ANTISENSE OLIGONUCLEOTIDES AS THERAPEUTICS FOR THE TREATMENT OF AGGRESSIVE BREAST CANCER

By

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ABSTRACT

There are many drugs currently available for the treatment of aggressive breast cancer. These include anthracyclines, taxanes, alkylating agents, anti-metabolites, plant alkaloids, nucleoside analogues and anti-hormonal agents. Unfortunately, even armed with this impressive arsenal, there has been little ground gained in terms of disease free years or reduced mortality for what is an essentially incurable disease in the metastatic state.

Clearly, we need to improve upon the therapies available for these patients. A key step toward this goal is the development of reproducible and relevant models in which newly developed drugs may be tested. This thesis outlines the characterisation of what is anticipated to be a powerful human xenograft model of aggressive breast cancer, that of the MDA435/LCC6 cell line. This cell line may be grown easily *in vitro*, as well as an ascitic or as a solid tumour in mice.

In order to have a major impact in the field of breast cancer treatment, it will not suffice to develop yet another cytotoxic agent. Instead, we must turn to the newer technologies, including gene targeted therapies, which target the molecular root of the disease. This work includes the use of both free antisense oligonucleotides (ODN), as well as those formulated within a lipid carrier. These encapsulated ODN are retained in the circulation for a longer period of time, are less susceptible to the actions of nucleases, and due to pharmacokinetic and pharmacodistribution properties of the liposomal carrier, result in enhanced tumour cell uptake of the ODN.

Finally, ODN, both free and liposome encapsulated, were administered to tumour bearing female SCID/Rag2m mice, either singly or in combination with a commonly used anti-cancer agent (doxorubicin). It is shown that ODN are capable of mediating the specific down-regulation of the target protein as well as impacting the rate of tumour growth.

It is the intention of the author to demonstrate the necessity for not only good models and newly developed and specifically targeted therapeutic agents, but that we must also consider the use of combination strategies in the treatment of aggressive breast cancer such that current mortality statistics may be improved.

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ABBREVIATIONS

aa Amino acids
AR Amphiregulin
β-CEL Beta-cellulin
CHE Cholesteryl hexadecyl ether
Chol Cholesterol
DAB Diaminobenzidene
DCIS Ductal carcinoma in situ
dH_2O De-ionised water
DMEM Dulbecco's modified eagle medium
DMSO Dimethylsulphoxide
DNA Deoxyribonucleic acid
DODAC Dioleyl dimethylammonium chloride
DODAP Dioleoyl dimethylammonium propane
DSPC Distearoyl phosphatidylcholine
DSPE Distearoyl phosphatidylethanolamine
EDTA Ethylenediamine tetraacetic acid
EGF Epidermal growth factor
EGFR Epidermal growth factor receptor
ELISA Enzyme linked immunosorbent assay
EPC Egg phosphatidylcholine
Epi Epiregulin
ER Oestrogen receptor
FBS Foetal bovine serum
FITC Fluorescein isothiocyanate
Flt-1 *fms*-like tyrosine kinase
H2A-ODN Anti-HER-2/neu antisense oligonucleotide
HB-EGF Heparin binding EGF
HBS HEPES buffered saline
HBSS Hank's balanced salt solution
HEPES Hydroxyethylpiperazine ethane sulphonic acid
HSP Hydrogenated soya phosphatidylcholine
IDC Infiltrating ductal carcinoma
KDR kinase-insert-domain-containing receptor
LCIS Lobular carcinoma in situ
MAPK Mitogen activated protein kinase
MPS Mononuclear phagocyte system
mRNA Messenger ribonucleic acid
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NP-40 Nonidet P-40
ODN Antisense oligonucleotide

PBS Phosphate buffered saline
PCNA Proliferating-cell nuclear antigen
PEG Poly [ethylene glycol]
PEG-C_{14}CER $PEG_{2000}C_{14}$Ceramide
PI Propidium iodide
PVDF Polyvinylidene fluoride
QELS Quasi-elastic light scattering
RNA ribonucleic acid
RNase Ribonuclease
SCR-H2A-ODN Scrambled control antisense oligonucleotide for HER-2/neu
SDS Sodium dodecyl sulphate
TEMED N,N,N',N'-Tetramethylethylenediamine
TGFα Transforming growth factor alpha
tPA Tissue type plasminogen activator
uPA Urokinase type plasminogen activator
VVO Vesicular vacuolar organelles
VEGF Vascular endothelial growth factor
VEGF-ODN Anti-VEGF antisense oligonucleotide
VPF Vascular permeability factor

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DEDICATION

This thesis is dedicated with much love to my daughters, Roxanne Nastassia and Rachel Emily.

For surely the light and courage that

emanate from their beings

has guided my soul and lent me strength

as I have ventured along this path.

Chapter 1

INTRODUCTION

1.1 Breast Cancer

Breast cancer is the most common type of cancer of women both in North America and world-wide. It is second only to lung cancer in cancer related deaths in women. In British Columbia, in the year 2000, 2300 women were estimated to be diagnosed with some form of breast cancer, and 630 women estimated to have died of this disease (NCIC Canadian Cancer Statistics, 2000). There is no single causative factor in breast cancer, but rather a number of causes, ranging from low to high associated risk, and including both genetic and non-genetic factors. Those factors which increase relative risk of developing breast cancer by only a small amount include first menses before the age of twelve and rapid establishment of regular cycles, late menopause (women who experience menopause before the age of 45 have half the breast cancer risk of women who go through menopause after the age of 55 (Henderson & Feigelson, 1997)), family history of postmenopausal breast cancer, and prolonged hormone use. Moderate risk factors include postmenopausal obesity, as well as any hyperplasia or abnormality on a previous breast biopsy. Factors associated with a 4-fold or greater increased risk of developing breast cancer include being a female, ageing, previous cancer in one breast, a family history of premenopausal breast cancer, and severe hyperplasia or atypia on a previous breast biopsy (Olivotto *et al.*, 1998; Henderson & Feigelson, 1997). In addition, there are environmental factors to be considered, such as exposure to x-ray radiation at an early age, which has been linked to

increased incidences of breast cancer (Doody *et al.*, 2000). It is clear that there are more environmental factors involved in the development and progression of breast cancer. This may be simply demonstrated by a comparison of the incidence rates among North American women as compared to Japanese women (Japanese women have an incidence of breast cancer one half that of their North American counterparts). However, after only one generation, the incidence among Japanese women living in North America is 75% that of Caucasian American women, and after several generations, the incidence rates are equivalent among the two groups of women (Stanford *et al.*, 1995). Conversely, there are factors associated with decreased risk of developing breast cancer, including increased parity, with the first birth at an early age, and lactation, with increased lactation resulting in decreased ovulatory cycles, and hence, decreased risk (Henderson & Feigelson, 1997), as well as partaking in regular exercise, and having a diet low in fat (Olivotto *et al.*, 1998).

A percentage of breast cancers are linked to specific genetic susceptibility, for example, mutations in the tumour suppressor genes BRCA1 and BRCA2. For malignant transformation, both copies of the gene must be either lost, mutated or inactivated. Females with the BRCA1 gene mutation have a 70% chance of developing breast cancer, compared to the approximately 11% risk of women in the general population, and this risk has been shown to be modified by non-genetic risk factors, such as parity (Narod *et al.*, 1995). Genes are also involved in the progression of breast cancer, and its prognosis. Examples of this are the tumour suppressor gene p53 (mutations in 15 - 35% of breast cancers)(Bernstein *et al*, 1999), the overexpression of the epidermal growth factor receptor (EGFR) and the oncogene HER-2/neu (amplified and/or overexpressed in up to 30% of

breast cancers)(Slamon *et al.*, 1987; Slamon *et al.*, 1989). Alterations in these and other genes can be very important predictive and prognostic factors in the treatment of individual cases of breast cancer. They also provide unique targets for the development of therapeutics in the fight against this disease, as discussed later in this chapter and in this thesis.

There are two main subcategories of malignant breast tumours; carcinomas and sarcomas. Of these two subtypes, carcinomas are by far the predominant tumour type. Currently, all breast carcinomas are classified on the basis of histological and/or cytological appearance, as well as gross findings such as site, size, shape, consistency, colour, and relationship to adjacent mammary and extramammary structures. Within the carcinomas, there are a variety of histological types, with most arising from the epithelial cells lining the terminal duct lobular units of the breast. Important among carcinomas is ductal carcinoma in situ (DCIS). In DCIS, the milk ducts become blocked with tumour cells, and calcium may be collected in the blocked ducts. There are two types of DCIS, namely solid or comedo, with comedo, identified by the large, irregular cells, being more likely to progress to invasion. Although not an infiltrating carcinoma, it is believed that the majority of infiltrating carcinomas arise from DCIS, especially from poorly differentiated, high grade DCIS. Of interest, the level of expression of the oncogene HER-2/neu within DCIS is also believed to be indicative of the likelihood of progression to infiltrating carcinoma of the breast (Olivotto *et al.*, 1998). A second type of non-invasive carcinoma is lobular carcinoma in situ (LCIS). This is differentiated from DCIS on the basis of tumour formation in the glands rather than ducts within the breast (Olivotto *et al.*, 1998).

Once tumour cells have broken through the epithelial lining of the ducts or glands and entered the fatty tissue of the breast, the tumour is classified as invasive, or infiltrating. The predominant type of infiltrating carcinoma is infiltrating ductal adenocarcinoma (IDC), which comprises 50 – 60% of all breast cancer cases (Rilke & Di Palma, 1998). These tumours are graded based on the degree of tubule formation (little or none being a higher grade tumour), nuclear pleomorphism, and mitotic index. With IDC, tumour emboli are often seen inside blood and lymph vessels within and outside the tumour area. The presence of such emboli within the lymphatics is a predictor of recurrence and metastatic spread (Henderson & Feigelson, 1997). IDC may be broken down into the subtypes tubular, colloid, mucinous, scirrhous and medullary, however treatment is generally the same for all types. (Olivotto *et al.*, 1998).

Other types of invasive breast cancer include i) inflammatory carcinoma, which is characterised by swollen, tender, reddened breasts, and is particularly aggressive, ii) lobular carcinomas, which account for approximately 15% of invasive breast cancer, and respond well to hormone based treatments, and iii) cancers that have their origin in parts of the breast other than the epithelium, including sarcomas, lymphomas and cystosarcoma phyllodes (Olivotto *et al.*, 1998).

1.2 Prognostic and Predictive Factors in Breast Cancer

Prognostic factors are those which relate to the risk of systemic recurrence and/or death following the primary therapy of a tumour. Predictive factors indicate how a tumour may

respond to a given therapy. A central aspect of this thesis concerns use of prognostic and predictive factors in guiding therapy.

1.2.1 Gross and Histopathological Factors

The classic prognostic factors in breast cancer include the histological type of the tumour, e.g., DCIS, LCIS or invasive carcinoma, as well as the components of the TMN tumour grading system. The TMN grading scheme includes the tumour size (T), the presence of distant metastases (M), and the involvement of lymph nodes (N). To address the nodal status primarily, if a patient has no cancer cells detectable in the axillary lymph nodes, this confers a good prognosis, and is referred to as node negative disease. Node positive disease encompasses any involvement of lymph nodes, whether it be only one node with detectable cancer cells, or more. The greater the number of positive lymph nodes, the poorer the prognosis for disease outcome. The presence of distal metastases is a poor prognostic factor, as is a larger tumour size at discovery. The mitotic index is a measure of the proliferation rate of the tumour. Other markers of proliferation are also employed, such as thymidine labelling index and measurement of cellular proteins expressed during specific parts of the cell cycle, for example, Ki67 (Pietilainen *et al.*, 1996) and proliferating-cell nuclear antigen (PCNA) (Ioachim *et al.*, 1996). As well, flow cytometry measurements of DNA are used to determine the proportion of tumour DNA in S-phase. With all of these measurements, the greater the proportion of the tumour that is proliferative, the poorer the overall prognosis. Other features of the TMN system include the presence of tumour fixation to the skin or underlying muscle, matting or fixation of the axillary nodes, and an inflammatory component, which also designate patients with a poor

prognosis. (Ravdin, 1997).

As well as utilising the TMN system, pathologists rely on histologic grading, which refers to a classification of breast cancer based on the nuclear grade of a tumour, the mitotic rate, and architectural differentiation. Those tumours that have pleomorphic nuclei are associated with a poorer prognosis, as are tumours with high mitotic indices and lack of cellular differentiation.

1.2.2 Molecular Factors

The discovery of new prognostic and predictive markers for breast cancer is exciting, and has opened the way for a new generation of targeted therapeutics for treating this disease. Several proteins have now been associated with more aggressive breast, as well as some other types of cancer. By looking at the combination of gross, traditional histopathologic markers, and these new molecular markers of aggressive disease, pathologists are able to obtain a much clearer picture of a patient's cancer, and are able to provide more individualised care to that patient. The following sections will discuss a select few of these new markers.

1.2.2.1 Cathepsin D

The oestrogen regulated lysosomal protease Cathepsin D has been proposed to play an important role in breast cancer invasion and metastasis (Han *et al.*, 1997). Because of this, patients with elevated cathepsin D levels, defined as those tumours with clearly detectable

cathepsin D levels (greater than or equal to 10% positive cells)(Gonzalez-Vela *et al.*, 1999) have an adverse prognosis (Solomayer *et al.*, 1998). As a protease, cathepsin D is likely to be involved in the degradation of extracellular matrix and therefore, tumour cell spread in circulatory and lymphatic systems. This same mechanism may also contribute to distant metastases. Within a tumour, positive staining of stromal cells for cathepsin D is often correlated with vascular density, another factor adversely related to prognosis (see section 1.2.2.2). Positive non–tumor cell staining is also associated with high grade tumour, high cell mitotic rate and oestrogen receptor (ER) negative status (all features of increased biological aggressiveness)(Gonzalez-Vela *et al.*, 1999). There is a trend of increased cathepsin D in the tumour cells of ER positive breast cancer, but there is disagreement in the significance of this cathepsin D, with some researchers disputing the relation to other clinicopathologic parameters (Gonzalez-Vela *et al.*, 1999), and others finding significant correlation between cathepsin D positive staining in tumour cells, and tumour size and nodal status in breast cancer metastases (Solomayer *et al.*, 1998). An interesting observation has been made by Solomayer *et al.* (1998), in their study of bone metastases, in that cathepsin D is found only on tumour cells, not on stromal cells in the bone marrow of patients with primary breast cancer. They feel therefore, that in the final steps of metastasis, cathepsin D in tumour cells, but not stromal, is involved in invasion.

1.2.2.2 Vascular Endothelial Growth Factor

Growing tumours are much more than passive clumps of cells. They are able to actively modulate their environment, inducing changes that allow advantageous conditions for growth. Since the continued growth of a tumour is dependent upon an adequate blood

supply, not surprisingly, one of the major microenvironment modifications that malignant cells have developed is a means of controlling their vasculature, including the secretion of factors that either inhibit or stimulate new blood vessel formation.

The formation of new vessels from pre-existing ones is known as angiogenesis, and involves a complex cascade of events and factors. One of the most important of these angiogenic factors is vascular endothelial growth factor (VEGF), a multi-functional cytokine and endothelial cell mitogen also known as vascular permeability factor (VPF). This alternative nomenclature is a reflection of the ability of VEGF/VPF to induce vascular permeability with a potency some 50,000 times that of histamine (Keck *et al.*, 1989; Senger *et al.*, 1990).

VEGF is a 34 – 42 kDa heparin-binding, dimeric, disulphide bonded glycoprotein that acts directly on endothelial cells by way of two specific class III receptor tyrosine kinases, fms-like tyrosine kinase (Flt-1) and kinase-insert-domain-containing receptor (KDR) (Senger *et al.*, 1993). Many tumour cells express and secrete VEGF at high levels, with the effect of inducing the formation of new vasculature to promote tumour growth.

The human VEGF gene is divided among eight exons, and as the result of alternative splicing of mRNA, yields four different VEGF isoforms of 121, 165, 189 and 206 amino acids (aa), respectively. Of these, the 121 and 165 aa isoforms are secreted, while the 189 and 206 aa isoforms remain bound to the cells. Finally, there are three additional forms of VEGF which have recently been described; VEGF-B, VEGF-C and VEGF-D. The

biological functions of these forms have not yet been fully elucidated, but may be involved in tumour spread through the lymphatics (Olufsson *et al.*, 1996; Joukov *et al.*, 1996; Lee *et al.*, 1996 Kurebayashi *et al.*, 1999).

VEGF is able to induce angiogenesis in two ways. Primarily, it does so by means of specific mitogenic activity on endothelial cells, but it also indirectly contributes to angiogenesis by effects on vascular permeability. One of the ways in which VEGF affects permeability is to enhance the activity of vesicular-vacuolar organelles (VVO) within the cytoplasm of endothelial cells. VVO are complex structures that span the endothelial cell cytoplasm and allow the passage of macromolecules, providing a pathway from the circulation into the tissues.

VEGF-induced hyperpermeability of tumour microvasculature allows for the accumulation of fluid and plasma proteins in the tumour interstitium. One of the extravasated proteins is plasma fibrinogen, the presence of which in the interstitium, leads to fibrin deposition and subsequent alteration of the tumour extracellular matrix. This altered matrix is ideal for, and promotes the ingrowth of fibroblasts, macrophages and endothelial cells, which serve to promote tumour growth (Bobik *et al.*, 1997). The deposition of an extravascular fibrin matrix is in itself, a potent angiogenic stimulus (Brown *et al.*, 1992), and also contributes to overall tumour growth.

Angiogenesis has also been related to the formation of metastases, in that risk of metastatic tumour is dramatically increased with the formation of a dense new vasculature (Bobik *et*

al., 1997). It is even possible in some cases to statistically correlate the appearance of metastases with the number of microvessels detected in the primary tumour (Martiny-Baron & Marmé, 1995).

The primary signal for angiogenesis in tumours is believed to be the release by tumour cells of diffusible signals toward pre-existing capillaries which commit resting endothelial cells to actively dividing and migrating (Plouet & Bayard, 1994). A further component of this theory is that cells at the tip of the migrating bud of endothelial cells are not dividing, but those behind them are, helping to push the bud toward the angiogenic stimulus. Figure 1.1 summarises some of the actions of the different forms of VEGF on the vascular endothelium. Endothelial cells stimulated by hypoxia, transforming events or cytokines are able to elaborate collagenases and plasminogen activators to aid in the local degradation of the basement membrane. Sprouting endothelial cells, which form the bud of a developing new vessel are able to induce the expression of the serine proteases; tissue type and urokinase type plasminogen activator (tPA and uPA, respectively) and metalloproteinase interstitial collagenases. This co-induction of PA and collagenases forms a pro-degradative environment which facilitates the migration of the endothelial cells through the tissue (Mignatti & Rifkin, 1996).

1.2.2.3 HER-2/neu

HER-2/neu (also known as c-erbB-2) is a proto-oncogene located on the long arm of chromosome 17, specifically in the area of 17q.12-21.32 (Popescu *et al.*, 1989). The HER-

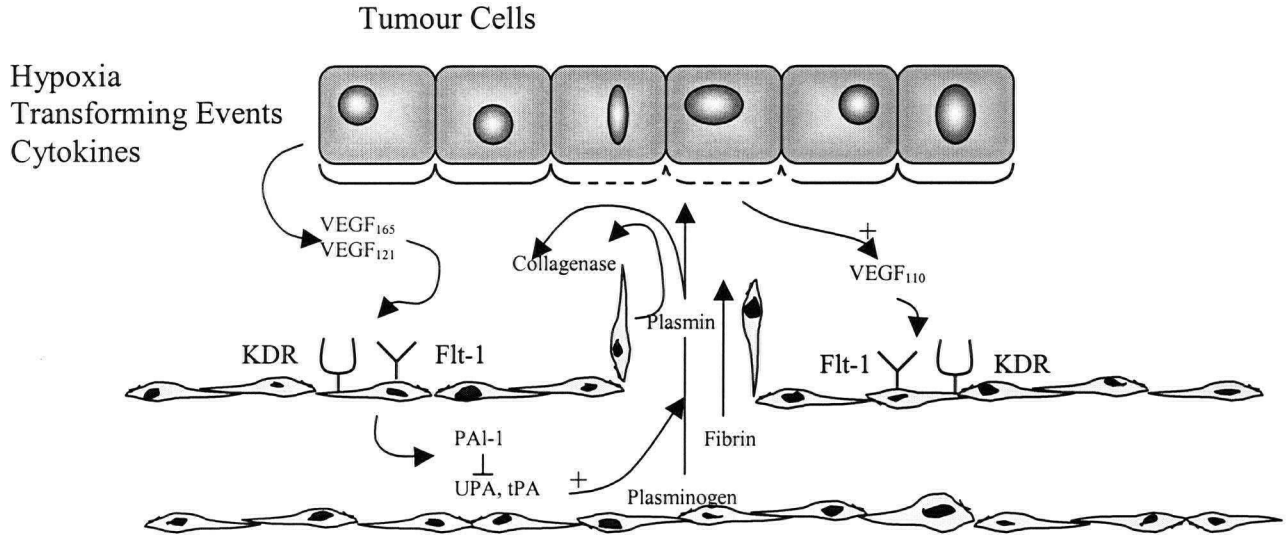

Figure 1.1 Actions of tumour-derived vascular endothelial growth factor on endothelial cells. Tumour cells secrete VEGF in response to various stimuli, which exerts a mitogenic affect on endothelial cells, as well as increasing intracytoplasmic [Ca^{2+}] levels. An additional effect is the formation of a prodegradative environment that facilitates migration and sprouting of endothelial cells by the co-induction of the serine proteases urokinase plasminogen activator (UPA) and tissue-type plasminogen activator (tPA) as well as collagenase. Plasminogen activation results in generation of plasmin, which may cleave extracellular matrix bound VEGF ($VEGF_{189}$ or $VEGF_{206}$) to release a diffusible proteolytic fragment ($VEGF_{110}$). The induction of plasminogen activator inhibitor (PAI-1) serves as a regulatory step to balance the proteolytic process. These actions are exerted through the interaction of VEGF with the class III receptor tyrosine kinases, KDR and Flt-1. (Adapted from Ferrara & Davis-Smyth, 1997).

