimulated by EGF with a maximal stimulation at 100ng/ml. MDA-MB-231 and MCF-7AdrR breast cell lines were not stimulated by EGF and growth of these cell lines was inhibited by high concentrations of EGF (Figure 7). These results are consistent with previous studies in these cell lines which show that only cell lines with a low level of EGF receptor, such as the MCF-7 cell line, are growth stimulated by EGF [Osborne, *et al*, 1980; Davidson, *et al*, 1987; Godden, *et al*, 1992].

In this thesis, membrane fluidity in breast cancer cell lines was measured using the technique of fluorescence recovery after photobleaching (FRAP). This technique measures fluidity on small areas on the cell surface, unlike the commonly used technique of fluorescence polarisation which measures an average fluidity over all membranes in the cell and all membrane domains [Van Blitterswijk, *et al*, 1981]. Two fluidity parameters are measured in FRAP: the percentage of molecules that are free to diffuse within the timescale of the experiment, and the rate of diffusion (D_L) [Axelrod, *et al*, 1976]. Three different lipid probes were used in this study since it has been demonstrated that different lipid analogues probe different membrane environments dependent on their chemical structure [Derzko, 1980].

The values obtained for lateral diffusion coefficients were within previously described ranges for lipid diffusion [Tocanne, *et al*, 1989]. However, in all cases the % recovery was low - typically less than 50%. Similar low percentage recoveries have been previously been reported for lipid diffusion in a variety of systems [Tocanne, *et al*, 1989; McKay, *et al*, 1991]. High immobile fractions may imply that these probes are located within small membrane domains. A probe confined to a domain which is small in size relative to the bleaching area will typically have a high immobile fraction since no reservoir for recovery of fluorescence exists [Zhang, 1993]. Significant differences in both D_L and mobile fraction were observed in the breast cell lines investigated which suggests that the different probes are located in different microdomains [Tocanne, *et al*, 1989].

Changes in membrane fluidity have been reported in almost all types of tumour but the implications of these alterations for tumour growth are poorly understood [Van Blitterswijk, 1984]. Previous studies of membrane fluidity in tumour cells have suggested that increased growth and metastatic potential in tumour cells is associated with increased fluidity [Deliconstantinos, 1987; Taraboletti, *et al*, 1989]. Thus the MDA-MB-231 cell line which is highly tumorigenic might be expected to

have more fluid membranes than the MCF-7 cell line [Clarke, *et al*, 1996]. However, results in this thesis suggest that MDA-MB-231 cell membranes are either less fluid than MCF-7 cells or no significant difference exists (Figures 11, 12 and 13). This may simply reflect the differences in the techniques used to assess membrane fluidity. On the other hand, a study of rat mammary carcinoma found no correlation between metastatic potential and membrane fluidity [Chatterjee, *et al*, 1976] and these results would caution against any general rule relating increased membrane fluidity with increased invasiveness. Indeed a significant decrease in lipid fluidity was observed in the drug resistant MCF-7AdrR cell line relative to its drug sensitive parent cell line MCF-7 (Figures 12 & 13). Lipid fluidity is influenced by both the lipid and protein content of the surrounding membrane - membrane fluidity may be decreased by an increase in protein content [Shinitsky, 1984]. In the MCF-7AdrR cell line both EGFR and p-glycoprotein are overexpressed [Vickers, *et al*, 1988]. Although these proteins represent only a small fraction of the total protein content of the membrane, such increases may have a significant impact on fluidity within localised membrane domains. Previous studies in drug resistant cell lines have also found a decrease in membrane fluidity [Ramu, *et al*, 1984; Callaghan, *et al*, 1992].

Originally, the aim of this study was to measure EGFR fluidity directly using the FRAP technique. This had been successfully performed in fibroblasts and in the A431 cell line which express highly elevated numbers of the receptor [Schlessinger, *et al*, 1978; Hillman, *et al*, 1982; Rees, *et al*, 1984]. Using FITC-labelled EGF and anti-EGFR antibodies we obtained values for EGFR lateral diffusion which agreed with these previous studies in the A431 cell line. However, measurement in the other cell lines proved impossible due to the low level of fluorescent signal achieved.

Effect of tamoxifen on membrane fluidity

The anticancer drug tamoxifen was developed as an antiestrogen and is proposed to act by competitively inhibiting the action of estrogens [Wakeling, *et al*, 1984]. However, numerous lines of evidence suggest that tamoxifen has actions unrelated to its ability to bind to the estrogen receptor. Previous studies have reported that in the complete absence of estrogens, tamoxifen, particularly at high concentrations, can inhibit the growth of breast cancer cells including those which are devoid of estrogen receptors [Reddel, *et al*, 1985; Langdon, *et al*, 1990]. Results in this thesis confirm this effect (Figure 18). A number of alternative actions have been described for tamoxifen which may explain this effect. In addition to the ER, tamoxifen also binds to a group of microsomal proteins called antiestrogen binding sites (AEBS). AEBS, which have been found in all cell types, have been proposed to mediate some of the alternate actions of tamoxifen [Wolf, *et al*, 1995]. Tamoxifen also inhibits the activity of the enzymes protein kinase C and the Ca^{2+}-calmodulin dependent cAMP phosphodiesterase [Lam, 1984; O'Brian, *et al*, 1986]. Tamoxifen induces secretion of the inhibitory factor TGFβ which is active against both ER positive and negative breast cancer cells [Knabbe, *et al*, 1987; Arteaga, *et al*, 1988].

A number of studies have previously reported that tamoxifen can have effects on the cell membrane. Tamoxifen is a highly lipophilic compound and has been shown to be incorporated into cell membranes and induce structural alterations in the bilayer [Custodio, *et al*, 1991, 1993a & 1993b]. Tamoxifen and related compounds are capable of adopting a conformation closely resembling the known modulator of membrane fluidity, cholesterol [Wiseman, *et al*, 1992]. In the membrane cholesterol acts to increase the rigidity and degree of order in fluid lipid domains via interactions between its hydrophobic rings and the fatty acid residues of membrane phospholipids [Shinitsky, 1984]. Thus addition of tamoxifen may be similar to increasing the cholesterol content of a membrane [Wiseman, *et al*, 1992].

Studies using the technique of fluorescence polarisation have confirmed that tamoxifen decreases membrane fluidity in both liposomes and breast cancer cells [Wiseman, *et al*, 1993a; Clarke, *et al*, 1989]. However, these results do not imply that tamoxifen would necessarily produce a similar effect when membrane fluidity is assessed by FRAP since, unlike FRAP, fluorescence polarisation measurements represent an average bulk fluidity of all membranes in the cell and do not distinguish between lipid domains [Van Blitterswijk, *et al*, 1981]. Studies have shown that results obtained by these two methods are not necessarily consistent [Kleinfeld, *et al*, 1981].

Using the FRAP technique, tamoxifen was shown to reduce lipid fluidity in MCF-7 breast cell membranes (Figures 14 and 15). The rate of lateral diffusion of AF18 was significantly decreased but the percentage of probe molecules free to diffuse was not significantly altered (Figure 14). A similar effect was observed with the other lipid probes used which suggests tamoxifen may be effective in reducing fluidity over several domains in the membrane (Figure 15). This effect is also apparently independent of steroid receptor status since it was observed in the steroid receptor negative cell lines MDA-MB-231 and MCF-7AdrR (Figure 16). This finding of a general effect on membrane fluidity in breast cancer cells by FRAP correlates well with previous fluorescence polarisation results [Clarke, *et al*, 1989].