2/neu oncogene was first isolated from DNA extracted from ethylnitrosurea-induced adrenal neuroglioblastomas of neonatal rats (Shih *et al.*, 1981). This gene encodes a 185 kDa transmembrane receptor tyrosine kinase (p185), which is a member of the Class I receptor tyrosine kinase (RTK) family (Fig. 1.2). Class I RTKs all possess a large, glycosylated extracellular ligand binding domain with two cysteine-rich sequences, a single hydrophobic transmembrane domain and a long cytoplasmic tail containing the kinase domain and C-terminally located autophosphorylation sites (Ben-Baruch *et al.*, 1998). The prototype member of the class I RTK family is the epidermal growth factor receptor (EGFR). EGFR is expressed at high levels in approximately one third of all epithelial cancers, including breast cancer. EGFR overexpression has also been implicated in a poor prognosis in breast cancer patients (Pawlowski *et al.*, 2000).

The HER-2/neu protein shares extensive sequence homology with EGFR (also known as HER-1), and in fact, the amino acids in the tyrosine kinase domain of the two receptors are more than 80 % identical (Schechter *et al.*, 1984; Yamamoto *et al.*, 1986) in the kinase domain, and approximately 45% homologous in the ligand-binding domain (Yamamoto *et al.*, 1986). At least in the case of EGFR, the ligand binding cleft of the receptor is confined to the portion linking the two cysteine-rich domains (Ben-Baruch *et al.*, 1998). Within the class I RTK family, in addition to EGFR and HER-2/neu, there are two other family members, namely HER-3 and HER-4, which are known to form both homodimers and heterodimers with both HER-1 and HER-2/neu.

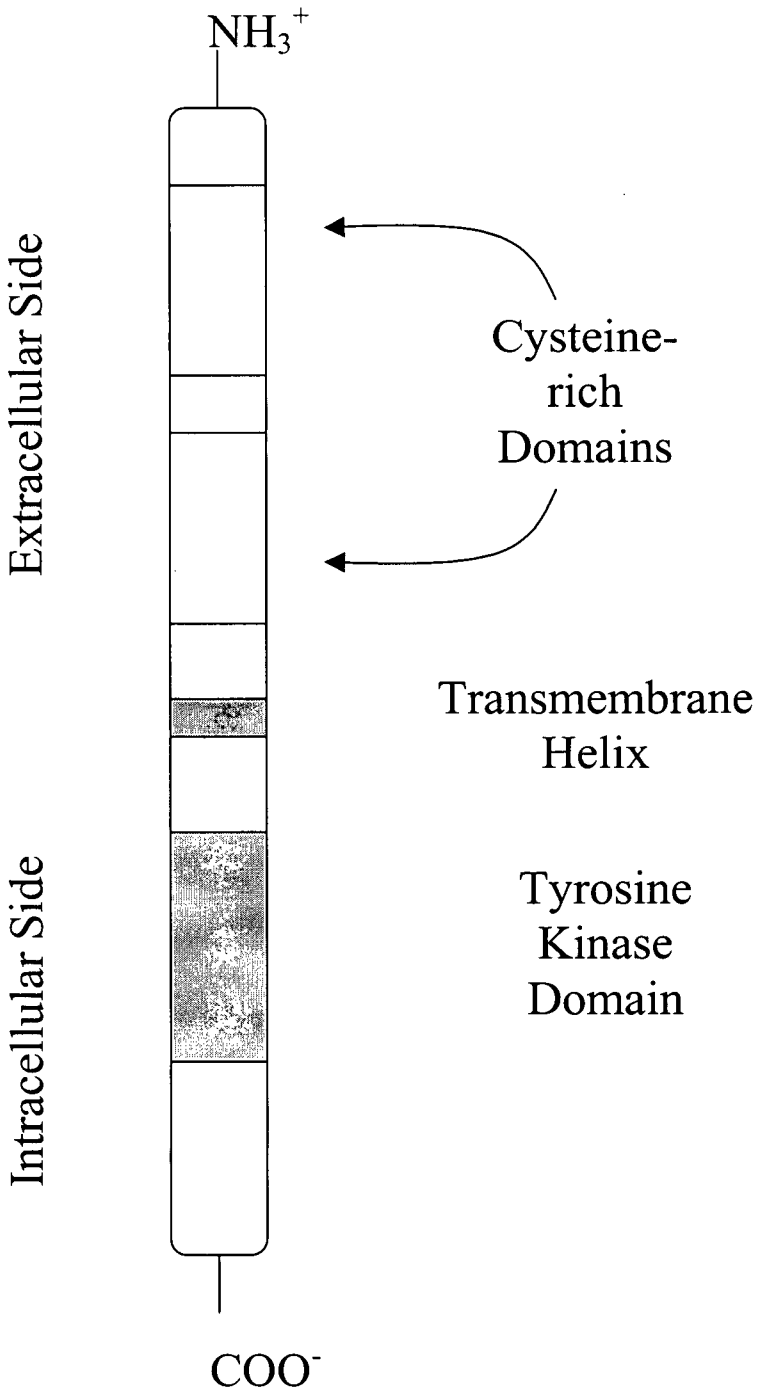

Figure 1.2 General structure of class I receptor tyrosine kinases. This class of RTKs includes the family members epidermal growth factor receptor (EGFR), HER-2/neu, HER-3 and HER-4. Indicated are the cysteine rich extracellular domain characteristic of this class, the transmembrane domain, and the tyrosine kinase domain of the intracellular portion of the receptor.

Although classified as a receptor, to date, there is no known ligand for p185, although it is known to mediate lateral signal transduction in all HER family members. This is by means of heterodimerization with EGFR (see Fig 1.3), and activation by binding of ligand (discussed further in section 1.2.2.3.1) to its receptor, or heterodimerization with HER-3 or HER-4, and binding of ligands to these receptors. Ligand binding results in activation of the receptor complex, trans-phosphorylation, and subsequent signal transduction.

HER-2/neu was first proposed to be a prognostic factor in breast cancer by Slamon *et al.* (1987), using a measure of the amplification of the gene itself. This initial study showed strong correlation between increased copy number of the gene and shortened disease free survival and overall survival in node positive patients. Many studies have been performed since 1987, using both gene amplification and immunohistochemical measurement of protein level as a measure of HER-2/neu level (Allred *et al.*, 1992; Press *et al.*, 1997; Ross *et al.*, 1998; Paik *et al.*, 1990; Pich *et al.*, 2000). These above mentioned studies, for the most part, support the contention that amplification and/or overexpression of HER-2/neu is associated with more aggressive disease, and enhanced metastatic potential in node positive patients.

Overexpression of HER-2/neu has been shown to correlate with resistance to a number of agents commonly used in the treatment of aggressive breast cancer, including cyclophosphamide, methotrexate and 5-FU (Piccart *et al.*, 1988; Mourali *et al.*, 1993). Intriguing data is available for p185 overexpressing breast cancers and treatment with increasing doses of doxorubicin. These studies have demonstrated significant improvement

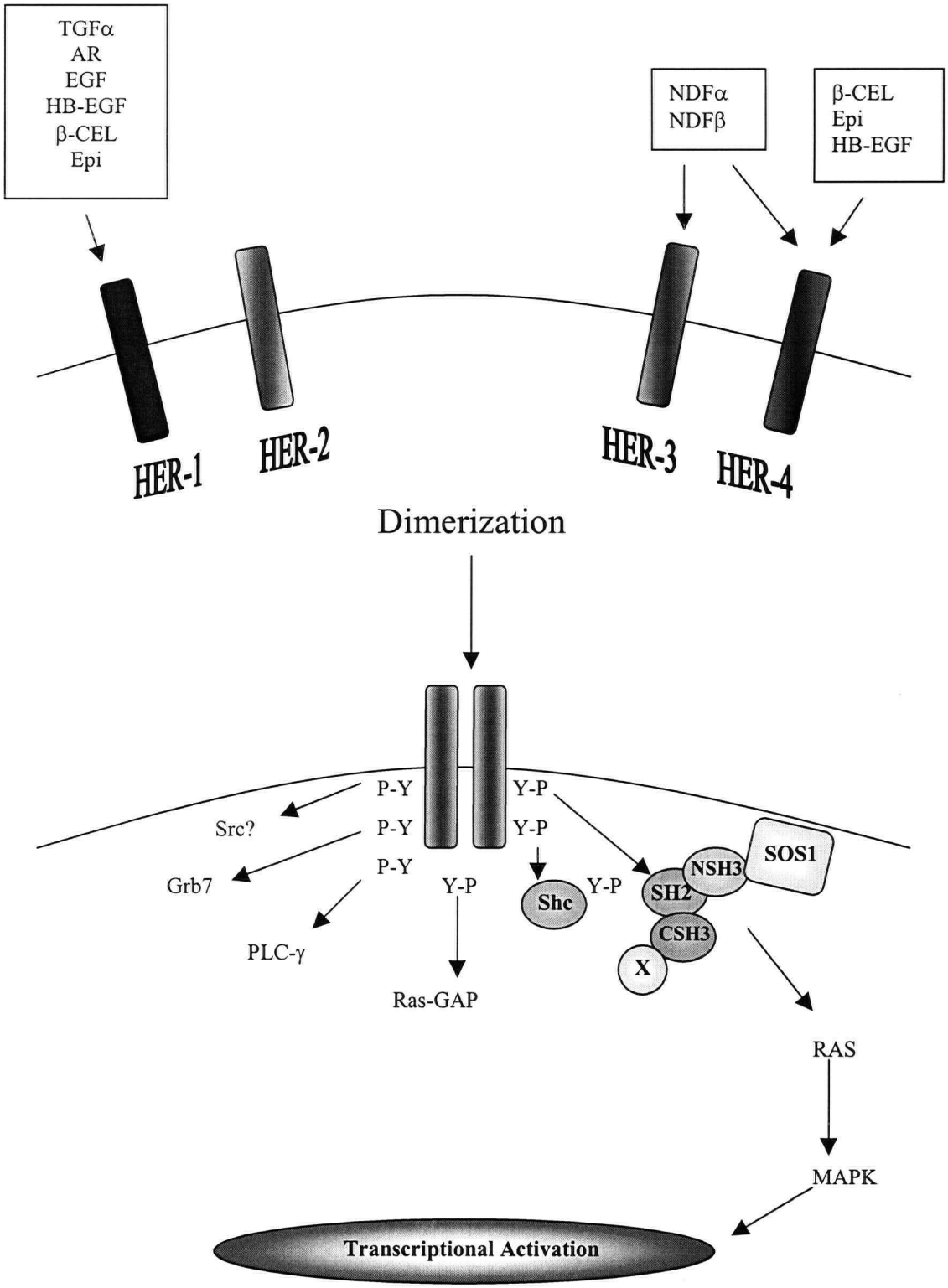

Fig. 1.3 Class I RTK ligands, homo- and heterodimerisation and signalling pathways. The four class I RTK family members are able to form either homodimers or heterodimers with any of the other family members. This leads to phosphorylation of tyrosine residues on the intracellular portion of the receptor, and subsequent signal transduction, culminating in specific gene regulation and cell cycle progression as outlined in the text.

in survival with increased doxorubicin (Paik *et al.*, 1998; Thor *et al.*, 1998) dose only in HER-2/neu overexpressing tumours, and not those with low or absent p185 expression. It is unclear as to whether this is due to increased sensitivity of p185 expressing tumours to doxorubicin, or whether the higher dose serves to overcome an intrinsic resistance in these tumours (Esteva-Lorenzo *et al.*, 1998).

1.2.2.3.1 HER-2/neu Signalling Pathways

There is a large family of EGF-related peptides which serve as ligands for the HER family of receptors. These ligands fall into three general groups: i) EGF, amphiregulin (AR) and transforming growth factor α (TGF-α), which bind EGFR; ii) beta-cellulin (β-CEL), epiregulin (Epi) and heparin binding EGF-like growth factor (HB-EGF), which bind both HER-1 and HER-4, and iii) neu differentiation factors (NDFs) or heregulins, which bind to both HER-3 and HER-4. Upon binding of ligand to receptor, there is promotion of receptor homo- and heterodimerization, and receptor activation (Fig 1.3).

Dimerisation leads to autophosphorylation of receptors on specific tyrosine residues in their cytoplasmic domains, which then provide docking sites for Src Homology 2 (SH2) and phosphotyrosine binding domain-containing proteins, including Shc, Grb2 and the p85 sub-unit of phosphatidylinositol kinase. This leads to signalling pathways such as the mitogen activated protein kinase (MAPK) pathway and the S6 kinase cascade (Olayioye *et al.*, 1998). There is, within the HER family of receptors, considerable overlap in the signalling pathways employed; however, there is some receptor preference for certain pathways, for

example, the Cb1 protein appears to couple exclusively to EGFR, whereas the Csk-homologous kinase binds only to HER-2. EGF- and NDF-activated EGFR associate with Shc, but only EGF-activated EGFR couples to Grb2, an observation explained by differential phosphorylation of EGFR (Olayioye *et al.*, 1998). This type of relationship applies to HER-2/neu as well, where phosphopeptide mapping of HER-2/neu has revealed dimerisation dependent phosphorylation (Olayioye *et al.*, 1998). When these preferences are coupled with the different receptor heterodimerization possibilities, the range of signalling can become quite diverse.

It has been shown that HER-2/HER-3 dimers are favoured over HER-2/HER-1 dimers (Olayioye *et al.*, 1998), and that in all the possible dimerisations, HER-2/neu is the preferred partner of the other HER-family receptors (Graus-Porta, 1997; Olayioye *et al.*, 1998). Because of this, HER-2/neu can be activated by EGFR, HER-3, HER-4 or by monoclonal antibody induced homodimerisation. Homodimer formation also leads to specific and distinct receptor phosphorylation patterns. As different adapter molecules have been mapped to specific tyrosine residues on the HER family members, and as a range of phosphorylation is possible due to the number of tyrosine residues which are phosphorylated at any given time, a broad range of signalling may be achieved via these different dimerisations (Olayioye *et al.*, 1998).

The diverse responses of HER-2/neu to activation include transformation, monoclonal antibody induced growth inhibition, ligand-induced growth stimulation or apoptosis. These varying results imply activation specific differences in the signalling capacity of HER-

2/neu (Olayioye *et al.*, 1998). These differences may result in unique responses of cells with overexpression of this protein to a given therapy, dependent upon the phosphorylation state, and the type of therapy, whether it be targeted to the protein, the mRNA or the DNA. It is critical that we develop an understanding of this potential variation of response before we can truly have a thorough knowledge of the mechanism by which the drugs that are being developed, are acting at a cellular level. In addition to this, although it is apparent that HER-2/neu is an ideal candidate for targeting of anti-cancer drugs, there is the potential for interaction of the protein with other members of the class I RTK family, thereby confounding the interpretation of results garnered with such therapies.

1.2.2.3.2 HER-2/neu in Normal Physiology

HER-2/neu protein expression has been identified by immunohistochemistry on epithelial membranes in the gastrointestinal, respiratory, urinary and reproductive tracts, and in the skin of both adult and foetal specimens. Generally however, the expression levels are higher in foetal specimens (Press *et al.*, 1990). Cells expressing p185 have been demonstrated as being derived from all three germ layers, and of polarised cells attached to basement membrane, endothelial and mesothelial cells were the only cells which were found not to express p185 (Press *et al.*, 1990). In studies with knockout (HER-2/neu$^{-/-}$) mice, HER-2/neu was found to be critical in the development of trabeculae in the heart, as well as the formation of synapses between motor neurons and muscles (Ben-Baruch *et al.*, 1998). In expression studies of human embryos and foetuses, from gestational week 5 to 8 there has been shown to be strongly positive staining for p185 in the developing

myocardium, as well as in the plasma membrane regions of the developing epicardium, whereas after gestational week 9, staining was not observed (Miosge *et al.*, 1997). A similar time dependent expression pattern was observed in muscle, with embryos of gestational weeks 4 through 10 demonstrating strong membrane staining for p185, while from gestational week 11 onwards, the immunoreaction ceased (Miosge *et al.*, 1997). Studies have also identified a key role for HER-2/neu in the development of trigeminal and facial motor nerves. Due to striking similarities between the HER-2/neu$^{-/-}$ mice and NRG$^{-/-}$ mice, and other knockout studies, these researchers concluded that the developmental role of HER-2/neu may be to augment signalling by NRGs, as well as to prolong the activity of EGFR ligands (Ben-Baruch *et al.*, 1998). As a generalisation, p185 expression during development is found mainly during the embryonic (to gestational week 9), and not the foetal (gestational week 10, on) period. The embryonic period is characterised by cell proliferation, cell differentiation and migration, whereas in the foetal period, growth and proliferation are not the predominant processes (Miosge *et al.*, 1997), implicating a role for p185 in these processes.

HER-2/neu is also known to play a role during the differentiation of normal mammary glands, as mRNA levels increase at the onset of proliferation and during the lobulo-alveolar development, and then decrease sharply in milk-producing glands. During the differentiation phase, post-transcriptional mechanisms, such as altered turnover of the protein, maintain high levels of p185 (De Bortoli & Dati, 1997).

With the above in mind, added to the cautions listed toward the end of section 1.2.2.3.1 is

that care needs to be taken in devising a therapeutic which targets proteins localised in normal, as well as cancerous tissues. Given the limited expression in the tissues of normal adults however, it can be suggested that HER-2/neu represents an almost ideal target for development of targeted therapy.

1.2.2.3.3 HER-2/neu Expression in Cancer

Many researchers have investigated HER-2/neu expression levels in a variety of human neoplasms, with sometimes conflicting results. These differing results may in part be due to alternate methods of tissue processing, such as freezing, paraffin embedding, length of time to staining, type of antibody used, etc. In addition, researchers may score tissue positivity on different scales, therefore leading to higher or lower reported HER-2/neu expression values. There is a now general consensus among researchers, that only staining which is present on the membranes of cells, and not intracytoplasmic, should be scored as positive staining for HER-2/neu. In fact, some researchers feel that intracytoplasmic staining may not even be representative of an immature precursor of p185, but rather, a distinct protein that is able to cross react with the antibodies used (Tannapfel *et al.*, 1996).

Having stated this, there have been reports of HER-2/neu amplification and/or overexpression in several tumour types, including gastric (Tannapfel *et al.*, 1996), colorectal (Tannapfel *et al.*, 1996; Yang *et al.*, 1996), transitional cell bladder (Tannapfel *et al.*, 1996), renal cell (Hofmockel *et al.*, 1997), ovarian (Schmitt *et al.*, 1996; Eltabbakh *et al.*, 1997), endometrial (Schmitt *et al.*, 1996), cervical (Nakano *et al.*, 1997; Sharma *et al.*,

1999), breast (Schmitt *et al.*, 1996) and non-small cell lung (Giatromanolaki *et al.*, 1996) carcinomas. Not all of these instances of overexpression are linked with an adverse prognosis; however, this does appear to be the case for at least breast and ovarian carcinomas.

1.2.2.3.3.1 HER-2/neu in Breast Cancer

The HER-2/neu gene is amplified and/or overexpressed in up to 30% of all human breast carcinomas and has been correlated with decreased overall survival and time to relapse in this patient population. This patient group has a significantly lower overall survival rate, and shorter time to relapse than those patients whose tumours do not overexpress HER-2/neu (Allred *et al.*, 1992; Borg *et al.*, 1990; Sjogren *et al.*, 1998; Slamon *et al.*, 1989). Moreover, this significance is retained even when adjustments are made for other prognostic factors (Slamon *et al.*, 1987), and is true for both node positive (Slamon *et al.*, 1987) and node negative (Andrulis *et al.*, 1998) breast cancers. Research into a mechanism for HER-2/neu action has shown that overexpression of HER-2/neu in cell lines increases the migratory capabilities of the cells, an important step in metastasis formation (Verbeek *et al.*, 1998).

When the overexpression of HER-2/neu in a significant sub-set of breast cancer patients is paired with the adverse prognosis related to this overexpression, it is apparent that HER-2/neu presents an ideal therapeutic target. Many researchers have embarked on the development of anti-HER-2/neu therapeutic strategies. The foremost among these are

discussed in section 1.3.

1.3 Therapeutic Strategies in the Treatment of Breast Cancer

Metastatic breast cancer is generally thought of as an incurable disease, with a median survival of 18 – 24 months (Miller & Sledge, 1999). With this in mind, it is sometimes difficult to decide whether to treat aggressively with curative intent, or to give palliative treatment only. Palliation may be achieved with several treatments, including hormonal manipulations, systemic chemotherapy, local radiotherapy, and immune-based biologic therapies (Miller & Sledge, 1999). These same treatments may, in a few cases, also lead to cures, however there is a definite need for development of novel agents which will expand the number of cures being obtained. Although there a number of prognostic markers such as HER-2/neu, that increase the potential for better treatment options, it is likely that these new therapeutics will only be used in combination with one or all of the existing treatment modalities. It is therefore useful to review these here.

1.3.1 Chemotherapeutics

A number of chemotherapeutic agents form the backbone of current therapy for aggressive breast cancer. These agents include anthracyclines, taxanes, cyclophosphamide, methotrexate and 5-fluorouracil (5-FU), and responses can be as high as 30% in previously untreated patients (Miller & Sledge, 1999). Newer agents include Navelbine (vinorelbine), gemcitabine and capecitabine. Table 1.1 lists the more commonly used agents, as well as briefly describing some of their mechanisms of action.

Because of the increased efficacy of anthracycline-containing regimens, they are the most commonly used regimens for both primary and metastatic breast cancer (Hortobagyi, 2000). The most common representative of the anthracyclines is doxorubicin. Therapy with this drug is limited acutely by myelosuppression, however both acute and chronic cardiotoxicity are of great concern. Since the cardiomyopathy is dose dependent, most protocols limit the lifetime dose of this drug to about 450 mg/m^2. Doxorubicin is usually given in combination with other drugs, such as cyclophosphamide and 5-FU.

The taxanes paclitaxel and docetaxel are relatively new anticancer agents which work by inhibiting microtubule disassembly by binding to dimeric tubulin. This results in cells being blocked in the G2 and M phases of the cell cycle (Miller & Sledge, 1999). Although these are plant alkaloids (derived from European and Western Pacific yew trees), taxanes used in cancer therapy are semi-synthetically manufactured. Importantly, these agents have anti-tumour activity in anthracycline resistant breast cancer. Response rates in metastatic breast cancer are equal to or greater than 30% with single agent paclitaxel, and over 40% with single agent docetaxel, and each of the taxanes are now being incorporated into combination chemotherapy regimens (Hortobagyi, 2000).

Nucleoside analogues are relatively new agents in the treatment of breast cancer. This group includes the deoxycytidine analogue, gemcitabine (difluorodeoxycytidine), as well as cytarabine. These drugs block the progression of cells from the G1 phase of the cell cycle through to the S phase (DNA synthesis). Additionally, cytarabine will act in the S phase to

kill cells undergoing DNA synthesis.

The most commonly used alkylating agent is currently cyclophosphamide. One of the advantages of cyclophosphamide over its counterparts, nitrogen mustard. and chlorambucil, is that is it available in both parenteral and oral formulations, and therefore can be used in a wide variety of dosage schedules (Carter, 1972). Cyclophosphamide is commonly used in conjunction with an anthracycline and another agent such as 5-FU in the treatment of metastatic breast cancer.

Vinorelbine, a norvinblastine analogue, also targets microtubules, and has reduced neurotoxicity as compared with vincristine due to its higher affinity for mitotic spindle microtubules, rather than axonal microtubules (Miller & Sledge, 1999). Vinorelbine is also better tolerated than vincristine, and has value as a salvage treatment (final treatment for patients non-responsive to other available therapies) in patients with prior anthracycline and taxane exposure (Miller & Sledge, 1999).

1.3.2 Hormonal Strategies

There are several agents used in the treatment of breast cancer that fall into this category, including tamoxifen, toremifene, raloxifene, anastrozole, mifepristone and others. Tamoxifen is a synthetic anti-oestrogen agent coming into common use in both the treatment of early breast cancer (Early Breast Cancer Trialists' Collaborative Group, 1998), as well as for the prevention of breast cancer (Fisher *et al.*, 1998). When all breast tumour

Table 1.1 Mechanisms of action of chemotherapeutic agents used in the treatment of aggressive breast cancer.

	Chemotherapeutic Agent	Mechanism(s) of Action
Anti-tumour Antibiotics	Anthracyclines (e.g., doxorubicin, epirubicin)	Intercalation within DNA; inhibition of topoisomerase II
	Mitomycin	Inhibition of DNA synthesis; RNA and protein synthesis inhibition at high doses
	Anthracenediones (e.g., mitoxantrone)	Intercalation within DNA
Plant alkaloids	Taxanes (e.g., paclitaxel, docetaxel)	Inhibition of microtubule disassembly by binding to dimeric tubulin
	Vinca alkaloids (e.g., vincristine, vinblastine, vinorelbine)	Inhibition of microtubules of the mitotic spindle
Nucleoside analogues	Gemcitabine, Cytarabine	Incorporation into DNA of intracellularly generated metabolite
Anti-metabolites	5-fluorouracil	Inhibition of thymidylate synthetase; Incorporation of FUTP into RNA and DNA
	Capecitabine	Prodrug leading to high tumour concentrations of 5-FU
	Methotrexate	Inhibition of purine synthesis
Alkylating agents	Cyclophosphamide	Prevention of cell division by cross-linking DNA strands
	Chlorambucil	Cross-linking of DNA and RNA strands, inhibition of protein synthesis

types are included in analyses, the overall response rate for tamoxifen as a single agent is approximately 30 – 35%. When evaluating ER and progestin receptor positive breast tumours only, the response rate is approximately 60 - 75% (Fornier *et al.*, 1999). Unfortunately, most breast cancer patients eventually become resistant to tamoxifen, and must rely on other strategies to fight their cancer (Fornier *et al.*, 1999). Other anti-oestrogens currently in clinical trial or approved for use in metastatic breast cancer are

toremifene, droloxifene and ICI 182780.

Other hormonal strategies are the use of i) aromatase inhibitors, which block the peripheral conversion of androstenedione to estrone (e.g., anastrozole letrozole, formestane and exemestane), and achieve major reductions in oestrogen levels without suppressing adrenal function, and ii) anti-progestins, an example of which is the abortion drug, RU 486 (Fornier *et al.*, 1999).

1.3.3 Molecular Targeting

The strategies discussed in section 1.3.2 are those which essentially target a specific component of breast cancer, namely, the oestrogen or progestin receptors. These drugs, while having a very specific mechanism of action, do not truly target themselves to the tissue in question. Many approaches of molecular targeting have been considered, including the use of monoclonal antibodies and small molecule inhibitors. The following section describes the use of antibodies directed against two members of the class I RTK family, HER-1 (EGFR) and HER-2/neu. This type of therapy represents a true targeting, as the therapy is concentrated at the site of action by the nature of the drug binding to specific ligands expressed on the tumour cell surface.

1.3.3.1 Antibodies

The most widely discussed antibody used in treatment of cancer is trastuzumab (Herceptin™). This is a humanised monoclonal antibody targeted to the extracellular

domain of the HER-2/neu protein, and has been shown in clinical trials to produce objective tumour responses in 15 – 21% of patients with HER-2/neu overexpression and who had relapsed following chemotherapy for metastatic breast cancer (Cobleigh *et al.*, 1998). It has been postulated that this antibody works by antagonising the function of the growth signalling properties of the HER-2/neu system. However it has also been suggested that it may signal immune cells to attack and kill tumour cells, and in addition, may enhance the cytotoxicity of other chemotherapeutic agents (Fornier *et al.*, 1999).

A potential concern with this therapy is that it may either cause cardiac dysfunction, or amplify the cardiac toxicity observed with the use of anthracyclines (toxicity observed in 6% of doxorubicin only patients versus 27% of patients treated with the combination of trastuzumab plus doxorubicin in a phase III clinical trial)(Ewer *et al.*, 1999). Trastuzumab has recently been granted approval in the United States and several European countries, and it is likely that it will be paired with anthracycline therapy; therefore this potential for enhanced cardiac toxicity needs to be further explored. One of the objectives of this thesis is develop an antisense oligonucleotide therapeutic targeting HER-2/neu, as does trastuzumab, and to pair this new therapy with cytotoxic agents such as doxorubicin. Ideally, this combination will be without the additional toxicity currently noted with the trastuzumab/doxorubicin combination.

Another antibody targeting one of the HER family members is currently in development. C225 is an anti-EGFR antibody designed for use in women whose breast tumours overexpress EGFR.

1.3.4 Gene Therapy

There are several aspects of gene therapy, including the enhancement of tumour immunogenicity by insertion of cytokine or co-stimulatory molecule encoding genes, or direct killing of tumour cells by insertion of tumoricidal genes, tumour suppressor genes, and genes encoding prodrug activating enzymes (Dachs *et al.*, 1997). In the work presented in this thesis, I have focused on the gene therapy approach of antisense oligonucleotides (ODN), short, single stranded segments of DNA. ODN are not a typical type of gene therapy in that they are not designed to correct gene defects or achieve insertion of new genes into tumour cells. These ODN thus share the specificity of whole gene approaches without the potentially deleterious insertion into the genome, as well as having other benefits as discussed below.

1.3.5 Antisense Oligonucleotides

Antisense oligonucleotides are relatively novel therapeutic drugs, and by their nature, are able to target the mRNA or DNA of many different proteins. ODN are short sequences of single stranded DNA, typically ranging from 12 – 30 bases in length. They are designed to prevent gene expression by interfering with some portion of the translation process of mRNA into protein. Theoretically, any transcribed gene is a candidate target for antisense inhibition, although any targeted sequence must be checked for homologies to other genes.

The affinity of any given ODN for the targeted mRNA results from the nature of the

hybridisation between the two. This is largely a result of Watson-Crick base pairing, as well as base stacking within the resultant double helix that forms. As the hybridisation length is increased, the affinity of binding increases accordingly, however, as RNA is able to adopt both secondary and tertiary structures, the theoretical binding affinities are rarely reached (Crooke, 1999). Also contributing to this lack of affinity is the complex nature of the cytoplasmic environment in which the hybrids are formed, with many protein interactions possibly obstructing complete hybridisation.

1.3.5.1 Types of antisense oligonucleotides

Native DNA has a phosphodiester backbone, and many ODN studies have utilised this same backbone. More recently, several backbone modifications have been made which improve the nuclease stability of the ODN, while still resembling natural nucleic acids as closely as possible. These include the replacement of one of the non-bridging oxygen molecules of the phosphodiester bond with a methyl group (methylphosphonate ODN), a methyl ester (phosphotriester ODN), a methyl ammonium group (phosphoroamidate ODN) or with a sulphur atom (phosphorothioate ODN) (Figure 1.4). There are also many modifications to antisense which involve the sugar moiety alone, or in combination with backbone modifications (Manoharan, 1999; Geary *et al.*, 2001). These modifications are designed to decrease the degradation of ODN due to both endo and exonucleases, as well as optimising tissue, plasma and target organ accumulation. The variability of different ODN with respect to size, sugar or backbone modifications may lead to subtle or more notable changes in pharmacokinetic properties such as plasma circulation time, tissue or disease site uptake, toxicity, as well as ultimately, efficacy.

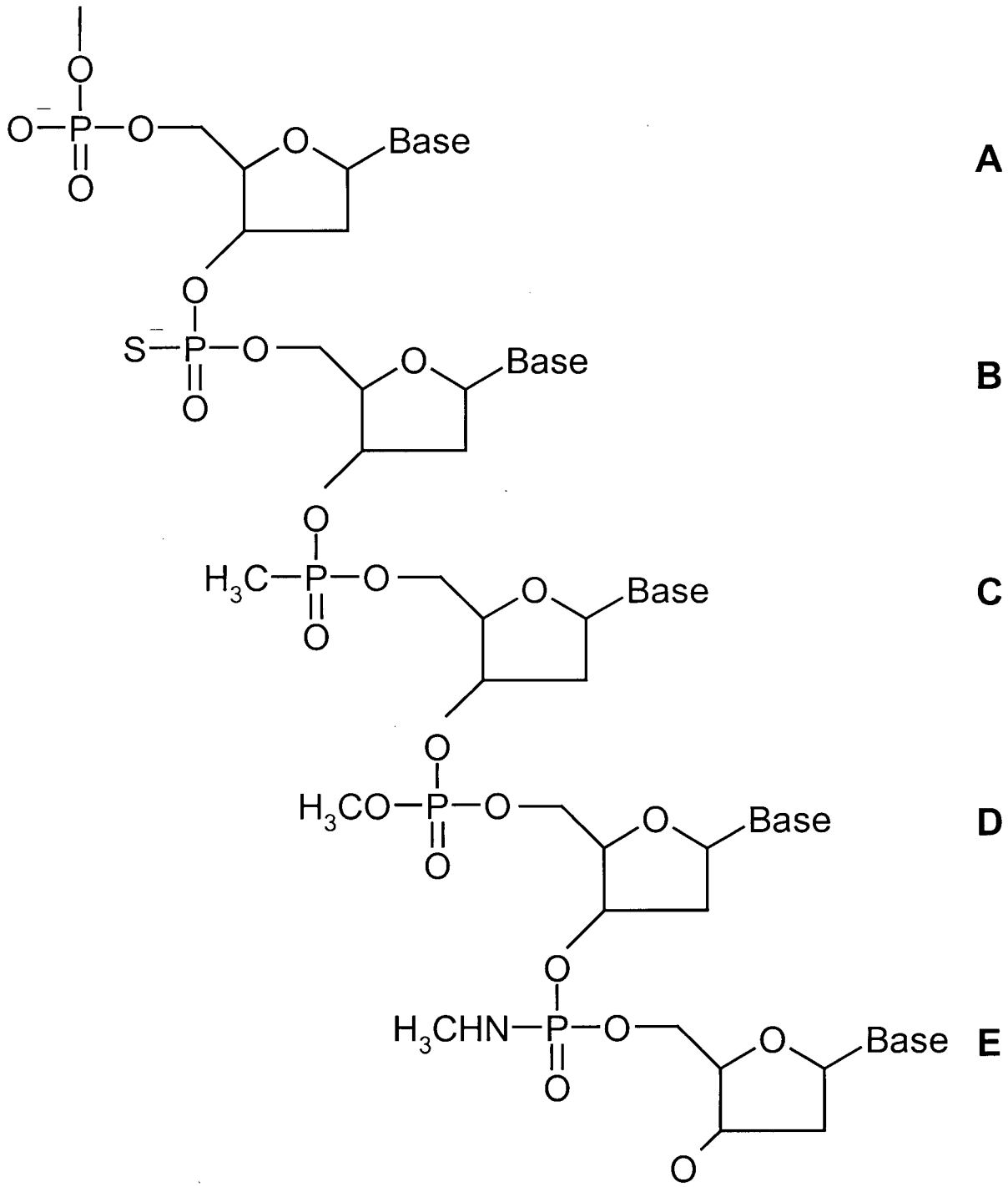

Fig 1.4 Native and common backbone modifications of antisense oligonucleotides (A) Native phosphodiester, (B) Phosphorothioate, (C) Methylphosphonate, (D) Phosphotriester, (E) Phosphoroamidate.

Phosphorothioate ODN, with which this thesis is concerned, are well absorbed from parenteral sites, display rapid distribution and prolonged (in comparison to phosphodiester ODN) elimination from tissue distribution sites, and are much more resistant to nuclease degradation (although some exonuclease chain-shortening of the antisense does occur). However, phosphorothioate ODN have other characteristics which may not be desirable, including a high capacity binding for proteins, in particular, albumin and alpha-2 macroglobulin (Brown *et al.*, 1994), are not orally available and are unable to cross the blood brain barrier (Nicklin *et al.*, 1998).

Similar to antisense oligonucleotides in both composition and specificity, are catalytic RNA molecules called ribozymes (Christoffersen & Marr, 1995; Probst, 2000). These molecules can be engineered to bind to specific RNA sequences and cleave them by one of two means; with a transesterification or a hydrolysis mechanism. They can also be designed to splice RNA and create new gene functions. Ribozymes tend to be much larger than antisense ODN due to the folding required to generate the catalytic sites. Thus, many nucleotides are in loops that do not interact with the target RNA. These molecules are being tested in several areas as therapeutics, including viral diseases such as HIV, and cancer (Christoffersen & Marr, 1995).

1.3.5.2 Mechanisms of action

There are several proposed (but largely still theoretical) mechanisms of antisense oligonucleotide action in the inhibition of protein translation (Figure 1.5). In some cases, the mechanism of action relies on the specific site within the mRNA that is targeted. For example, if an ODN is directed toward a sequence of pre-mRNA that is required for splicing, the mechanism of action would be described as being that of inhibition of the production of mature mRNA by sequence specific inhibition (Crooke, 1999). Other mechanisms of blocking the processing of pre-mRNA include the blocking of polyadenylation of the 3'- terminus or 5'-capping of pre-mRNA with methylguanosine (Crooke, 1999), which are necessary for stabilisation of the RNA, and transport into the cytoplasm (Schlingensiepen & Schlingensiepen, 1996). In a similar fashion, many ODN are targeted toward the coding region of mRNA, and are thought to sterically block the progression of the ribosome, and therefore halt translation (Schlingensiepen & Schlingensiepen, 1996). Initiation of protein translation may be blocked by antisense targeted toward the translation initiation codon, the promoter region, or a region close to either of these sites. In addition to these site specific inhibition mechanisms, both phosphodiester and phosphorothioate antisense oligonucleotides are thought to rely on the action of the ubiquitous enzyme, RNaseH. This enzyme specifically recognises a DNA-RNA hybrid, and cleaves the RNA portion of that hybrid. Sugar modified ODN such as 2'-fluoro or 2'-O-methyl, or methylphosphonate ODN are not thought to act as substrates for RNaseH activity (Crooke, 1999), additionally, RNaseH is believed to only cleave the RNA strand if five to eight contiguous base pairs of an RNA-DNA duplex form (Woolf *et al.*, 1992).

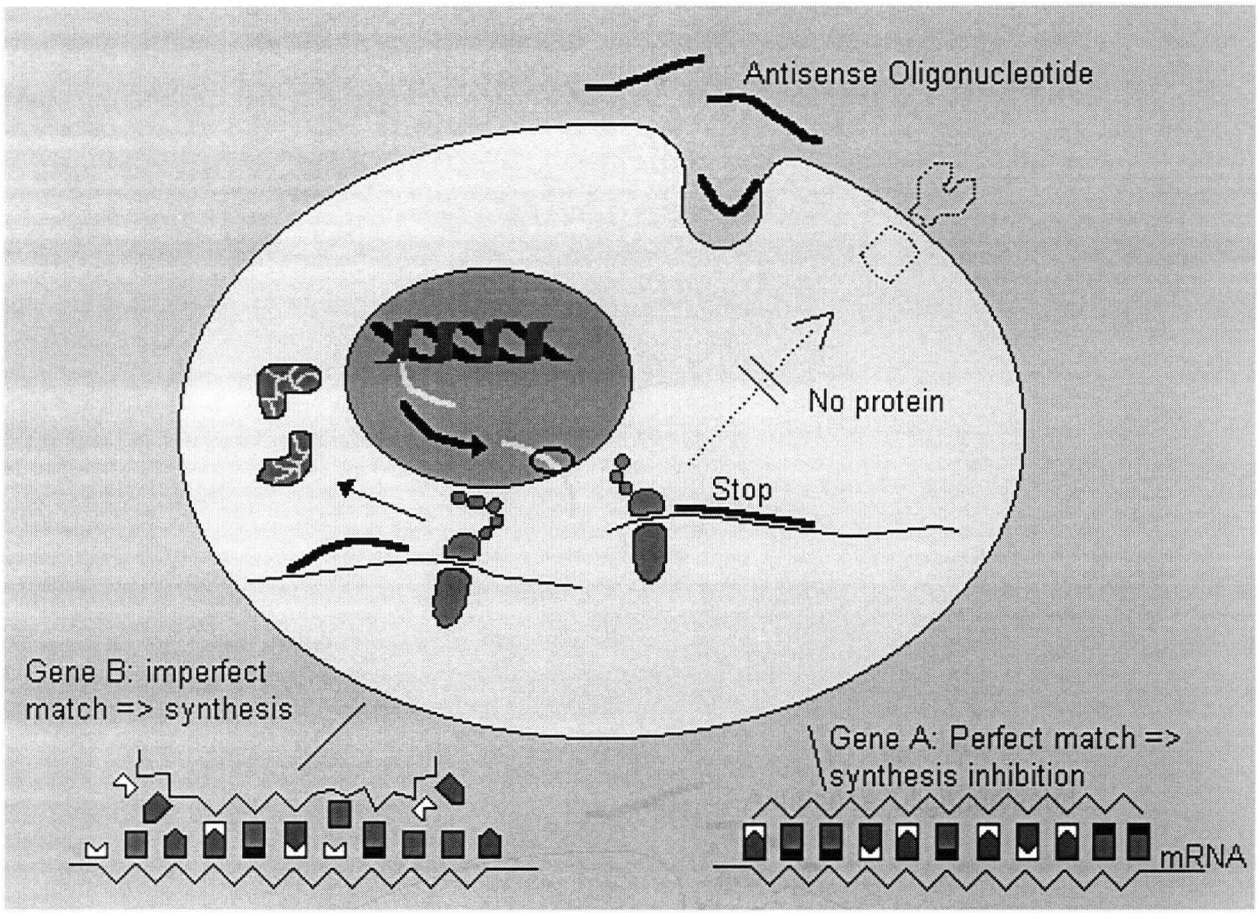

Figure 1.5 Proposed mechanisms of action of antisense oligonucleotides in cells: inhibition of processing of pre-mRNA and transport into the cytoplasm or complimentary base pairing with mature mRNA in the cytoplasm, leading to lack of protein production by steric hindrance, blockage of ribosomal machinery or RNaseH activation.

As antisense oligonucleotides are a relatively new form of therapy, there is still some disagreement in the literature as to what constitutes adequate control(s) for these agents. It has been argued that no suitable controls may be defined to conclusively demonstrate that a given biological effect results from a sequence specific ODN mechanism (Neckers & Lyer, 1998). These authors state that although appropriate controls include a sense control, a scrambled control and a mismatch control, these sequences may not contain biologically active motifs found in the antisense sequence. Further, should mismatch controls be incorporated into experiments, these sequences may not control for hybridisation specificity of the active ODN. They conclude by stating that each individual antisense sequence may be considered a unique drug, for which no adequate control exists. Other authors disagree with this viewpoint, listing controls other than altered ODN sequences, including agents such as small molecule inhibitors of protein function, monoclonal antibodies, expression of dominant negative proteins or the use of gene knockouts (Giles, 2000). The work presented in this thesis includes the use of a single antisense control sequence, specifically the scrambled control for the active anti-HER-2/neu ODN. It was felt that as an experimental end point, the definitive demonstration of lack of protein expression in the case of active, but not control, sequence antisense, should provide a measure of satisfaction that the antisense was having a sequence specific effect, an end point considered to be imperative by Stein and Krieg (1994). In addition, bearing in mind the above arguments, it was unclear as to whether additional sequence controls would strengthen the case for specificity of action. A later chapter (Chapter 6) does include the use of a monoclonal antibody against HER-2/neu as a control treatment, which, as stated above, may indeed be a more relevant control for this type of experiment.