The effect of the cholesterol, 17β-estradiol and ICI 182,780 on AF18 fluidity was examined in the MCF-7 cell line. These compounds also significantly reduced the lateral diffusion of the AF18 probe with no significant difference between the effects of cholesterol, 17β-estradiol or ICI 182,780 and tamoxifen (Figure 17). These results contrast with previous studies in liposomes and breast cancer cells where significant differences were observed between the effects of tamoxifen, 17β estradiol and cholesterol. 17β-estradiol was shown to be more effective than tamoxifen in decreasing membrane fluidity in liposomes while cholesterol was less

effective than tamoxifen [Wiseman, *et al*, 1993a]. 17β estradiol was also demonstrated to have a greater effect on membrane fluidity than tamoxifen in ER negative breast cancer cells. However in ER positive cells, no significant difference was found between the effects of micromolar concentrations of tamoxifen or 17β estradiol on membrane fluidity [Clark, *et al*, 1989].

Effect of tamoxifen on EGFR function

It is possible that tamoxifen's effect on membrane fluidity could directly influence the functioning of the membrane-associated EGFR. A decrease in membrane fluidity may alter the physical state of the membrane surrounding the receptor and directly affect its activity, or, the increase in viscous drag within the lipid matrix could impede the receptor's ability to dimerise. Since activation of the EGFR is dependant on its dimerisation we sought to relate tamoxifen's membrane effects to its effect on EGFR function.

Of the three breast cell lines studies, only MCF-7 cells exhibited a growth response to EGF. Using 100ng/ml EGF, the effect of tamoxifen on EGF-stimulated cell growth was examined. Addition of tamoxifen at concentrations ranging from 10^{-8} to 10^{-6}M reduced stimulation of growth by EGF with a maximal effect seen using 10^{-6}M tamoxifen (Figure 20). This growth inhibition could be due to stimulation of production of TGFβ by tamoxifen [Knabbe, *et al*, 1987]. However, addition of neutralising anti-TGFβ antibodies only partly reversed the effect of tamoxifen which suggests that tamoxifen is affecting EGFR function by a mechanism other than stimulation of TGFβ production.

The ability of tamoxifen, and its metabolite 4-hydroxytamoxifen, to antagonise growth factor stimulation of cells has previously been described by a number of authors [Vignon, *et al*, 1987; Freiss, *et al*, 1990a; Wosikowski, *et al*, 1993] although some studies found no effect on EGF-stimulated cell growth [Cormier, *et*

al, 1989]. The steroidal antiestrogens ICI 164,384 and ICI 182,780 have also been shown to inhibit EGF and IGF-1 stimulated growth [Wakeling, 1989; Wosikowski, *et al*, 1993]. Antiestrogens have also been shown to interfere with growth factor induction of some, but not all responsive genes [Chalbos, *et al*, 1993; Wosikowski, *et al*, 1993]. These differences in observations may be due to differences in cell culture conditions. All studies were performed in the absence of the weak estrogen phenol red but differences exist in the use of treated fetal calf serum. Although charcoal-stripped FCS has had all detectable steroids removed, it may remain a source of undefined growth promoting substances. Under serum-free conditions tamoxifen, droloxifene (3-hydroxytamoxifen) and ICI164,384 all inhibit EGF and IGF-1 stimulated proliferation of MCF-7 cells [Wosikowski, *et al*, 1993].

It has been suggested that tamoxifen's effect on EGF stimulated growth could be due to a alterations in receptor expression [Vignon, *et al*, 1987]. A decrease in the EGFR receptor expression or its affinity for EGF could explain the observed reduction in growth response to EGF. Studies have demonstrated that tamoxifen treatment of MCF-7 cells produces a dose-dependent decrease in expression of the EGFR after 5 days in culture [Berthois, *et al*, 1989]. Conflicting studies suggest that inhibition of EGF-stimulated growth is actually associated with an increase in high affinity EGF binding sites [Freiss, *et al*, 1990b]. Membrane fluidity changes have previously been shown to alter the affinity of the insulin receptor for its ligand [Neufeld, *et al*, 1986]. Figure 21 shows the result of a whole cell EGFR binding assay. In this study, no effect was seen on EGF binding after tamoxifen treatment. The effect of tamoxifen on response to EGF is therefore unlikely to be due to either a decrease in receptor expression or high affinity ligand binding.