1.3.5.3 Pharmacokinetics and Toxicology

Antisense ODN are presumed to be taken up by cells through endocytosis, and accumulate in an endosomal-lysosomal compartment. Following this stage, some of the ODN is able, through an as of yet undefined mechanism, to gain access to the cytosol and then the nucleus of the cell, in which places it is able to interact with both cytosolic and nuclear RNA species. As this thesis outlines experiments performed with phosphorothioate ODN, this section of ODN pharmacokinetics will have the same focus. Following either intravenous or subcutaneous injections or infusions, phosphorothioate ODN have a biphasic plasma half life, with a T½α (distributional phase) of a few minutes to one hour, depending on dose, and a T½β (elimination phase) of up to several hours (Juliano *et al.*, 1999). Phosphorothioate oligonucleotides circulate in plasma bound to plasma proteins that protect them from filtration (Levin, 1999). ODN are distributed widely throughout the tissues of the body (excluding the central nervous system and testes), primarily in the liver and kidney, accounting for the majority of ODN clearance (Levin, 1999), then to a lesser extent in the spleen, bone marrow and lymph nodes, then other tissues. Liver and kidney concentrations of ODN appear to saturate as the dose is increased; however this may be attenuated by prolonging the infusion time, suggesting that saturation is a function of the dose rate as well as the dose (Levin, 1999). Unlike traditional small molecule drugs, which are mainly metabolised in cytochrome P-450 dependent pathways, ODN are metabolised by nucleases. Although 3'-exonuclease removal of bases is the predominant pathway for metabolic degradation, both 5'- and 3'-exonuclease excision may occur in tissues, but endonuclease digestion is not commonly observed (for phosphorothioate ODN) (Levin, 1999). Exonuclease digestion of at least a single nucleotide of intravenously administered,

naked phosphorothioate ODN has been observed in up to 40% of the administered dose after only 5 minutes, however this does not increase substantially over time, such that after 4 hours, the predominant form of ODN is the parent compound, and not the shortened chains (Glover *et al.*, 1997). Elimination of antisense oligonucleotides is mainly via the kidneys (Juliano *et al.*, 1999).

Toxicity of antisense oligonucleotides may be sequence dependent or independent. Sequence dependent effects are for the most part, centred on sequences containing G quartets, and CpG motifs. ODN containing G quartets (4 contiguous guanosine bases) are known to bind proteins, and are able to form tetrameric structures, altering the activity of the antisense (Levin, 1999). Unmethylated CpG motifs, especially when flanked 5' by two purines and 3' by two pyrimidines, are known to be potently immunogenic, with the ability to induce B cell proliferation and release of cytokines IL-6, -12 and interferon-gamma (Krieg, 1995). Rodents are particularly sensitive to the effects of the inclusion of a CpG motif in phosphorothioate ODN. In addition to mitogenic activation of B cells, this motif induces a dose-dependent splenomegaly, lymphoid hyperplasia and diffuse multi-organ mixed mononuclear cell infiltrates. At doses above 10 mg/kg, the kidney, heart, lung, thymus and pancreas have infiltrates of monocytes, lymphocytes and fibroblasts. When plasma concentrations exceed 40 – 50 μg/ml, there is a transient activation of complement, primarily through the alternative pathway, which is thought to be mediated through the binding of ODN to Factor H, a negative regulatory factor of this pathway (Levin, 1999). At this dose, phosphorothioate ODN are able to transiently inhibit clotting times, as measured by the activated partial thrombin time. This effect is also highly transient, and

parallels the concentration of ODN in the plasma (Henry *et al.*, 1997). It is important to note however, that these toxicities are observed at doses that are substantially higher than those required to achieve a pharmacologic response, and indeed, are higher than those used in clinical trials (Levin, 1999).

The above discussion has focused primarily on the *in vivo* toxicities of ODN, in which carrier systems are not typically utilised. *In vitro* applications however, require the use of helper lipids to assist the ODN in entering the cultured cells. The lipid systems most commonly used for this purpose consist of a cationic lipid that is able to bind to negatively charged ODN, and a helper lipid which aids in binding of the lipid/ODN complex to the cell surface, and mediates internalisation of the complex. This thesis explores the use of carrier systems in *in vivo* applications to determine if the encapsulation of ODN into liposomes will be beneficial in increasing the exposure of tumour tissue to the ODN, and also to increase internalisation of that ODN once the target site is achieved.

1.3.5.4 ODNs Targeting HER-2/neu

As HER-2/neu presents such an attractive target for anti-cancer therapeutic strategies, it is not surprising that several groups have developed ODN targeting HER-2/neu mRNA. Published references to ODN mediated downregulation of HER-2/neu appear in the literature as early as 1994 (Colomer *et al.*), in which up to a 60% reduction in HER-2/neu protein levels was achieved in an *in vitro* setting with several breast cancer cell lines. Further *in vitro* experiments with the human breast carcinoma cell lines SK-BR-3 (Vaughn *et al.*, 1995), BT474 and T47D (Liu & Pogo, 1996; Roh *et al.*, 1998) confirmed these early

results, using antisense targeting different areas of the HER-2/neu mRNA, and achieving as high as 90% protein inhibition as well as growth inhibition and accumulation of cells in the G1 phase of the cell cycle.

More recently, HER-2/neu targeting antisense has been taken into *in vivo* models, with Uedo *et al.* (1999) demonstrating an inhibition of carcinogenesis in a rat model following treatment with anti-HER-2/neu ODN. These authors surmised that the inhibition was due to the combination of decreased cell proliferation and increased apoptosis resulting from ODN treatment. Roh et al (1999; 2000(a); 2000(b)) have performed extensive work with HER-2/neu targeted ODN, including *in vivo* studies with human breast cancer xenografts in nude mice. This work supports earlier experiments in that cells in *in vitro* studies are arrested in G0/G1 and appear to undergo apoptosis, and importantly, *in vivo* work showed synergism between the ODN treatment and cytotoxic (doxorubicin) therapy. They conclude that by downregulating the HER-2/neu protein, the tumour cells are rendered more sensitive to the actions of cytotoxic agents, therefore making anti-HER-2/neu targeted ODN a potentially powerful therapeutic. These studies were all performed with xenograft models which had a high level of HER-2/neu overexpression, and indeed, Liu and Pogo (1996) suggest that anti-HER-2/neu targeted ODN should only be effective in breast cancer cells with elevated HER-2/neu.

The work presented in this thesis expands upon the work by the above researchers by the inclusion of xenograft models with low expression of HER-2/neu. I will demonstrate the ability of a HER-2/neu targeted ODN to be efficacious in such a model, thereby

demonstrating the potential for a beneficial therapeutic effect in the absence of elevated expression levels.

1.4 Liposomes

Liposomes are phospholipid vesicles with bilayer membranes which, depending upon the lipid composition, are relatively impermeable to both amphipathic and highly water soluble molecules at physiological temperature. When liposomes were first explored as potential delivery vehicles, they were formed from synthetic and naturally occurring phospholipids with sufficient cholesterol to stabilise the structures *in vivo*. In general, these formulations were eliminated relatively quickly by the cells of the mononuclear phagocyte system (MPS). Later modifications to liposomes included the addition of a moiety that conferred steric stabilisation such as the ganglioside GM1 or the commonly used polyethylene glycol (PEG)-conjugated lipids. Liposomes with these additions to the formulation are eliminated less rapidly by the MPS due to the exclusion of macromolecules such as opsonins from the peri-liposomal space, and have different bio-distribution patterns. It has more recently been suggested that the primary effect of steric stabilisation is due to elimination of surface-surface interaction that can lead to liposome aggregation or liposome-cell interactions (Johnstone *et al.*, 2001).

This section of the introduction will review liposomes as being a clinically viable, well characterised delivery system that has been used extensively to deliver ODN in *in vitro* settings, as well as preliminary *in vivo* research.

1.4.1 Liposomes as Drug Delivery Vehicles

As liposomes have an internal aqueous space, it is possible to entrap therapeutic agents such as small molecule drugs or diagnostic dyes, within them. The encapsulation of drugs in liposomes alters the pharmacokinetic and biodistribution properties of the entrapped agents (Gabizon *et al.*, 1994; Gabizon *et al.*, 1998). The extent and character of these alterations depends on the lipids used in the liposome formulation (acyl chain length and charge), as well as the overall size and lipid dose of the administered liposomes (reviewed in Tardi *et al.*, 1996). For example, the inclusion of cholesterol in liposome formulations has been shown to prevent lipoprotein-induced vesicle destabilisation and concomitant release of the encapsulated drug (Scherphof *et al.*, 1978; Kirby *et al.*, 1980). This leads to increased circulation longevity of that drug and enhanced bioavailability. When negatively charged phospholipids, such as phosphatidylserine, phosphatidic acid or phosphatidylglycerol are included in the formulation, liposomes are rapidly cleared from the circulation by the cells of the mononuclear phagocyte system (Schroit *et al.*, 1985); however, rapidly cleared formulations may still affect drug parameters such as, in the case of doxorubicin, reduced cardiotoxicity (Rahman *et al.*, 1990). There is a need to balance the enhanced circulation longevity with the ability to make the drug bioavailable, or, to design liposomes such that once they have reached the target site, whether it be a site of inflammation, tumour or other, the drug is released, resulting in a local depot of drug where required.

Liposomes represent ideal drug delivery systems for tumours as the microvasculature in tumours is typically discontinuous, having pore sizes varying between 100 to 780 nm

(Hobbs *et al.*, 1998). This allows vesicles the size of liposomes (approximately 100 – 150 nm) to extravasate within the tumour tissue to provide locally concentrated drug delivery, a primary role of liposomal formulation. Accumulation within the tumour tissue has the additional benefit of decreased drug accumulation in non-target tissues, and hence lower drug associated toxicities.

Drugs may be loaded into liposomes in several manners. A common, but relatively inefficient loading technique is that of passive loading. In this technique, lipids which have been dried down to a film are hydrated with a buffer solution containing the drug that is to be encapsulated. This will typically result in up to 20% of the drug in solution being encapsulated in the liposomal interior, but may also be substantially lower than this. Alternative mechanisms of drug loading have therefore been devised (refer to sections 1.4.2.1 and 1.4.2.2 for examples). Once loaded, drugs may partition into the aqueous interior of the liposome if they are hydrophilic, or may be sequestered within the lipid bilayer, in the case of hydrophobic drugs (Fig. 1.6).

1.4.2 Liposomes as Drug Delivery Vehicles in the Treatment of Solid Tumours, in Particular, Breast Cancer

The anthracycline antibiotic doxorubicin is one of the most important and widely used anti-tumour agents. Produced by the fungus *Streptomyces peucetius* var. caesius, doxorubicin has broad activity against a range of human neoplasms, including leukemias and lymphomas, breast, ovarian, stomach, bladder and bronchogenic carcinomas, and soft tissue sarcomas.

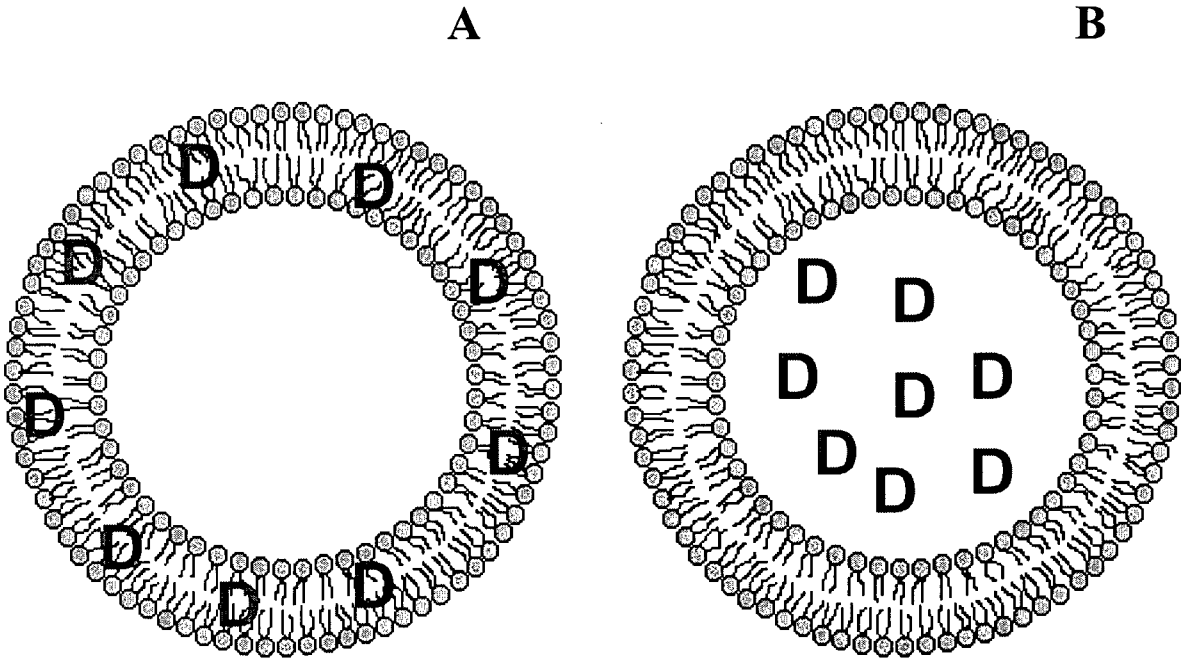

Fig 1.6 Partitioning of drugs (D) into the lipid bilayer or the central aqueous core of liposomes. (A) Hydrophobic drugs partition into the lipid bilayer, (B) Hydrophilic drugs sequester in the central aqueous core.

Currently, anthracycline containing combination therapy is the standard of care for treatment of breast cancer, although there is much excitement in the areas of the taxanes and monoclonal antibodies (Lebwohl & Canetta, 1999). These are attractive agents for combination therapy due to minimal overlapping toxicities with anthracyclines. In clinical trials of advanced breast cancer, the objective response rates for conventional doxorubicin treatment are approximately 42% and 30% in untreated and previously treated patients, respectively (Henderson & Canellos, 1980).

The clinical activity of doxorubicin, as with other anthracyclines, is limited by cardiomyopathy. This, in turn, is related to the total cumulative dose of the drug and is principally due to free radical damage to myocytes (Ferrans *et al.*, 1997; Muller *et al.*, 1997). Damage occurs due to build-up of the anthracycline within the heart muscle, DNA intercalation and free radical formation, which result in damage to myocardial cells. Pathologically, this is caused by lysis of both the thick and thin myofilaments leading to myofibrillar loss, and dilatation of the sarcoplasmic reticulum, resulting in vacuolisation of myocytes (Ferrans *et al.*, 1997).

The typical acute dose limiting toxicity for doxorubicin is that of a high incidence of myelosuppression, typically leukopenia and thrombocytopenia. This is usually a transient phenomenon, with the leukocyte nadir observed by 10 to 14 days post treatment, and recovery to normal cellularity by 21 days (Cancer Care Handbook, 1999). In severe cases, this may lead to neutropenic fever and sepsis, requiring hospitalisation. Mucositis, including both stomatitis and pharyngitis which may lead to ulceration has been associated

with continuous infusion of doxorubicin.

An almost universal occurrence of alopecia accompanies treatment with conventional doxorubicin (Dorr & Fritz, 1982). This is typically evident 3 to 4 weeks post initiation of therapy, with re-growth occurring 2 to 5 months after therapy, or in some cases, during therapy. Acute nausea and vomiting are very common, with many patients requiring the use of antiemetics to control these side effects. As well, rare doxorubicin induced toxicities include ulceration and necrosis of the colon, and neuropathy.

The primary aim of doxorubicin encapsulation in liposomes has been to decrease non-specific organ toxicity. Liposomes are able to direct the doxorubicin away from sites with tight capillary junctions such as the heart muscle, and the gastrointestinal tract. They are instead accumulated within organs rich in cells of the mononuclear phagocyte system (MPS). It should be noted however, that when liposomes contain doxorubicin the cells of the MPS are affected such that they can no longer accumulate liposomal systems. Although this "MPS toxicity" has been noted in animals, as measured by a change in liposome elimination profiles (Parr *et al.*, 1993), similar effects have not been observed in humans. Regardless MPS toxicity may be of some concern in cases with increased risk of opportunistic infections such as in AIDS patients, especially due to depletion of liver macrophages, which have been shown in animal models to take up to 2 weeks to be restored (Daemen *et al.*, 1995). Interestingly, the first formulation of doxorubicin approved for use in humans was based on its use in the treatment of Kaposi's Sarcoma, an AIDS related cancer. Liposomal formulations containing polyethylene-glycol (PEG) modified

lipids have even greater blood circulation time as a result of diminished liposome-cell interactions, however these formulations have also been shown to exhibit MPS toxicities. Regardless of whether enhanced circulation lifetime of a liposomal formulation of doxorubicin is due to subtle changes in lipid composition, inclusion of doxorubicin which affects MPS cell function, or the use of PEG-modified lipids, increased circulation longevity of the liposomes allows enhanced extravasation across the leaky endothelium of solid tumours, providing a local depot for drug release (Papahadjopoulos *et al.*, 1991).

In order for liposomal formulations of doxorubicin to present an advantage in therapy over the free agent they must be able to enhance anti-tumour activity at the maximum tolerated dose without a concurrent increase in toxicity. The increased accumulation of liposome-encapsulated doxorubicin in tumours, and decreased accumulation in sensitive non-target organs can enhance the therapeutic index of the drug, but it is argued here that the primary benefits occur as a consequence of reduced toxicity rather than enhanced therapeutic activity. It is important to recognise that such conclusions have been reached on the basis of data obtained in animal models of cancer and current Good Laboratory Practices (cGLP) toxicity studies. The real benefits of liposomal encapsulation of doxorubicin on the drug's therapeutic index will only be appreciated following extensive clinical studies. In this regard, it is notable that arguments in support of clinical use of the two approved liposomal formulations of doxorubicin are different (reviewed in Waterhouse *et al.*, 2001). Advocates of one formulation highlight improved anti-tumour activity while advocates of the other emphasise reduced toxicity.

The above discussion of liposomal doxorubicin has highlighted the fact that liposomal drug formulations are pharmaceutically viable therapeutics. Importantly, recent efforts have combined liposomal doxorubicin with trastuzumab in the treatment of patients with HER-2/neu overexpressing breast cancer. It is hoped that the combination of trastuzumab with the liposomal doxorubicin will ameliorate the cardiotoxicity noted when trastuzumab is combined with free doxorubicin, while maintaining the increased efficacy of the combination over that of doxorubicin as a single agent (Slamon *et al.*, 1998). Examination of the properties of liposomal doxorubicin assist in illustrating the benefits and limitations to lipid-based drug delivery as applied to formulations of antisense oligonucleotides, which will be explored further in the body of this thesis.

1.4.2.1 Caelyx (Doxil)

The pegylated (Stealth) liposomal doxorubicin has been in use in the U.S. since 1995 under the brand name Doxil and is marketed in Canada and Europe under the name Caelyx. This formulation is comprised of hydrogenated soya phosphatidylcholine (HSP) and cholesterol (Chol). The Doxil liposomal formulation also contains the hydrophilic polymer polyethylene glycol. The PEG moiety, linked to a distearoyl phosphatidylethanolamine (DSPE) lipid anchor, extends out from the liposomal surface to create a hydrated barrier or shield which protects the liposomes (see section 1.4 for a review of the benefits of PEG inclusion in liposomal formulations). The Doxil formulation is loaded with doxorubicin in an active fashion using a $(NH_4)_2SO_4$ gradient across the lipid bilayer to draw the drug to the liposomal interior, where it then precipitates with sulphate (Fig. 1.7). These liposomes

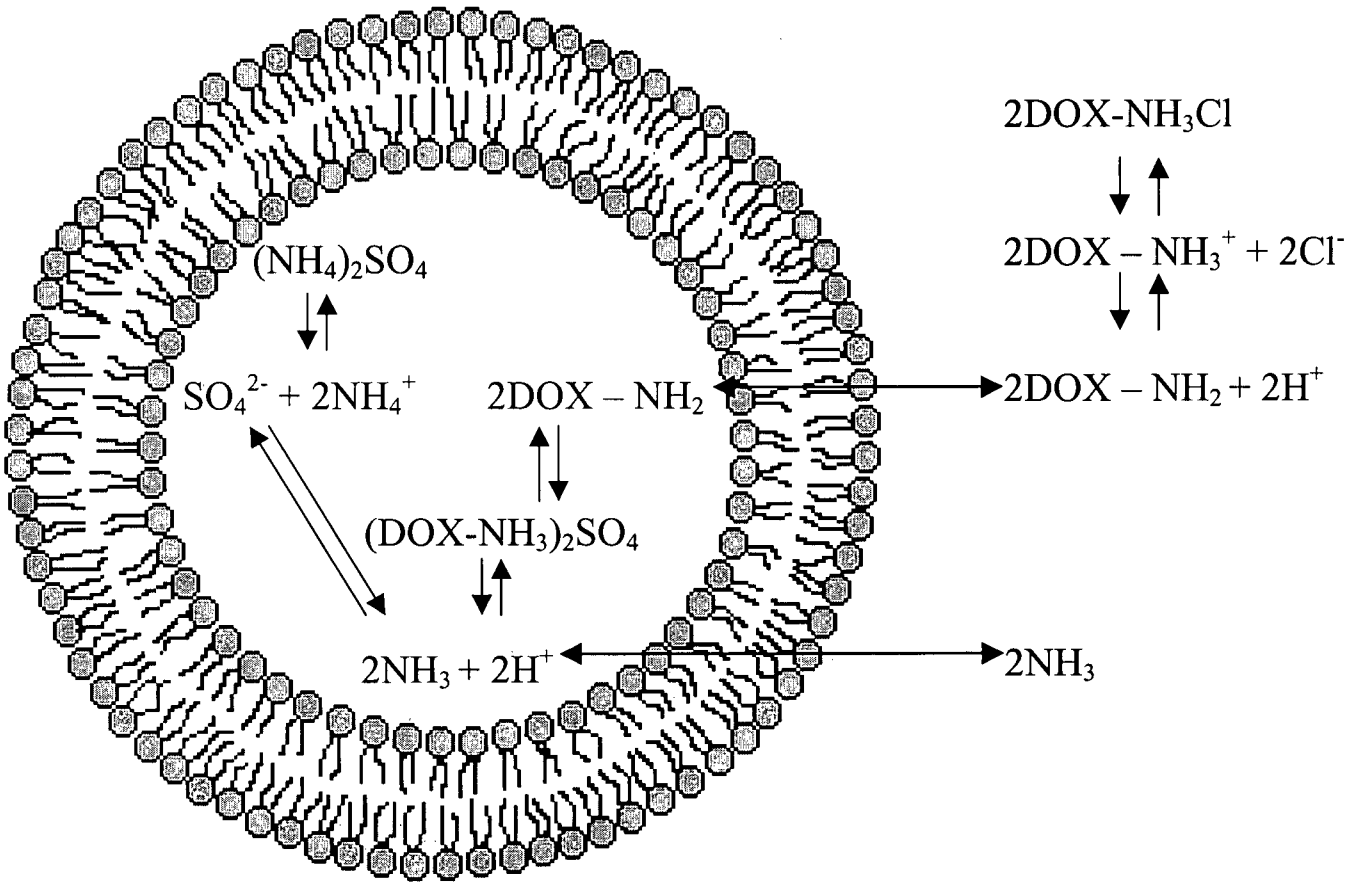

Figure 1.7 Ammonium sulphate gradient method of loading of doxorubicin into liposomes. Liposomes are formed in the desired concentration of ammonium sulphate. An ammonium sulphate gradient across the bilayer is formed by dialysis. Doxorubicin hydrochloride is added to the liposomal dispersion, crosses into the interior of the liposome, where it is trapped in a gel-like precipitate form.

have an altered distribution pattern as compared to liposomes without PEG modified lipids. They have a tendency to exhibit decreased accumulation in the liver and spleen and a reduced plasma elimination rate.

1.4.2.2 Myocet

The second liposomal doxorubicin formulation is comprised of egg phosphatidylcholine (EPC) and cholesterol (Myocet, given community marketing authorisation from the European Commission in August, 2000 for the treatment of metastatic breast cancer). In pre-clinical and phase I studies, Myocet was shown to have a circulation half life of approximately 3 times that of free doxorubicin (Cowens *et al.*, 1993), although these data were derived from a large range of α plasma elimination half lives. In addition, the encapsulation of doxorubicin in liposomes was associated with decreased toxicity of the drug, notably decreased cardiomyopathy and gastrological toxicity (Cowens *et al.*, 1989; Kanter *et al.*, 1993). The Myocet formulation also employs an active entrapment process for doxorubicin (Fig. 1.8): liposomes are prepared in an acidic buffer (pH 4.0 300 mM citrate), then sodium carbonate is added to increase the pH outside the liposomes to approximately 7.3. When these liposomes are combined with doxorubicin and heated briefly, the doxorubicin crosses the lipid bilayer and becomes protonated in the liposome interior. Once doxorubicin becomes protonated it has difficulty crossing the lipid bilayer which results in trapping efficiencies of over 99% (Mayer *et al.*, 1986).

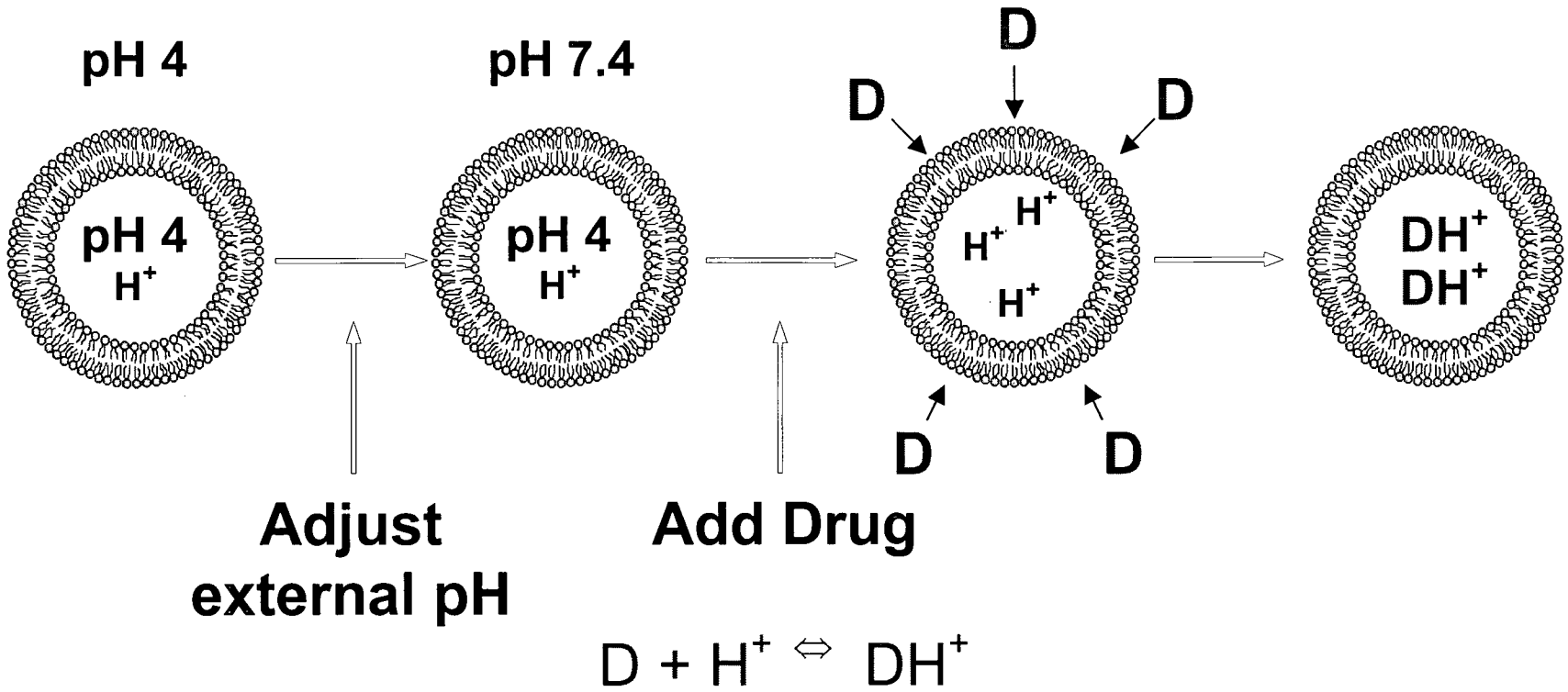

Figure 1.8 pH gradient method of loading drugs (D) into liposomes. Liposomes are formed in acidic buffer, then the external buffer is adjusted/exchanged to neutral pH, thereby creating a pH gradient across the bilayer. Drugs cross through the bilayer in response to the gradient, become protonated in the liposomal interior, and are subsequently trapped within the liposomes.

1.4.3 Liposomal Targeting

Liposomal targeting refers to a means of increasing the amount of drug delivered to a specific target site. There are several possible approaches that can be considered if the objective is to target liposomes to tumours. These have included the attachment of small molecules, sugar molecules, serum proteins, and antibodies or antibody fragments (Drummond *et al.*, 1999). These moieties are either conjugated to the lipids prior to formation of the liposomes, or may be added to pre-formed liposomes in order to target the liposomes and encapsulated drugs to internalising cellular receptors.

For a certain conjugate to be valuable as a targeting agent, several parameters must be met. These include: i) the targeting ligand must be relatively specific to cancer (or other pathology) cells, ii) the epitope bound should result in internalisation of the liposome, iii) the incorporation of targeting molecules should not increase clearance of the liposomes by tissues other than the tumour (i.e., the MPS)(reviewed in Drummond *et al.*, 1999).

An example of a targeting approach which meets many of these parameters involves use of HER-2/neu as a target. Anti-HER-2/neu immunoliposomes have been constructed by conjugation of the Fab' fragments of a humanised monoclonal anti-HER-2/neu antibody to PEG-DSPE. These studies have demonstrated that doxorubicin loaded into liposomes with the conjugated Fab' fragments was delivered to an intracellular compartment within the tumour to a much greater extent than liposomes with no conjugate (Park *et al.*, 1997). *In vitro* studies with these liposomes ascertained the total liposome uptake to be approximately 23,000 liposomes per tumour cell with the conjugated Fab' fragment, as

opposed to virtually undetectable uptake levels of liposomes with no targeting moiety (Kirpotin *et al.*, 1997). When these liposomes were loaded with doxorubicin, the therapeutic index of that drug was significantly increased in xenograft models of human breast cancer, both by increasing its anti-tumour efficacy and by reducing the systemic host toxicity (Park *et al.*, 1997).

1.4.3.1 Liposomal Oligonucleotides

As discussed in section 1.3.5.1, antisense oligonucleotides are sensitive to the degradative actions of nucleases. This is true more so for unmodified phosphodiester ODN, however exonuclease digestion does occur with phosphorothioate ODN as well. In order to offset this degradation, as well as to increase cellular uptake and intracellular delivery, several groups have designed liposomal carriers for ODN (Tari *et al.*, 1994; Puyal *et al.*, 1995; Meyer *et al.*, 1996; Tari *et al.*, 1996; Semple *et al.*, 2000; Stuart *et al.*, 2000). For the delivery of ODN, liposomes may be formulated such that the ODN are encapsulated in the aqueous interior, or bound to cationic lipids on the liposomal surface. The use of cationic lipids enables ODN to be complexed with pre-formed liposomes, rather than having to be encapsulated within them. This may be disadvantageous however, as the large size and positively charged nature of these complexes result in rapid elimination. Maurer *et al.*, (1999) have suggested that the ideal liposomal carriers for gene based drugs are small (< 100 nm), long circulating vesicles that will accumulate at sites of tumours and inflammation. These liposomes should also be serum stable, and not be recognised by the cells of the MPS. This last may be accomplished by the inclusion of PEG-grafted lipids in the liposomal formulation, as has been demonstrated by Meyer *et al.*, (1998), as stabilising

the liposomes and allowing similar amounts of encapsulation of ODN as non-PEG-containing liposomes.

The principle advantages of liposomal encapsulation of ODN are the extended circulation longevity and protection from the actions of nucleases both in the serum and intracellularly. It has been demonstrated (Stuart *et al.*, 2000), that encapsulation of ODN led to a serum half life of approximately 12 hours, as compared to less than 10% of injected dose of free ODN being detectable in plasma 30 minutes post-injection. Nuclease protection assays have also been employed to demonstrate enhanced stability of ODN when formulated into liposomes (Semple *et al.*, 2000).

1.5 Thesis Objectives

The objectives of the work presented in the body of this thesis were threefold. Firstly, to develop and characterise a xenograft model of aggressive human breast disease. Secondly, to use that model in the testing of novel antisense oligonucleotide sequences, designed to target specific molecular defects associated with aggressive breast disease, namely HER-2/neu and vascular endothelial growth factor. Finally, these oligonucleotide therapeutics were combined with conventional cytotoxic agents with the view that the combination may prove to have additive or synergistic effects, thus leading to more efficacious treatment than with either agent alone.

1.5.1 Hypotheses

There are several basic hypotheses which culminate in the overall objectives of the thesis. These are listed below:

- The encapsulation of antisense oligonucleotides into liposomal carrier formulations will lead to enhanced circulation longevity of the antisense molecules.
- The encapsulation of antisense oligonucleotides into liposomal carrier formulations will lead to enhanced tumour site delivery of the antisense molecules.
- The enhanced circulation longevity and tumour site delivery of the encapsulated antisense oligonucleotides will lead to more efficacious treatment of a model of aggressive human breast disease.
- The combination of conventional cytotoxic therapies (e.g., doxorubicin) with encapsulated antisense oligonucleotides will lead to additive or synergistic effects, such that greater therapeutic advantage will be seen than with either agent alone.

1.5.2 Summary of Novel Research and Impact in the Field of Breast Cancer Research

This thesis will describe the development and characterisation of the MDA435/LCC6 model as one which is representative of aggressive human breast disease, and is also versatile in the sense that it may be grown as a solid or as an ascitic tumour in immunocompromised mice (discussed in detail in chapter 4). This will greatly aid further research in the area of therapeutics for the treatment of aggressive breast disease, as it allows both rapid screening of new agents, and assessment of pharmacodynamic and

efficacy characteristics.

Importantly, the work described herein details the pharmacodynamic properties of both free and liposome-encapsulated antisense in the MDA435/LCC6 model, setting the groundwork for future research with this relatively novel and powerful therapeutic tool. Also, the preliminary characterisation of the molecular profile of the model allows for a better understanding of the effects of various therapeutics, such that we may be better able to intelligently design combination therapies in the treatment of breast cancer.

Chapter 2

MATERIALS AND METHODS

2.1 Chemicals and Oligonucleotides

Taxol (Bristol Myers Squibb Company, Montreal, QC), Doxorubicin hydrochloride (Faulding (Canada) Inc., Vaudreuil, QC), Vincristine sulphate (David Bull Laboratories, Vaudreuil, QC, Canada), and mitoxantrone hydrochloride (Lederle Laboratories Division, Cyanamid Canada, Montreal, QC), were obtained from the British Columbia Cancer Agency (Vancouver, BC, Canada).

Anti-HER-2/neu antisense oligonucleotide (H2A-ODN) and scramble control sequences (SCR-H2A-ODN) were from Hybridon Inc. (Milford, MA). H2A-ODN has been previously demonstrated to achieve downregulation of p185 *in vitro* (Vaughn *et al.*, 1996). Sequences of antisense oligonucleotides were as shown in table 2.1. H2A-ODN and VEGF-ODN targeted the 15 bases immediately upstream of the initiation codon of human HER-2/neu mRNA, and –3 to +18 of the human vascular endothelial growth factor mRNA coding region, respectively, while SCR-H2A-ODN was a scrambled control sequence for H2A-ODN, having the same base content as H2A-ODN, but in a randomly scrambled order. [^{3}H]-VEGF-ODN and [^{3}H]-H2A-ODN were obtained from TriLink Biotechnologies, Inc. (San Diego, CA). Fluorescein isothiocyanate (FITC) labelled VEGF-ODN was obtained from the Nucleic Acid and Protein Services (NAPS) Unit at the University of British Columbia. Any chemicals not otherwise noted, were from Sigma

(Oakville, ON, Canada).

Table 2.1 Sequences and backbone modifications of antisense oligonucleotides

Oligonucleotide	Sequence	Backbone
H2A-ODN	5'- GGT GCT CAC TGC GGC	Phosphorothioate
SCR-H2A-ODN	5'-GGT CGA TGC CGC GTC	Phosphorothioate
VEGF-ODN	5- AGA CAG CAG AAA GTT CAT GGT	Phosphorothioate

2.2 Cell Culture

SK-BR-3, MCF7, BT-474 human breast carcinoma cells, HL-60 human leukaemia and A431 human squamous carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). MDA-MB-435 breast carcinoma cells were from the tumour repository of the National Cancer Institute (Frederick, MD). Dr. Dihua Yu, of the University of Texas M.D. Anderson Cancer Centre generously donated HER-2/neu- CMV plasmid-transfected MDA-MB-435 (MDA-MB-435^{HER2}) cells. Expression levels of HER-2/neu were confirmed in these cells prior to use by western analysis, as described below. MDA435/LCC6 cells were a gift from Dr. Robert Clarke, Georgetown University (Washington, DC). DoHH2 human follicular lymphoma cells were donated by Dr. Brown (Genta Inc., Berkeley Height, NJ). SK-BR-3 cells were maintained in McCoy's 5A medium with 10% foetal bovine serum (FBS), MCF7, DoHH2 and HL-60 were maintained in RPMI 1640, 10% FBS and 2 mM L-glutamine. All other cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS and 2 mM L-glutamine. Cultures were sustained at 37°C and in an atmosphere of 5% CO_2, typically in 25 cm^2 or 75 cm^2 tissue culture treated plastic cell culture flasks (Becton Dickinson, Franklin Lakes, NJ). All media, Hank's Balanced Salt Solution Modified (HBSS), and

trypsin-ethylenediamine-tetraacetic acid (trypsin-EDTA) were obtained from StemCell Technologies Inc. (Vancouver, BC, Canada).

Sub-culturing of cell lines was performed as follows: media was aspirated from flasks, and cells gently washed with 2 to 5 mL of HBSS. HBSS was aspirated, and replaced with 0.5 to 1.0 mL of trypsin-EDTA. Flasks were gently rocked to ensure even coverage of trypsin-EDTA over cells, then incubated at room temperature for up to 2 minutes. Ten mL fresh BSA-containing media was added to halt the action of trypsin. The resultant detached cells were gently mixed, then resuspended in fresh media and in a new flask in a 1 in 10 dilution.

Determination of cell number was by use of a hemocytometer slide. Briefly, cells in suspension were mixed in a 1:1 (vol:vol) ratio with 0.4% trypan blue (Sigma), a dye which is not taken up by live cells. Hence, when viewed under a microscope, dead cells appear blue in colour. Live cells were counted visually and cell concentration per mL determined considering that each square of the hemocytometer with a cover slip in place, represents a total volume of 0.1 mm^3, or 10^{-4} cm^3. Since 1 cm^3 is approximately equivalent to 1 mL, the following equation was used to relate back to cell concentration:

$$\text{Cells/mL} = (\text{count per square})(\text{dilution factor})(10^4)$$

2.3 *In Vitro* Growth Characterisation

For cell growth studies, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Cells were plated at 1000 cells per well in 96 well plates in 200 μL per well of appropriate media with 10% FBS and 2 mM L-glutamine, then each day

for 7 days, a plate was assessed for cell growth. MTT was diluted 1:5 (vol:vol) in DMEM (10% FBS and 2 mM L-glutamine). Each well had 50 μL diluted MTT added and plates were incubated at 37°C for four hours. Following aspiration, 150 μl of dimethylsulphoxide (DMSO) was added to each well to solubilise the cellular generated formazan, and plates were shaken for ten minutes. Absorbance was determined at 570 nm using an MRX Microplate Reader (Dynex Technologies Inc., Chantilly, VA)

2.4 *In Vitro* Cytotoxicity

For cytotoxicity studies, cells were plated at 2500 cells per well in 96 well plates and incubated overnight at standard conditions. The following day, differing concentrations of the drugs taxol, doxorubicin or vincristine were added. Included in each plate was a row of wells with media only (no cells), and cells plus media, with no added drug, for assay standards. Plates were incubated for 3 days, following which, a MTT assay was performed as described in section 2.3 and wells assessed for cell viability at the different drug concentrations.

2.5 *In Vitro* Oligonucleotide Delivery

Delivery assays were performed to ensure the ODN were efficiently being delivered to the intracellular compartment. Approximately 1 X 10^4 MDA435/LCC6 or BT-474 cells were plated in each chamber of 8 well chamber slides, and incubated overnight in DMEM with 10% FBS and 2 mM L-glutamine. The following day, cells were treated with VEGF-ODN complexed with dioleyl-dimethylammonium chloride (DODAC)/dioleoyl

phosphatidylethanolamine (DOPE) liposomes (50:50, mol:mol)(Inex Pharmaceuticals Corp., Burnaby, BC, Canada) at an antisense concentration of 800 nM, and a positive to negative (lipid to ODN) charge ratio of 1.3. The correct amounts of lipid and ODN to be combined for this ratio were determined according to the relationship of 3.08 nmol negative charge per 1 μg DNA, and 1 nmol positive charge per nmol DOPE. Lipid and ODN were combined in serum-free DMEM at 4°C, with the addition of a trace amount of FITC labelled VEGF-ODN for visualisation. Following a 30 minute incubation at 4°C, the mixture was warmed to 37°C for 5 minutes prior to being added to cells. Each well had a total of 200 μL ODN/liposome complex in media added. Slides were incubated 4 hours at 37°C and 5% CO_2, then rinsed in PBS and mounted. Slides were viewed with both phase contrast microscopy and fluorescent microscopy. Resulting images were overlaid to determine positioning of fluorescence (i.e., intra- or extra-cellular).

2.6 *In Vitro* Oligonucleotide Mediated Downregulation of HER-2/neu

MDA-MB-435, MDA-MB-435^{HER2}, BT-474 or SK-BR-3 cells were plated at 3 X 10^5 to 1 X 10^6 cells per 25 cm^2 tissue culture flask and allowed to adhere overnight. Antisense (H2A-ODN or SCR-H2A-ODN) was complexed with DODAC/DOPE liposomes at a positive to negative charge ratio of 1.3 to 1.0, using the ratio of 3.08 nmol negative charge per μg oligonucleotide as described in section 2.5, to determine correct mixtures. For complex formation, antisense and liposomes were dissolved separately in serum free media, then combined in the appropriate amounts. Solutions were mixed then incubated on ice for 30 minutes, and warmed to 37°C for 5 minutes prior to addition to cells. Media was

aspirated and replaced with H2A-ODN- or SCR-H2A-ODN-liposome complexes at an antisense concentration of 0.35 μM. Cells were incubated 24 hours, washed with HBSS, then incubated with media containing 10% FBS and 2 mM L-glutamine for a further 72 hours at 37°C and 5% CO_2. Determination of p185 levels was by Western analysis or flow cytometry.