EGFR tyrosine kinase activity which is essential for receptor functioning, is activated in a process involving dimerisation of receptors [Schlessinger, 1988]. Any effect which restricted the ability of the receptor to diffuse laterally may

reduce this process. Using an EGFR tyrosine kinase assay, the effect of tamoxifen was examined in isolated cell membranes. Preincubation of cells with tamoxifen had no significant effect on the receptor's tyrosine kinase activity (Figure 22). However, this assay was performed on isolated receptors and under these conditions interactions may occur between receptors without the constraining effect of membrane fluidity. To exclude this possibility, a whole cell assay was performed and the effect on EGFR autophosphorylation was examined (Figure 23). Autophosphorylation is necessary for the maximal biological activity of the receptor and would be expected to be decreased if receptor dimerisation was inhibited [Honegger, *et al*, 1987; Helin, *et al*, 1991]. Incubation of MCF-7 cells with tamoxifen at concentration known to inhibit membrane fluidity was shown to decrease receptor autophosphorylation. Tamoxifen has previously been shown to decrease EGFR autophosphorylation in MCF-7 cells treated for 5 days with tamoxifen [Freiss, *et al*, 1990b]. The effect was also observed in ER negative cells indicating that is independent of the ER mediated actions of tamoxifen.

Unfortunately, using the existing FRAP equipment, EGFR mobility could not be directly measured in cells with low receptor numbers. Since EGFR could be labelled in the A431 cell line, this cell line was used to investigate the effect of tamoxifen on both lipid and EGFR fluidity. Using the AF18 probe, tamoxifen was demonstrated to decrease lipid fluidity in this cell line. A decrease in fluidity was also observed using anti-EGFR antibodies after tamoxifen treatment (Figure 24). This suggests that EGFR mobility is directly affected by tamoxifen and that this may also occur in breast cell lines.

The effect of tamoxifen on cellular responses to EGF stimulation may therefore be due to its effect on the cell membrane. Tamoxifen, adopting a molecular conformation similar to cholesterol, would insert into the cell membrane, increase the degree of lipid order and have a rigidifying effect on membrane fluidity.

Membrane proteins such as EGFR would then exhibit reduced lateral diffusion due to the greater viscosity and resistance of the membrane. Thus, on ligand binding, receptor dimerisation would be restricted, resulting in a decrease in receptor autophosphorylation. Since phosphorylation of the receptor is essential for recruitment of downstream proteins containing SH2 domains, signalling via the receptor would be reduced resulting in a reduction in the growth response to ligand binding.

Studies have previously demonstrated that the activity of the insulin receptor is sensitive to decreases in membrane fluidity. In adipocytes, insulin signalling is sensitive to stabilisation of membrane fluidity by membrane active peptides. These peptides stabilise the membrane in a non-specific manner and this decrease in fluidity inhibits insulin receptor autophosphorylation in intact cells [Epand, *et al*, 1991]. In vivo decreases in insulin receptor autophosphorylation and activation in diabetic rats, is associated with increased membrane viscosity [Nadiv, *et al*, 1994].

Alterations in membrane fluidity may also influence the functioning of other membrane receptors involved in the regulation of breast cancer proliferation. The EGFR forms heterodimers with related receptor molecules such as c-erbB-2 [Rajkumar, *et al*, 1994]. Such heterodimers have an increased tyrosine kinase activity and may be more effective in tumour promotion than EGFR homodimers [Wright, *et al*, 1992a]. Heterodimerisation may be similarly affected by alterations in membrane fluidity. The insulin like growth factors, IGF-I and IGF-II are potent mitogens for breast tissue and IGF-1 receptor are frequently expressed by primary breast tumours[Stewart, *et al*, 1992; Klijn, *et al*, 1993]. Tamoxifen and related compounds have been shown to antagonise IGF-1 stimulation of growth of breast cancer and other tumour types [Wakeling 1989; Newton, *et al*, 1994; De Cupis, *et al*, 1995]. This effect may be due to a decrease in cell surface binding of the IGF-1 receptor [Freiss, *et al*, 1990b; De Cupis, *et al*, 1995].

Membrane associated enzymes may also be affected by an alteration in the physical properties of the membrane. The activity of the enzyme adenylate cyclase is highly dependent on membrane fluidity [Houslay, 1985]. A decrease in fluidity by tamoxifen could decrease the activity of the enzyme which would result in reduced cellular levels of cAMP. Low levels of cAMP are inhibitory to the growth of some tumour cells [Dumont, *et al*, 1989]. However the physiological relevance of this effect is unclear since tamoxifen has also been demonstrated to directly inhibit the cAMP phosphodiesterase which under certain circumstances would increase the levels of cAMP [Lam, 1984].

The degree of fluidity of a membrane can also affect the expression and exposure of receptors, adhesive molecules and antigens [Shinitsky, 1984]. Enrichment of tumour cells with cholesterol, which would decrease membrane fluidity, has been shown to be associated with an increase in exposure of membrane-associated proteins [Skornick, *et al*, 1984]. This could have profound implications both for the process of metastasis and for recognition of tumour cells by the immune system.

Metastasis of tumours requires interactions between the disseminating tumour cells and the basement membrane and tissues. These interactions are mediated by adhesive molecules and alteration in membrane fluidity could alter the exposure and binding capacity of these molecules. An early step in the metastatic process - interaction between the tumour cells and the endothelium has been shown to be influenced by the membrane fluidity of the endothelial cells. Reduction in the cholesterol content of endothelial cells, which would increase membrane fluidity, results in enhanced adhesion of tumour cells. This effect can be mimicked with a membrane fluidising agent, the anaesthetic procaine [Ramachandran, *et al*, 1986]. This may explain why increased fluidity is correlated with increased tumorigenicity and invasive potential [Taraboletti, *et al*, 1989]. By decreasing fluidity tamoxifen could may reduce adhesion and metastasis.

Alterations in the surface expression of membrane proteins caused by alteration in membrane fluidity may also have implications for the effectiveness of the immune system in dealing with tumours [Shinitsky, 1984]. Tumours may escape immune surveillance because of masking of cell surface antigens but cryptic antigens may be exposed by alterations in membrane fluidity. In this study, tamoxifen treatment produced no alteration in the cell surface binding of EGF. Other studies have shown that tamoxifen alters the cell surface exposure of receptors such as the IGF-1 receptor [Freiss, *et al*, 1990b]. Since one area investigated in tumour therapy is the use of immunotherapy targeted against tumour specific antigens, tamoxifen treatment could enhance the efficacy of such therapy [Davies, *et al*, 1996].

If tamoxifen does indeed have a general effect on tumour cell growth due to its membrane action, breast cancer patients not previously targeted for antiestrogen therapy and other tumour types may benefit tamoxifen treatment. The estrogenic actions of tamoxifen remain a problem since these are the cause of many of the serious side effects associated with tamoxifen such as increased risk of endometrial cancer and retinopathy [Wolf, *et al*, 1995]. However, steroidal antiestrogens which lack the estrogenic effects of tamoxifen and estradiol, also have a membrane stabilising effect which may contribute to their anticancer effects.