2.6.1 Western Blotting to Assess HER-2/neu Protein Levels

For determination of HER-2/neu levels by western blot, cell lysates were prepared from cells grown in culture. For the lysate generation, all steps were performed on ice. Lysis buffer (1 mL per 25 cm^2 flask) containing 150 mM NaCl, 0.1 mM sodium azide (Fisher Scientific, Fairlawn, NJ), 0.1% sodium dodecyl sulphate (SDS)(Bio-Rad), 1% Nonidet P-40 (NP-40) and 0.5% sodium deoxycholate, with Boehringer Manheim complete mini protease inhibitor cocktail tablets (1 tablet per 10 mL lysis buffer)(BH Canada, Laval, QC, Canada) was added to flasks. After a twenty-minute incubation, flasks were gently scraped with a cell scraper and lysates transferred into microfuge tubes. Lysates were spun down at 10,000 rpm for 10 minutes. Supernatants were collected and stored at minus 20°C until needed.

Protein concentration was determined by the Bio-Rad-P protein assay as per manufacturer's instructions. Equivalent amounts of protein were diluted 1:1 in 2X loading buffer (0.25 M tris, pH 6.8, 10% glycerol, 10% β-mercaptoethanol, 0.2% bromophenol blue). Samples were heated in a 95°C water bath for 5 minutes, then loaded on 7.5 %

polyacrylamide gel. Acrylamide and methylenebisacrylamide, prepared in a 28:2 weight ratio in dH_2O, were from GibcoBRL (Grand Island, NY). Gels were prepared as follows. For each mini-gel, 1.5 mL 30% acrylamide, 1.5 mL resolving gel buffer (90.8 g tris, 0.4% SDS to final volume of 500 mL in dH_2O) and 3.0 mL dH_2O were combined. To this, 10 μL 10% ammonium persulphate and 2 μL N,N,N',N'-Tetramethylethylenediamine (TEMED) were added to initiate polymerisation of the acrylamide. This mixture was pipetted to within approximately 3 cm of the top of the glass plates of the mini-gel apparatus, overlaid with water and allowed to set for a minimum of 30 minutes. Once the gel was polymerised, the water was removed and replaced with the stacking gel. Solutions required were as follows: stacking buffer, 30.3 g tris, 0.4% SDS to final volume of 500 mL; stacking gel mix, 25 mL stacking buffer, 16 mL 30% acrylamide, 59 mL dH_2O; stacking gel, 5 ml stacking gel mix, 10 μl 10% APS, 3 μL TEMED. The stacking gel was pipetted over the polymerised resolving gel, a comb inserted, and allowed to polymerise for a minimum of 30 minutes. Samples were pipetted into the wells created by the comb. A full range Rainbow™ recombinant protein molecular weight marker (Amersham Canada Limited, Oakville, ON, Canada) was run along with samples. Gels were run in buffer consisting of 30 g tris, 144 g glycine, 10 g SDS per 1 litre dH_2O. Voltage was set to 86V, and gels were typically run for about 70 minutes. Proteins were transferred overnight at 4°C and 22 V, onto Immobilon™-P (Millipore, Bedford, MA) polyvinlyidene fluoride (PVDF) membranes. For the western blot, polyclonal rabbit anti-c-erbB-2 (HER-2/neu)(Zymed Laboratories, Inc., South San Francisco, CA) and mouse anti-actin antibodies in phosphate buffered saline (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 per 1 litre in dH_2O)(PBS) with 1% (w/v) casein, were incubated with the

membrane for 2 hours. After 5 three-minute washes in PBS, the membrane was incubated with anti-mouse and anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Promega, Madison, WI) in PBS with 1% casein for one hour. Membrane was again washed five times in PBS. Detection was with ECL™ western blotting detection reagents (Amersham Canada Limited), as per manufacturer's instructions. Membranes were placed next to BioMax Light Film (Kodak), and film exposed for 10 – 40 seconds. Following development of films, protein was quantified using densitometric analysis (MolQuant software on Personal Densitometer SI (Amersham Pharmacia Biotech, Inc., Piscataway, NJ)).

2.6.2 Flow Cytometric Analysis

HER-2/neu and bcl-2 levels were assessed in parental MDA-MB-435 and MDA435/LCC6 cells by flow cytometry. Cells in culture were trypsinised briefly to release them from the flask, then washed in PBS with 0.1% bovine serum albumin (PBSB). Cells were resuspended at a concentration of 10^7 cells per millilitre PBSB with 10% human serum, and incubated on ice for 10 minutes. Following the blocking step, cells were divided into aliquots of 5 X 10^5 cells per tube for staining. Controls were left unstained, stained with secondary antibody only (goat anti-mouse IgG FITC, Becton Dickinson, San Jose, CA), or stained with mouse IgG1 isotype control (Serotec, Raleigh, NC). The primary antibody was TAB250, mouse anti-human HER-2/neu (Zymed), which recognises an epitope on the extracellular portion of the protein. Cells were incubated with primary antibody for 30 minutes on ice, washed twice with PBSB, and then incubated 45 minutes with secondary antibody. Following staining, cells were washed three times with PBSB and resuspended

in 300 µl PBSB with 0.3 µg/ml propidium iodide (PI). Alternatively, a single step staining method was utilised, using an anti-HER-2/neu FITC antibody (Becton Dickinson, Franklin Lakes, NJ). Following staining, cells were washed three times with PBSB and resuspended in 300 µl PBSB with 0.3 µg/ml PI. Cells to be stained for bcl-2 expression were fixed in formaldehyde (Polysciences, Warrington, PA), permeabilised in 1% Tween-20, and incubated with blocking reagent (500 µg/ml RNAseA, 20% human serum in PBST with 0.5% Tween-20) prior to incubation with FITC-conjugated anti-bcl-2 antibody (Dako, Mississauga, ON), with the addition of a positive, DoHH2 (Kluin-Nelemans *et al.*, 1991), and negative, HL-60 control cell lines for comparison. Analysis was on the Epics® Elite ESP flow cytometer (Beckman-Coulter, Miami, FL).

2.7 *In Vitro* Oligonucleotide Mediated Downregulation of VEGF

On day 1, 1 X 10^5 MDA435/LCC6 cells were plated in each well of a 12 well tissue culture treated plate. Cells were incubated overnight in DMEM with 10% FBS and 2 mM L-glutamine at 37°C and 5% CO_2. The following day, cells were treated with VEGF-ODN complexed with DODAC/DOPE liposomes (complexes prepared as described in section 2.6) in final ODN concentrations of 0.25 µM, 0.5 µM and 0.75 µM. Controls were FBS containing media and cells, serum-free media and cells, lipid-only treatment (no ODN). Cells were incubated with the complexes for 24 hours, then media was removed, wells washed, and replaced with serum-containing DMEM. Cells were then allowed to recover for 24 hours prior to assessment of VEGF levels in the supernatants.

2.7.1 Determination of VEGF Levels

A Quantikine® human VEGF enzyme linked immunosorbent assay (ELISA) kit from R&D Systems (Minneapolis, MMN) was used as per manufacturer's instructions to quantify VEGF levels in cell culture supernatants, ascites fluid and serum samples. Briefly, standards and samples were pipetted into microplate wells that had bound anti-VEGF antibody. Wells were washed then an enzyme-linked VEGF specific antibody added to wells. Following further washing, a substrate solution was added to the wells, and colour developed in proportion to the amount of VEGF bound in the initial step. Colour development was stopped, and level determined by spectrophotometry at 450 nm. To assess levels of VEGF in cells grown in culture, cells were plated in 25 cm^2 tissue culture flasks. When cells were confluent, supernatant was collected and assayed. Serum samples were used in the ELISA without dilution, and ascites fluid was diluted up to 100-fold in PBS, as necessary, prior to determination of VEGF levels.

2.8 Liposomal Oligonucleotide Formulation

Liposomal antisense was prepared following the method of Semple *et al.* (2000). Stock lipid solutions were prepared in 100% ethanol. distearoylphosphatidylcholine (DSPC) (Northern Lipids Inc., Vancouver, BC), cholesterol (Sigma) and DODAP (Avanti Polar Lipids, Alabaster, AL) were prepared at 20 mg/mL, while PEG_{2000} C_{14}Ceramide (PEG-C_{14}CER)(Northern Lipids Inc.) was prepared at 50 mg/mL (see Figure 2.1 for molecular structure of lipids utilised). Lipids were combined in a molar ratio of

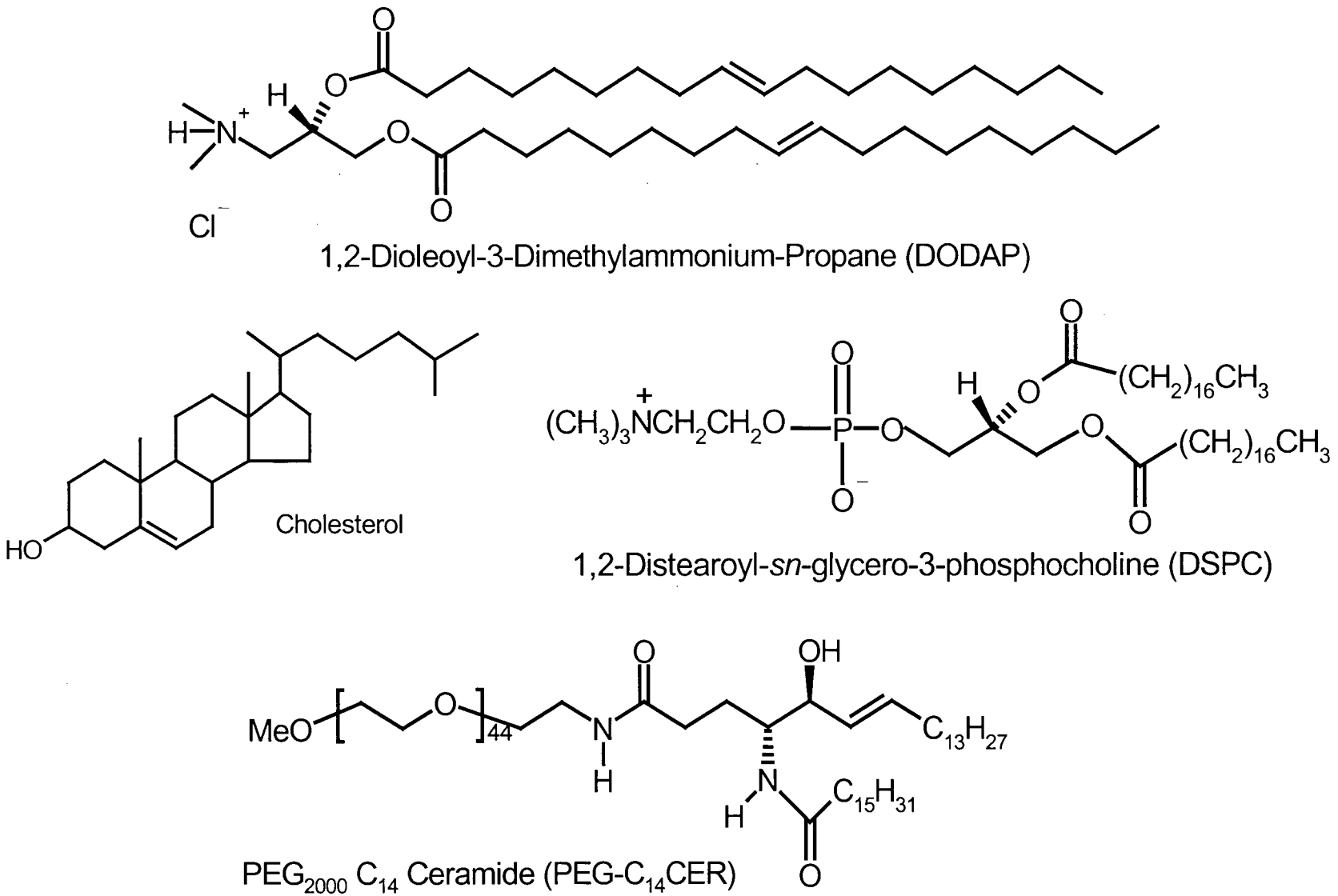

Figure 2.1 Lipids used in the formulation of liposomal antisense oligonucleotides.

2.0:2.5:4.5:1.0 (DODAP:DSPC:Chol:PEG-C_{14}-CER). As required, [^{14}C]-CHE was added to the dissolved lipids as a non-exchangeable or metabolisable marker (Pool *et al.*, 1982). Antisense was dissolved in 300 mM citrate buffer, pH 4.0 at a concentration of 3.33 mg/mL, with the addition of [^{3}H]-H2A-ODN or VEGF-ODN for quantitation as required. Antisense and lipid solutions were briefly heated to 60°C, then combined in a weight to weight ratio of 0.2 to 1.0, antisense being added drop-wise to lipids in ethanol while mixing vigorously. Mixtures were subjected to 5 freeze-thaw cycles of immersion in liquid nitrogen followed by thawing at 65°C and then extruded ten times through three stacked 100 nm pore size polycarbonate membranes (Nucleopore, Pleasanton, CA) using a thermobarrel equipped extrusion device (Northern Lipids Inc., Vancouver, BC). Following extrusion, samples were dialysed against 300 mM citrate buffer, pH 4.0 for two hours to remove ethanol, then against pH 7.5 HEPES buffered saline (HBS) for a minimum of 12 hours at 4°C. Liposomes were then passed over a DEAE agarose anion exchange column (BioRad, Mississauga, Ontario, Canada) equilibrated in HBS to remove unencapsulated antisense.

As necessary, lipid and antisense concentrations were determined by [^{14}C] and [^{3}H] counts respectively, by mixing samples with 5 mL of Pico-Fluor 40 scintillation cocktail (Packard, Groningen, The Netherlands), and counting with a Packard 1900 scintillation counter (Meriden, CT). Typical specific activities were 900 dpm/μg for antisense and 130 dpm/μg for lipid.

Encapsulation of antisense was determined using scintillation counts or by Bligh and Dyer

assay as follows. A 10 µl aliquot of liposomal antisense or water (for blank) was added to 240 µl distilled water. This was combined with 750 µl $CHCl_3$/MeOH (1.0:2.1 vol:vol) and 100 µl MeOH. The mixtures were vortexed until clear, and absorbance determined at 260 nm. Concentration was determined according to the following calculation:

Concentration = (A_{260})(Extinction co-efficient of antisense, ε)(dilution)

Where ε = 36.02 µg/OD_{260} unit for H2A-ODN

and 31.18 µg/OD_{260} unit for VEGF-ODN

2.9 Liposomal Oligonucleotide Characterisation

Prior to any *in vivo* experimentation with liposomal oligonucleotides, it was important to characterise the formulation with respect to size, encapsulation, efficiency and integrity of the encapsulated antisense. This ensured a minimum of unknown variables in any given experiment, as well as allowing for reproducibility of preparations and safety of any animals used.

2.9.1 Size

Liposomes were diluted to approximately 0.5 mg/mL in HBS, then analysed by quasi-elastic light scattering, using a Nicomp 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA), operating at 632.8 nm. The particle sizer was set at 23°C for analysis, the photopulse rate was set to between 350 and 450 kHz and the high voltage adjust was set between 650 and 850 volts. Samples were analysed until greater than 500,000 readings had been collected. The size of the liposomes was typically about 130 nm in a Gaussian

distribution pattern.

2.9.2 Antisense to Lipid Ratio

As required, phosphate and ODN assays were performed to determine concentrations of lipid and ODN in the final liposomal preparations, or, lipid and ODN concentrations were assessed by dpm counts as outlined in section 2.8. For the phosphate assay, two solutions were required, Fiske reagent (150 g $NaHSO_3$, 5 g Na_2SO_3, 2.5 g 1-amino-2-napthol-4 sulphonic acid, to 1 litre in dH_2O, filtered and stored in dark), and ammonium molybdate reagent (4.4 g ammonium molybdate, 40 mL concentrated sulphuric acid, made up to 2 litres in dH_2O). Samples, in the 25 – 200 nmol phosphate range, along with standards of 0, 50, 100 and 200 nmol phosphate, were pipetted into thick walled glass tubes and heated at 180°C with 700 μL perchloric acid for 2 hours. Tubes were brought to room temperature, then 700 μL Fiske reagent, and 7 mL ammonium molybdate reagent were added. Tubes were immersed in boiling water for 20 minutes to allow the colour reaction to occur. Absorbance of the solutions was determined at 815 nm after the samples had equilibrated to room temperature. Concentration was determined by comparison to a standard curve. Antisense concentration as determined by Bligh and Dyer assays was as described in section 2.8. ODN to lipid ratio (wt/wt) was then calculated.

2.9.3 Oligonucleotide Integrity

Liposomal ODN and free VEGF-ODN and H2A-ODN were run on 12% denaturing polyacrylamide gels. A 40% stock solution of acrylamide containing 2% bisacrylamide

was prepared in dH_2O. A 10X concentrated tris-borate-EDTA (TBE) buffer was prepared by dissolving 109 g tris base, 55 g boric acid and 7.5 g disodium EDTA in 1 litre dH_2O. The gel was then prepared by adding 15 mL of the acrylamide stock solution to 21 g of urea, 5 mL concentrated TBE buffer, 11 mL dH_2O, 300 μL 10% APS and 30 μL TEMED. The mixture was poured into a BioRad mini-gel unit, a comb inserted, and the gel allowed to polymerise for 60 minutes. Following polymerisation, the gel was transferred to an electrophoresis unit with the reservoir filled with TBE. Samples consisted of 2 μg liposomal ODN added to bromophenol blue tracking dye. Gels were electrophoresed at 250 V for approximately 30 minutes.

Following electrophoresis, the gels were transferred to glass dishes for staining. Gels were soaked in a 25% (w/v) trichloroacetic acid solution for 10 minutes, then washed in dH_2O. Following this, gels were incubated in a 0.1% (w/v) silver nitrate solution for 20 minutes. Gels were again rinsed, then developed in sodium carbonate, formaldehyde solution (3.75 g Na_2CO_3, 64 μL formaldehyde in 125 mL dH_2O). When bands were sufficiently dark, the reaction was stopped by addition of 1% (v/v) acetic acid. Gels were then photographed as required.

2.10 Liposomal Oligonucleotide Stability Over Time at 4°C, 21°C and 37°C

A single batch of liposomal VEGF-ODN was prepared as detailed in section 2.8. Lipid was labelled with [^{14}C]-CHE and ODN was labelled with [^{3}H]-VEGF-ODN for quantitation. The prepared liposomes were separated into three sterile vials, and one vial each stored at 4°C, 21°C (room temperature) or 37°C. Liposomal ODN was characterised

over a period of 22 weeks for size, encapsulation and ODN integrity. Size was analysed on the NICOMP 270 submicron particle sizer as described in section 2.9.1.

ODN to lipid ratio was performed by scintillation counts of liposomes both before and after running aliquots over DEAE Bio-Gel 100 – 200 mesh mini anion exchange columns to separate any released antisense from liposomal antisense. Encapsulation was determined according to the following equation:

$$\frac{\text{Post-column ODN concentration}}{\text{Pre-column ODN concentration}} \text{ X } 100\% = \text{Percent encapsulation}$$

Samples of liposomal ODN were run on 12% denaturing polyacrylamide gels which were then silver stained as described in section 2.9.3. Controls of free VEGF-ODN and H2A-ODN were run with samples to indicate ODN lengths.

2.11 Immunohistochemical Visualisation of VEGF, p185, bcl-2 and Cathepsin D in Solid Tumour Sections

Xenograft human breast carcinoma or epitheloid tumours grown in female SCID/Rag2m mice were removed from the animals, fixed in 4% buffered formalin for a maximum of 7 days, embedded in paraffin, and 5 μm sections on glass slides prepared (Wax-It, Aldergrove, BC).

Preliminary steps for VEGF, p185 and Cathepsin D staining were the same and were as follows. Slides were de-paraffinised in two changes of xylene, then hydrated in a 100%, 95% then 70% ethanol series. Endogenous peroxidase activity was blocked by immersing

the slides in a 0.3% (v/v) solution of H_2O_2 in methanol. Slides were then washed in dH_2O, followed by three changes of PBS. The next step was incubation of the sections with primary antibody, diluted in PBS for one hour. The primary antibody used for VEGF staining was mouse anti-human VEGF monoclonal antibody, clone 26503.111 from R&D Systems, for p185 an antibody cocktail was used as described in Paik *et al.*, (1991): a 1 in 150 dilution of polyclonal rabbit anti-c-erbB-2 in PBS as described in section 2.6.1.1, and a 1 in 30 dilution of monoclonal mouse anti-HER2 (c-erbB-2) antibody in PBS (Zymed Laboratories). For Cathepsin D, the primary antibody was a 1 in 5 dilution in PBS of mouse monoclonal anti-human Cathepsin D (Zymed Laboratories).

Blocking agents and secondary antibodies were from the Vector Laboratories Vectastain® Elite mouse and/or rabbit ABC kits (Burlingame, CA). Visualisation was with ImmunoPure® Metal Enhanced DAB Substrate kit (Pierce, Rockford, IL). Positive controls for staining were: sections of A431 tumours for VEGF, SK-BR-3 tumours for p185, and for bcl-2, follicular lymphoma cells (DoHH2 cells). Slides were counterstained with Gill's hematoxylin (2 g hematoxylin, 200 mg sodium iodate, 17.6 g aluminium sulphate, 750 mL dH_2O, 250 mL ethylene glycol, 20 mL glacial acetic acid), dehydrated in ethanol, cleared in xylene and mounted with Permount (Fisher Scientific). Sections for bcl-2 staining were sent out to the British Columbia Cancer Research Centre. Briefly, immunostaining was performed using polyclonal anti-Bcl-2 antibodies (rabbit polyclonal anti-Bcl-2; Pharmingen, San Diego, CA) after microwave antigen retrieval (Gascoyne *et al.*, 1997).