EGFR internalisation

A number of studies have suggested that the rate of internalisation of EGFR and other receptors is related to the ability of the receptors to diffuse laterally within the lipid bilayer [De Diego, *et al*, 1991; Paccaud, *et al*, 1993]. This would imply that decreases in membrane fluidity by tamoxifen treatment could alter rates of internalisation. In this study no differences were found in the rates of internalisation in breast cell lines under normal growth conditions (Figure 25). This finding is similar to previous studies where breast cancer cells were found to have the same kinetics of internalisation [Mueller, *et al*, 1994]. In the A431 cell line

however, internalisation was incomplete, possibly due to saturation of the coated pits needed for endocytosis [Wiley, 1988]. In this study no difference was found in internalisation rates after treatment with tamoxifen, estradiol or ICI182,780 at concentrations which would reduce membrane fluidity (Figure 26). This may reflect a difference in location between membrane domains altered by tamoxifen and the location of the EGFR. However such an explanation seems unlikely given that in the A431 cell line, tamoxifen treatment can be shown to slow the lateral diffusion of the EGFR (Figure 24). Schlessinger (1983) suggested that lateral and rotational diffusion of receptors was not rate-determining for internalisation of the EGFR.

Tamoxifen and drug resistance

In this study tamoxifen had no effect on the adriamycin sensitivity of the MCF-7 cell line or the multi-drug resistant cell line MCF-7AdrR (Figures 28 & 29). This result is in contrast to previous findings which have shown that tamoxifen, and related compounds, are capable of decreasing drug efflux from liposomes and reversing the multidrug resistant phenotype in cancer cells [Kayyali, *et al*, 1994; Di Vincenzo, *et al*, 1996]. Multidrug resistance has been shown to be reversed by both increases and decreases in membrane fluidity [Callaghan, *et al*, 1993; Drori, *et al*, 1995]. This has been suggested to be due to increased membrane resistance to conformational change which would alter the activity of p-glycoprotein [Wadkins, *et al*, 1993]. It has been suggested that the use of membrane fluidising agents to overcome resistance may be an advantage since cholesterol treatment induced a small sub population of cells to develop a more resistant phenotype [Leibovici, *et al*, 1996]. Alternate mechanisms of drug resistance may also exist since not all cells which exhibit the multidrug resistant phenotype express p-glycoprotein. The MCF-7AdrR cell line has been suggested to exhibit alternate mechanisms of resistance which may account for the inability of tamoxifen to reverse doxorubicin resistance in this cell line [Zyad, *et al*, 1994].

Future Work

The work described in this thesis has shown that the anticancer drug tamoxifen decreases membrane fluidity in breast cancer cells and also decreases the activity of the EGFR possibly due to a reduction in the lateral diffusion of the receptor. A number of further areas of study are suggested by these results.

Existing FRAP equipment restricts the use of the technique to measuring EGFR mobility in cell lines with elevated levels of receptor expression. Direct measurement of EGFR mobility in breast cancer cells with low numbers of receptor may prove possible in the future either by improvement in the sensitivity of the FRAP apparatus or by newer techniques such as single particle tracking or modification of confocal microscopy techniques [Storrie, *et al*, 1996; Bayley, *et al*, 1995].

Tamoxifen decreased membrane fluidity after 24 hours. Longer incubations (48, 72 and 96 hours) produced identical results but it may be useful to examine changes in membrane fluidity over a shorter time course. It would also be worth investigating the effects of the long term exposure to tamoxifen (greater than six months) used in the treatment of breast cancer patients. Another study would involve determination of the length of time required for tamoxifen-treated cells to revert to "pre-tamoxifen" levels of membrane fluidity after withdrawal of tamoxifen from the medium.

Tamoxifen treatment may have implications for immune recognition of tumour cells since alterations in membrane fluidity may affect the surface expression of tumour cell antigens. Preliminary experiments were performed in this laboratory to investigate changes in cell surface carbohydrate expression by tamoxifen by looking for changes in the binding of fluorescent lectins such as concanavalin A. Although very little change was observed in lectin binding after tamoxifen

treatment, further studies with a wider range of lectins may provide greater insight into this area.

In this study, tamoxifen was found to have no effect on the sensitivity of the drug resistant MCF-7AdrR cells to adriamycin. This contrasts with previous studies which have demonstrated that tamoxifen can reverse the action of p-glycoprotein. Tamoxifen's effect on p-glycoprotein function could be studied using fluorescent dyes such as rhodamine 123 which act as substrates for p-glycoprotein.

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