2.12 *In Vivo* Growth Characterisation of MDA-MB-435 and MDA-MB-435^{HER2} Human Breast Carcinoma Cells

Female SCID/rag2m mice (bred internally at the B.C. Cancer Agency, Joint Animal Facility) were injected sub-cutaneously (s.c.) on the back with five million MDA-MB-435 or MDA-MB-435^{HER2} cells, in a total volume of 50 µl. Tumour growth over time was assessed every two to three days with callipers. To calculate tumour size in grams, the formula, length (mm)/2000 X width^2(mm)/2000, was used. All animal studies were completed in accordance with the current guidelines of the Canadian Council of Animal Care and with the approval of the University of British Columbia animal care committee.

2.13 *In Vivo* Growth Characterisation of MDA435/LCC6 and MDA435/LCC6^{HER2} Human Breast Carcinoma Cells

MDA435/LCC6 and MDA435/LCC6^{HER2} cells were grown as either solid tumours or ascites tumours, in female SCID/Rag2m mice as detailed in sections 2.13.1 and 2. These cells were used as a rapidly growing alternative to the parental MDA-MB-435 with reproducible patterns of growth both *in vitro* and *in vivo*.

2.13.1 Solid Tumours

Mice were injected s.c. on the back with 5 X 10^6 or s.c. in the mammary fat pad with 2 X 10^6 MDA435/LCC6 or MDA435/LCC6^{HER2} cells suspended in a total volume of 50 µL HBSS per injection. Tumour growth was assessed over time as in section 2.11.

2.13.2 Ascites Tumours

Mice were injected intraperitoneally (i.p.) with 2 X 10^6 MDA435/LCC6 or MDA435/LCC6^{HER2} cells suspended in HBSS in a total volume of 200 μL per injection. On days 7, 14, 21 and 28 groups of 4 mice were terminated by CO_2 asphyxiation. Ascites fluid was collected by peritoneal lavage. For early time-points, a known volume of HBSS was injected into the peritoneum prior to lavage. Total peritoneal fluid volume was determined. Cells were counted with a hemocytometer, and both cells/mL and total peritoneal cell count determined.

2.14 Oligonucleotide Pharmacokinetics

The pharmacokinetics of free antisense ODN were compared to those of liposomal ODN for both intravenous and intraperitoneal (i.p.) injections. ODN and lipid levels, as judged by radioactivity, were determined in serum and tissue (kidney, heart, spleen, liver, lungs and muscle) in both tumour free and MDA435/LCC6 tumour bearing female SCID/Rag2m mice.

2.14.1 Plasma Elimination of anti-VEGF and anti-HER-2/neu Oligonucleotides

To determine the circulation longevity of the liposome encapsulated antisense in comparison to free antisense, groups of four female tumour free SCID/Rag2 mice were injected i.v. or i.p. with a single dose of either free or liposomal antisense at an antisense dose of 10 mg/kg, and a lipid dose ranging from 50 to 100 mg/kg. Samples were prepared

with [^{3}H]-VEGF-ODN or [^{3}H]-H2A-ODN and [^{14}C]-CHE lipid label for quantitation. Blood was collected by tail nick at 1 and 4 hours, and by cardiac puncture at 24 hours. Serum antisense and lipid levels were assessed by radiometric assay.

This experiment was repeated in tumour-bearing mice. Female SCID/Rag2m mice were injected i.p. on day 0 with 2 X 10^6 MDA435/LCC6 cells. On day 17, free or liposomal antisense was injected, and samples collected, as for tumour-free mice.

2.14.2 Tissue Distribution of anti-VEGF and anti-HER-2/neu Oligonucleotides in Tumour-Free Mice

For tissue distribution of liposomal antisense, female SCID/Rag2m mice were injected i.v. with [^{3}H]-VEGF-ODN or [^{3}H]-H2A-ODN and [^{14}C]-CHE labelled liposomal antisense, at an antisense concentration of 10 mg/kg. At 1, 2, 4, 24 and 48 hours, groups of 4 mice were sacrificed. Organs collected were liver, spleen, lung, kidney and muscle. All organs were weighed prior to processing. Tissues (with the exception of the spleen) were made into a 30% homogenate in water using a Polytron tissue homogeniser. Aliquots of 200 µL homogenate were transferred to scintillation vials. Spleens were collected directly into scintillation vials. Solvable® (500 µl) was added to each vial to dissolve tissues. Vials were incubated at 50°C overnight, then cooled prior to addition of 50µL 200 mM EDTA, 25 µl 10 M HCl and 200 µL 30% H_2O_2. This mixture was incubated at room temperature for one hour prior to addition of 5 mL scintillation cocktail. Samples were analysed for antisense and lipid by scintillation counting. This was repeated in mice bearing MDA435/LCC6 ascites tumours, with the addition of heart muscle.

2.14.3 Peritoneal Fluid Oligonucleotide Accumulation and Tumour Cell Association in MDA435/LCC6 Ascites Tumour-Bearing Mice

For peritoneal cavity distribution of liposomal antisense, female SCID/Rag2m mice were injected i.v. with [^{3}H]-VEGF-ODN or [^{3}H]-H2A-ODN and [^{14}C]-CHE labelled liposomal antisense, at an antisense concentration of 10 mg/kg. At 1, 2, 4, 24 and 48 hours, groups of 4 mice were sacrificed. Peritoneal lavages were performed by first injecting 3 ml HBSS into the peritoneal cavity, mixing, then collecting fluid and cells. Total volume collected was measured, and extrapolated back to total fluid volume in the peritoneal cavity prior to lavage. Aliquots were taken for scintillation counting for determination of lipid and ODN concentration within the peritoneal fluid.

For determination of cell-associated antisense levels, on day 17, the peritoneal fluid antisense and lipid distribution was determined as described above, as well as cell count determined by hemocytometer counting. In addition, the fluid and cells collected by peritoneal lavage were centrifuged at 1200 rpm for 10 minutes to spin down the peritoneal exudate cells. The fluid was removed, and cells washed twice in HBSS. Cells were processed as tissue samples (see section 2.14.2), and associated ODN determined by radiometric assay.

2.15 *In Vivo* Efficacy of Anti-VEGF Oligonucleotides in MDA435/LCC6 Ascites Tumour Bearing Mice

For efficacy studies, ascites tumours were established as above. Mice were treated with a total of 10 - 15 (3 - 5 per week) injections of free or liposomal H2A-ODN or VEGF-ODN at a dose of 10 mg/kg, initiated day 3 post-tumour cell inoculation. At completion of injections, peritoneal fluid and cells were harvested by peritoneal lavage. Total peritoneal fluid volume and VEGF levels were assessed, as were total cell count, both human and mouse (distinguished with the aid of an anti-Thy 1 mouse specific antibody (Becton Dickinson)), and HER-2/neu expression levels in peritoneal cells. For mice treated with VEGF-ODN, serum VEGF levels were also assessed.

2.16 *In Vivo* Efficacy of Anti-HER-2/neu Oligonucleotides in MDA-MB-435 and MDA-MB-435^{HER2} Solid Tumour Xenografts

Female SCID/Rag2m mice were injected s.c. on day zero with five million MDA-MB-435 or MDA-MB-435^{HER2} cells in a total volume of 50 µl. Mice were treated with saline; free doxorubicin i.v. on days 3, 7 and 11 post-tumour cell inoculation at a dose of 7.5 mg/kg; free ODN (H2A-ODN or SCR-H2A-ODN) i.p. three times per week for a total of 15 injections at a dose of 10 mg/kg, commencing on day 3 post-tumour cell inoculation; liposomal ODN (prepared as outlined in section 2.8) i.v. three times per week for a total of 15 injections at 10 mg/kg, commencing on day 3 post-tumour cell inoculation or a combination of free doxorubicin and liposomal ODN with routes of administration and dosages as above.

This experiment was repeated in female SCID/Rag2m mice bearing established MDA-MB-435 or MDA-MB-435^{HER2} tumours. Tumours were initiated in mice as above, and treatments were started day 54 post-tumour cell inoculation. Tumour measurements and tissue processing were the same for the two groups.

Tumour growth over time was assessed every two to three days with callipers once tumours became measurable. When tumour size exceeded one gram, animals were terminated, and tumours collected in formalin. Tumours were embedded in paraffin, and 5 μm sections prepared for immunohistochemical staining.

2.17 *In Vivo* Efficacy of Anti-HER-2/neu Oligonucleotides in MDA435/LCC6 Ascites Tumour-Bearing Mice

MDA435/LCC6 ascites tumours were initiated in female SCID/Rag2m mice by injection of 2 X10^6 cells i.p. On day 3 post tumour cell inoculation, treatment was initiated with either free or liposomal H2A-ODN or SCR-H2A-ODN. A control group of mice were treated with empty liposomes at a lipid dose equivalent to that of the liposomal ODN groups. Liposomal ODN was prepared as in section 2.8. Antisense was administered i.p. at a dose of 10 mg/kg, three times per week for a total of 15 injections. Dilutions were in saline, to a total volume of 200 μL. On day 21 post-tumour cell inoculation, mice were terminated by CO_2 asphyxiation. Peritoneal lavages were performed as detailed in section 2.14.4 for determination of total peritoneal fluid and cell count. Peritoneal tumour cells were washed in HBSS and stained with FITC labelled anti-HER2 antibody as described in section

2.6.1.2, and p185 expression levels determined by flow cytometry. Aliquots of 50,000 cells from untreated and liposomal H2A-ODN treated mice were spun onto slides (cytospin), air dried, and stained with a modified Wright-Giemsa stain (British Columbia Cancer Agency) to visually assess cell type and morphology.

2.18 Statistical Analysis

To determine significance of results, statistical analysis was performed with ANOVA one way post-hoc comparisons, using STATISTICA software. This was done both for individual time points, and for area under the curve analysis. Statistics were performed for both tumour size comparison, as well as pharmacokinetic properties of free and liposomal antisense.

Chapter 3

ANTI-HER-2/NEU ANTISENSE OLIGONUCLEOTIDES, ALONE OR IN COMBINATION WITH CONVENTIONAL CYTOTOXIC DRUGS, IN THE TREATMENT OF THE MDA-MB-435 AND MDA-MB-435^{HER2} XENOGRAFT MODELS OF HUMAN BREAST CANCER

3.1 Introduction

Therapeutic applications utilising HER-2/neu as a target are numerous and include: i) use of humanised antibody and HER-2/neu binding recombinant peptides to block intracellular signalling (Cobleigh *et al.*, 1998) (section 1.3.3.1); ii) anti- HER-2/neu hammerhead ribozyme mediated downregulation of HER-2/neu (Juhl *et al.*, 1997); iii) antisense oligonucleotide mediated suppression of HER-2/neu expression (Saxon *et al.*, 1999) (section 1.3.5.4); as well as iv) vaccine development to elicit immune responses to HER-2/neu positive cells (Cefai *et al.*, 1999). The most advanced treatment strategies targeting HER-2/neu have been those using the recombinant humanised monoclonal antibody, trastuzumab, which targets the extracellular domain of the HER-2/neu protein.

A relatively novel modality of therapy that I, and many others (Dachs *et al.*, 1997; Saxon et al., 2000) have been pursuing involves the specific downregulation of oncoproteins using antisense oligonucleotides. Those rationalising the development of ODNs have forwarded elegant mechanisms of activity; however recent investigations have led to some puzzling questions about what governs biological activity of ODNs. First, ODNs are very potent immune stimulators and therapeutic activity can be attributed to systemic immune effects

rather then to ODN/mRNA interactions (Hacker, 2000). Second, in order for ODNs to be active, delivery to the inside of a target cell must be achieved efficiently. In tissue culture, ODNs are not active unless given in association with a delivery system such as cationic liposomes (Monia *et al.*, 1996). *In vivo* however, ODN can be active when given in free form (Skorski *et al.*, 1994), raising questions about what governs biological activity of ODNs. Although studies assessing the mechanism of ODN activity *in vivo* are critically important to the further advancement of this technology, those of us who are searching for better agents for treatment of aggressive, life-threatening diseases, are primarily concerned about proving therapeutic activity in a clinical setting. The studies described in this chapter summarise preliminary research testing the therapeutic activity of a HER-2/neu targeted ODN, with an immediate goal of assessing its activity in combination with conventional cytotoxic drugs. This is described for both ODN administered in free form, and a liposomal formulation. This latter was in hope of extending the circulation longevity of the ODN, and hence increasing the tumour exposure to the drug.

Before these studies could be initiated, appropriate models had to be established for both *in vitro* and *in vivo* testing, as outlined in section 1.5. This was true for both basic delivery of the ODN to the target cells, as well as demonstrating efficacy, or, in the case of ODN, the specific downregulation of the target message, in this case, the mRNA coding for the protein p185. The models used in these studies were xenograft tumours comprised of MDA-MB-435 human breast carcinoma cells or MDA-MB-435^{HER2} cells. The latter are MDA-MB-435 cells that have been genetically engineered by transfection with a CMV-HER-2/neu plasmid to overexpress the HER-2/neu protein (Tan *et al.*, 1997). MDA-MB-

435 cells are oestrogen receptor negative, invasive and metastatic cells derived from a pleural effusion in a 31-year old woman (Cailleau *et al.*, 1974). This chapter will begin by describing the growth characteristics of these cells, with a focus on the impact of overexpression of the HER-2/neu oncogene.

The antisense molecules used in these studies were 15 base oligonucleotides, with phosphorothioate backbone modifications. The sequences of the antisense oligonucleotides are shown in table 2.1, with H2A-ODN being the active anti-HER-2/neu antisense, and SCR-H2A-ODN being a control sequence, having the same base composition, but in a different order.

Initial experiments were designed to establish the efficiency of ODN delivery to cells in an *in vitro* setting. Once this was accomplished, measurements of protein produced following *in vitro* ODN treatment were performed. As *in vivo* experiments utilised a liposomal ODN formulation, this formulation was characterised with respect to size, encapsulation and integrity of encapsulated antisense, over a period of 22 weeks, and at three different temperatures.

This chapter culminates in a description of efficacy experiments of H2A-ODN in human xenograft tumour models, both as a single agent, and in combination with the commonly used cytotoxic anti-cancer drug, doxorubicin. As doxorubicin was being used as a "gold standard" for tumour response to treatment, it was important to ensure that the two cell lines responded to this drug in similar fashion. Therefore, cytotoxicity assays were

performed in an *in vitro* setting prior to establishment of the xenograft models.

3.2 Results

3.2.1 *In Vitro* Growth of MDA-MB-435 and MDA-MB-435^{HER2} Cells

The *in vitro* growth rates of MDA-MB-435 and MDA-MB-435^{HER2} cells were directly compared in order to assess what, if any, impact, the overexpression of HER-2/neu had on the growth of these cells. As shown in Figure 3.1A, the HER-2 transfected line grew substantially more rapidly than did the non-transfected, parental MDA-MB-435, with doubling times of 1.5 and approximately 3 days, respectively.

3.2.2 *In Vivo* Growth of MDA-MB-435 and MDA-MB-435^{HER2} Cells

Having demonstrated an *in vitro* growth advantage associated with increased levels of HER-2/neu, the next experiment was the subcutaneous injection of these cells into groups of 4 female immune compromised mice, to see if the same advantage would be apparent *in vivo*. It was expected that the overexpression of HER-2/neu would confer a growth advantage *in vivo*, as overexpression of this protein in human breast cancer is associated with a more aggressive disease (Slamon *et al.*, 1987). Surprisingly, as may be seen in Figure 3.1B, this was not the pattern observed with the MDA-MB-435^{HER2} cells. Instead, the growth curves of these two solid tumours were remarkably similar up to 100 days. It may therefore be stated that increased HER-2/neu protein due to transfection of the HER-2/neu gene provided a growth advantage *in vitro* but not *in vivo*.

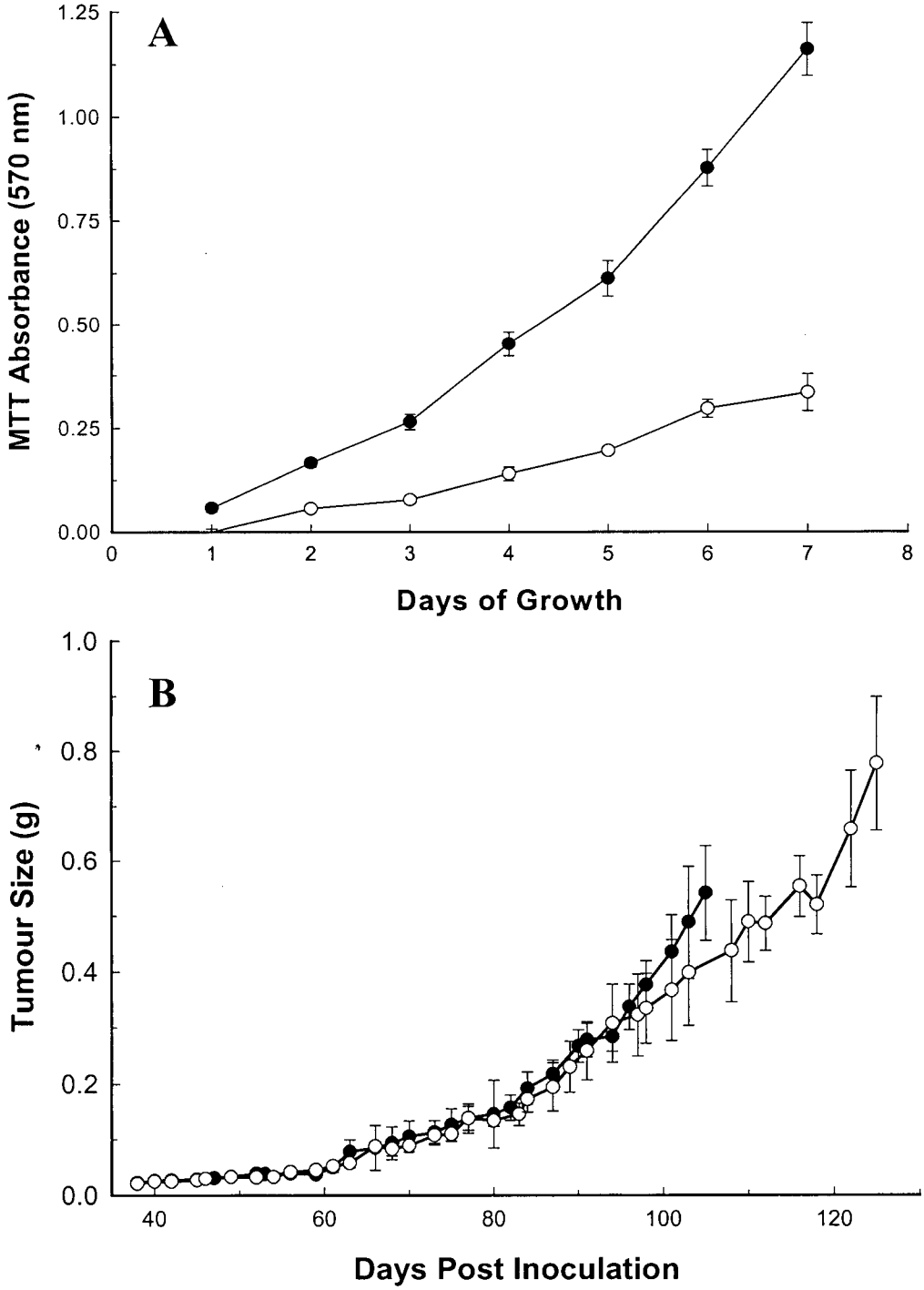

Fig 3.1 *In vitro* (A) and *In vivo* (B) Growth of untreated MDA-MB-435 and MDA-MB-435^{HER2} tumour cells. Data points in (B) represent the mean ± standard error of data from a minimum of 4 animals. *In vitro* assays were in 96 well plates, using an MTT assay as described in section 2.3. For *in vivo* growth, female SCID/Rag2m mice were inoculated s.c. day 0 with 5 X 106 tumour cells, and tumour measurements made over time. (○) MDA-MB-435; (●) MDA-MB-435^{HER2}

3.2.3 p185 Expression in Solid MDA-MB-435 and MDA-MB-435^{HER2} Tumours

The tumours from the mice that were inoculated with either the MDA-MB-435 or MDA-MB-435^{HER2} cells were removed from the mice on day 105 (MDA-MB-435^{HER2}) or day 125 (MDA-MB-4345), processed and stained immunohistochemically for p185 expression as described in section 2.10. Figure 3.2A shows the absence of positive staining for p185 in MDA-MB-435-derived tumours, as compared to the characteristic "fishnet" pattern staining seen in Figure 3.2B in the MDA-MB-435^{HER2}-derived tumour sections, indicating that these cells do indeed produce p185 in a readily detectable manner in xenograft tumour models.

3.2.4 ODN Delivery to Cells *In Vitro*

Prior to initiating experiments to test the ability of the HER-2/neu targeted ODN to downregulate protein production *in vitro*, it was useful to demonstrate that the delivery system used (DODAC/DOPE/ODN complexes) was capable of mediating intracellular delivery of the ODN. This was accomplished by combining a small amount of FITC labelled antisense with the complexes, then visualising the cells under fluorescent light following incubation. Figure 3.3A shows a phase contrast microscopic photograph of human breast carcinoma cells viewed under a 10X objective. Figure 3.3B shows the same field of cells as viewed under fluorescent light. In this figure, the green represents areas of ODN concentration. These two pictures have been overlaid in figure 3.3C to show the localisation of ODN within the cancer cells, indicating successful delivery using the DODAC/DOPE liposomes. Also notable in Figure 3.3C is the heterogeneous nature of

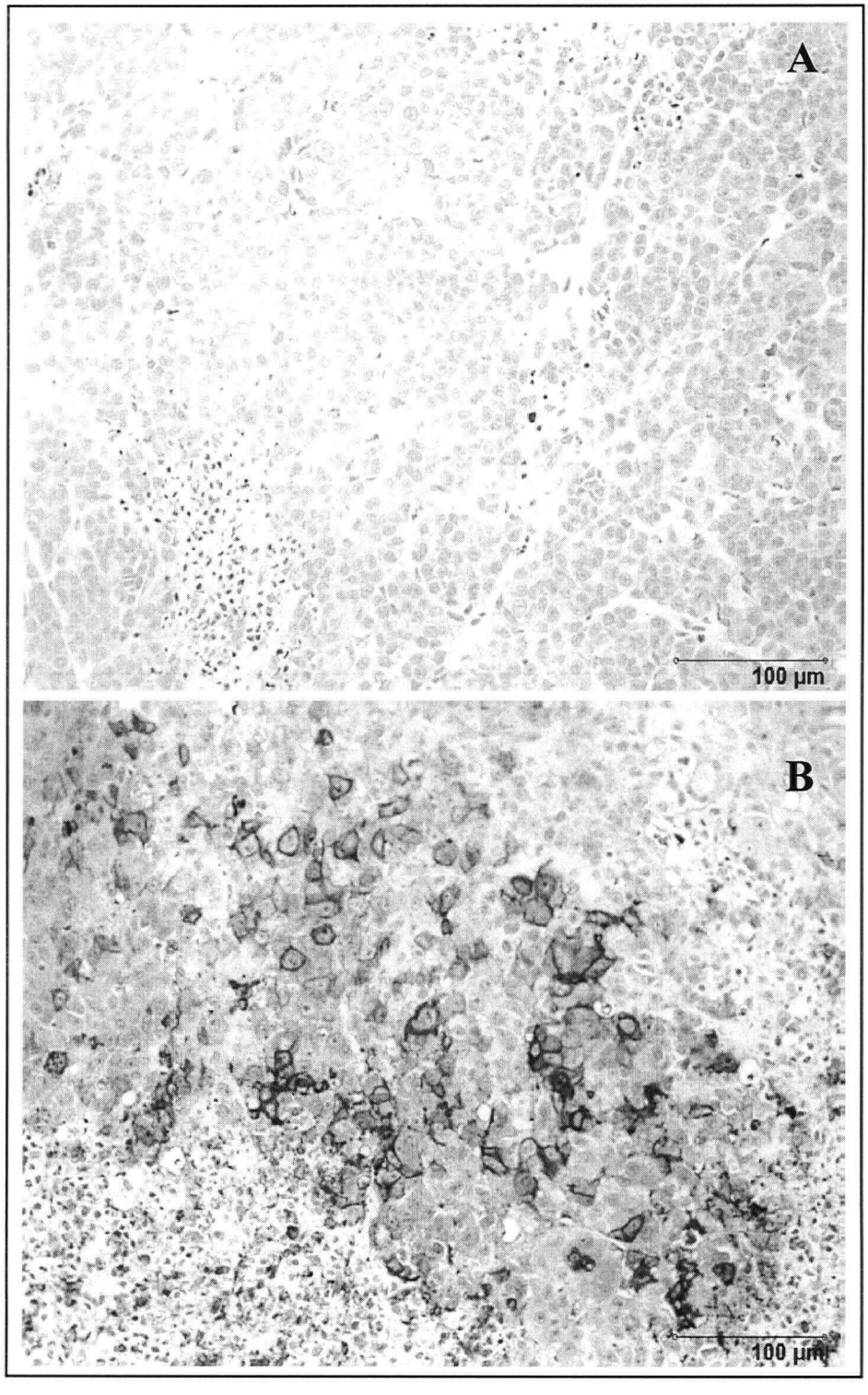

Figure 3.2 Immunohistochemical staining of HER-2/neu protein in solid tumour sections from (A) MDA-MB-435 and (B) MDA-MB-435^{HER2}-derived tumours grown subcutaneously in female SCID/Rag2m mice. Tumours were removed from mice, formalin fixed, paraffin embedded and sectioned. Sections were stained for HER-2/neu protein using an anti-HER-2/neu antibody cocktail as described in section 2.11.

ODN delivery, with some cells showing strong nuclear accumulation, while other cells do not appear to have taken up much ODN at all and some have a more cytoplasmic distribution.

3.2.5 *In Vitro* Downregulation of p185 with H2A-ODN in both the MDA-MB-435^{HER2} Cell Line, and SK-BR-3, a Breast Cell Line Which Exhibits High HER-2/neu Levels Due to Over-Amplification of the Gene

Anti-HER-2/neu ODN was initially tested for its ability to downregulate the HER-2/neu protein in a cell line with high expression of the gene. Using DODAC/DOPE liposomes as delivery agents, SK-BR-3 human breast carcinoma cells were incubated with H2A-ODN for a period of 24 hours prior to assessing the activity of the sequence. A downregulation of 67% was observed for SK-BR-3 cells allowed to recover in fresh, serum-containing DMEM for 72 hours post treatment. If cells were harvested earlier, downregulation was not as pronounced. This is illustrated in Figure 3.4A, where 48 hour recovery was equated with only 33% downregulation of protein as measured by a flow cytometric approach. This figure was derived from flow cytometric analysis of a HER-2/neu protein expression on a minimum of 10,000 cells, with the mean value being shown.

Table 3.1 shows representative p185 to actin ratios, from control (no treatment) HER-2/neu transfected cells, as well as cells treated with cationic liposome only, cationic liposome/H2A-ODN complexes and cationic liposome/SCR-H2A-ODN complexes. Densitometry analysis of western blots indicates cationic liposome/H2A-ODN mediated downregulation of p185 in MDA-MB-435^{HER2} to be greater than 80%. Of note, is that

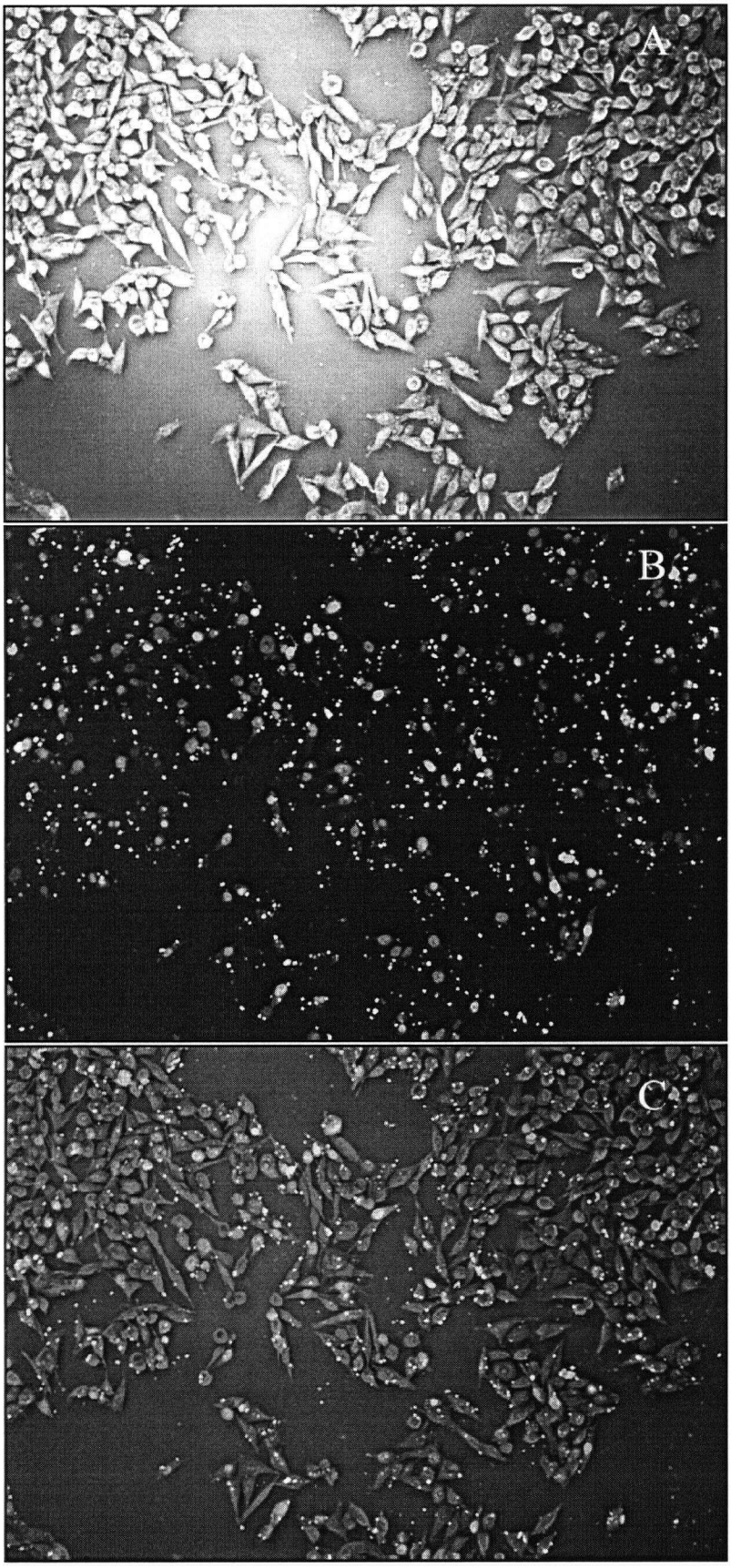

Figure 3.3 *In vitro* delivery of FITC-labelled VEGF-ODN to human breast carcinoma cells. (A) Phase contrast view under 10X objective of cells treated with VEGF-ODN complexed with DODAC/DOPE liposomes; (B) Fluorescent view of same field; (C) Overlay of Figs. 3.3A and 3.3B showing accumulation, largely within the nucleus, of fluorescently tagged ODN in the cells.

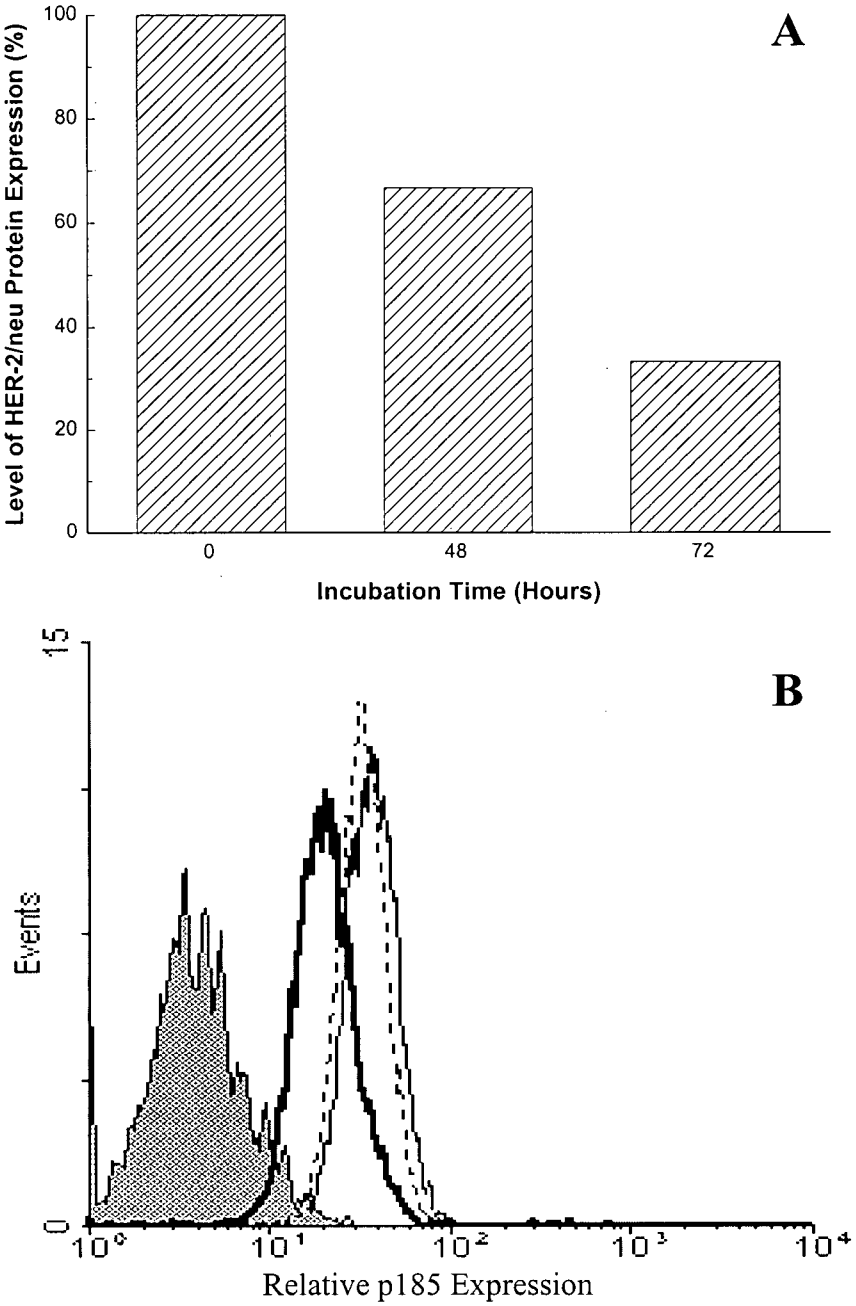

Figure 3.4 p185 expression in human breast carcinoma cells following treatment with cationic liposome/ODN complexes, as assessed by flow cytometry. (A) SK-BR-3 cells treated with cationic liposome/H2A-ODN complexes at antisense concentration of 0.35 μM for 24 hours, then allowed to recover for 48 or 72 hours; (B) Flow cytometric profile of p185 levels in MCF-7 cells. The shaded area represents IgG1 isotype control stained cells. The dashed line represents cells treated with empty liposomes only; the solid thin line is the staining of cells following treatment SCR-H2A-ODN, and the solid thick line represents cells treated with H2A-ODN. Data is from a 72 hour recovery. Antisense concentration was 0.35 μM.

the cationic liposome/SCR-H2A-ODN treatment also resulted in downregulation of p185 (see Table 3.1), but this was not to the same extent as seen with cationic liposome/H2A-ODN treatment. Both formulations resulted in less than 20% reduction in cell viability. Table 3.1 also shows results obtained using the flow cytometric analysis of p185 levels in the strongly HER-2/neu overexpressing SK-BR-3 cells following treatment with liposome/H2A-ODN complexes. Again, the treatment with antisense caused downregulation of p185, with only 40% of control expression remaining in these cells. For MDA-MB-435 cells, levels of p185 were too low for accurate determination of downregulation in this manner. Similar experiments performed with MCF-7 cells, which have approximately 3-fold higher expression of HER-2/neu than MDA-MB-435, resulted in a 50% downregulation of p185 as analysed by flow cytometry (Figure 3.4B). Although ODN/cationic liposome complexes show good delivery and activity *in vitro*, this form of delivery is not a systemically viable option due to rapid plasma elimination of these highly charged large particles (Devine *et al.*, 1997).

Table 3.1 p185 to actin ratios in DODAC/DOPE +/- ODN treated MDA-MB-435^{HER2} cells, and relative p185 expression levels in SK-BR-3 cells following treatment with DODAC/DOPE +/- ODN. Column 2: data derived from a representative western blot showing the p185 to actin ratio as a percentage of the control following 24 hour incubation of MDA-MB-435^{HER2} cells with empty liposomes, lipsome/H2A-ODN complexes, or liposome/SCR-H2A-ODN complexes, at an antisense concentration of 0.35 μM. Treatment was followed by a 72 hour recovery incubation in fresh, serum containing media. Column 3: results from a representative *in vitro* treatment of SK-BR-3 cells with liposome/H2A-ODN complexes or liposome/SCR-H2A-ODN complexes; p185 levels determined by flow cytometry, and presented as a percentage of p185 expression in control, untreated cells.

Treatment	p185/Actin in MDA-MB-435^{HER2} Cells	Relative % p185 Expression in SK-BR-3 Cells
Control	100.00	100.00
Liposomes only	86.37	Not assessed
Liposome/H2A-ODN complex	9.93	41.02
Liposome/SCR-H2A-ODN complex	46.86	95.80

3.2.6 Treatment with Free Doxorubicin in Mice Bearing MDA-MB-435 or MDA-MB-435^{HER2} Tumours

MDA-MB-435 and MDA-MB-435^{HER2} tumours were initiated as described in section 2.11. To define treatment strategies using the selected pre-clinical models, mice were treated following s.c. injection with MDA-MB-435 or MDA-MB-435^{HER2} either early (on day 3 post-tumour cell inoculation) or late (on day 54 post tumour cell inoculation). Mice were treated with free i.v. doxorubicin on days 3, 5 and 11 (early schedule; Fig. 3.5A) or days 54, 58 and 62 (late schedule; Fig. 3.5B) post tumour cell inoculation, at a dose of 7.5 mg/kg, which is the maximal tolerated doxorubicin dose for these mice. When treatment was initiated prior to tumours becoming established, doxorubicin was able to delay the tumour growth in both MDA-MB-435 (Figure 3.6A) and MDA-MB-435^{HER2} (Figure 3.6B) derived tumours. Once tumours had become established in the mice however, treatment with doxorubicin was ineffective in delaying the further growth of either MDA-MB-435 (Figure 3.6C) or MDA-MB-435^{HER2} (Figure 3.6D) tumours in these mice. No significant difference in the doxorubicin sensitivity was noted between the parental cell line and the HER-2/neu transfected cell line.

3.2.7 Treatment with Free H2A-ODN or SCR-H2A-ODN in Mice Bearing MDA-MB-435 or MDA-MB-435^{HER2} Tumours

Antisense therapy was explored in these models using both free (unencapsulated) or liposomally encapsulated ODN. Treatment with free SCR-H2A-ODN at a dose of 10 mg/kg (a well tolerated dose that has proven effective with other ODNs used for treatment

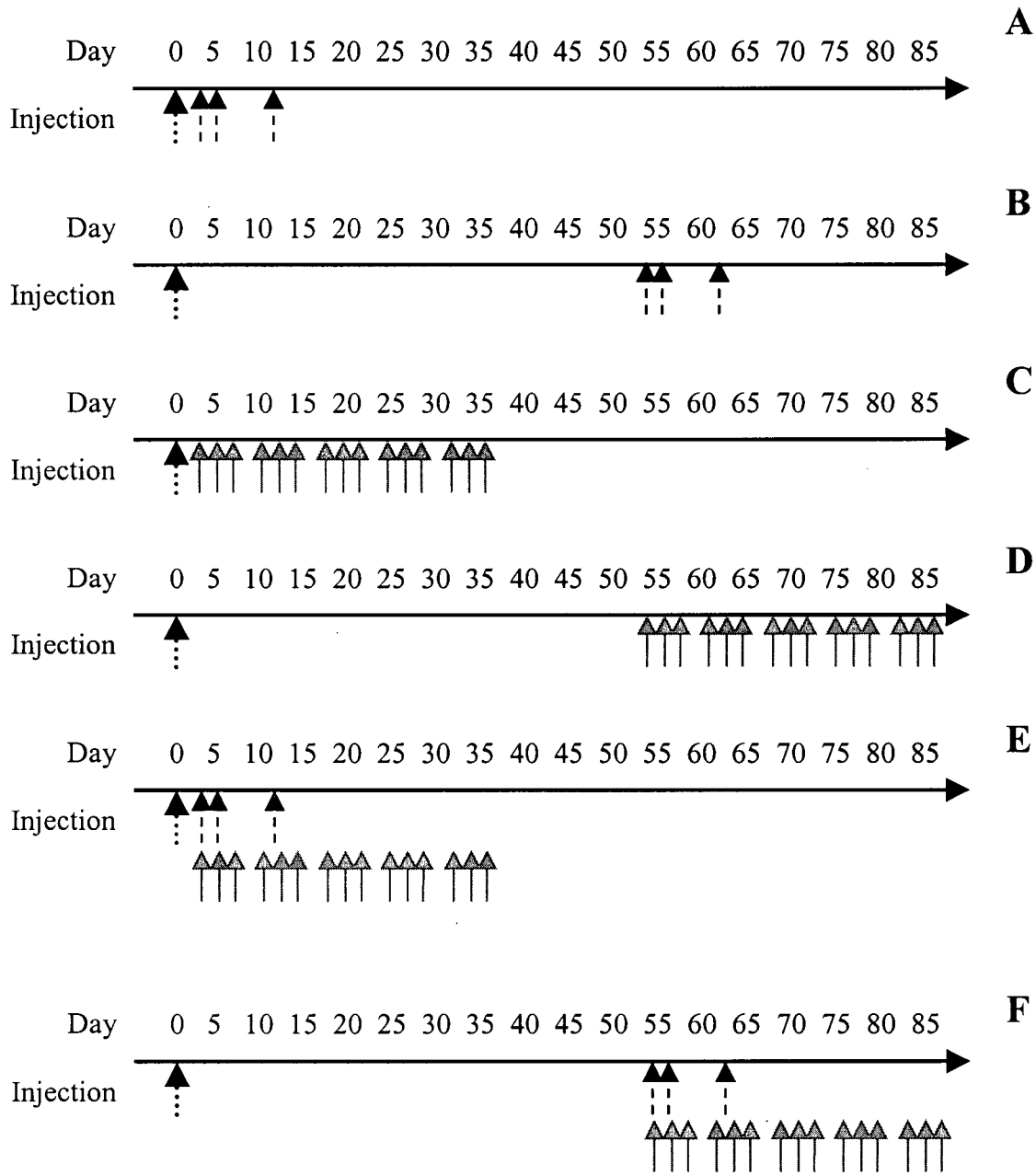

Figure 3.5 Inoculation and injection schedules for treatment of MDA-MB-435 and MDA-MB-435HER2 derived tumours with doxorubicin, free H2A-ODN or SCR-H2A-ODN, liposomal H2A-ODN or SCR-H2A-ODN, or a combination of doxorubicin plus antisense oligonucleotides. Thick dotted arrows represent inoculation of mice with tumour cells; thin dashed arrows represent treatment with doxorubicin at 7.5 mg/kg; thin solid arrows represent treatment with either free or liposomal ODN at 10 mg ODN per kg body weight. (A, C & E: early treatment schedule; B, D & F: late treatment schedule; E & F: combination treatment schedules).

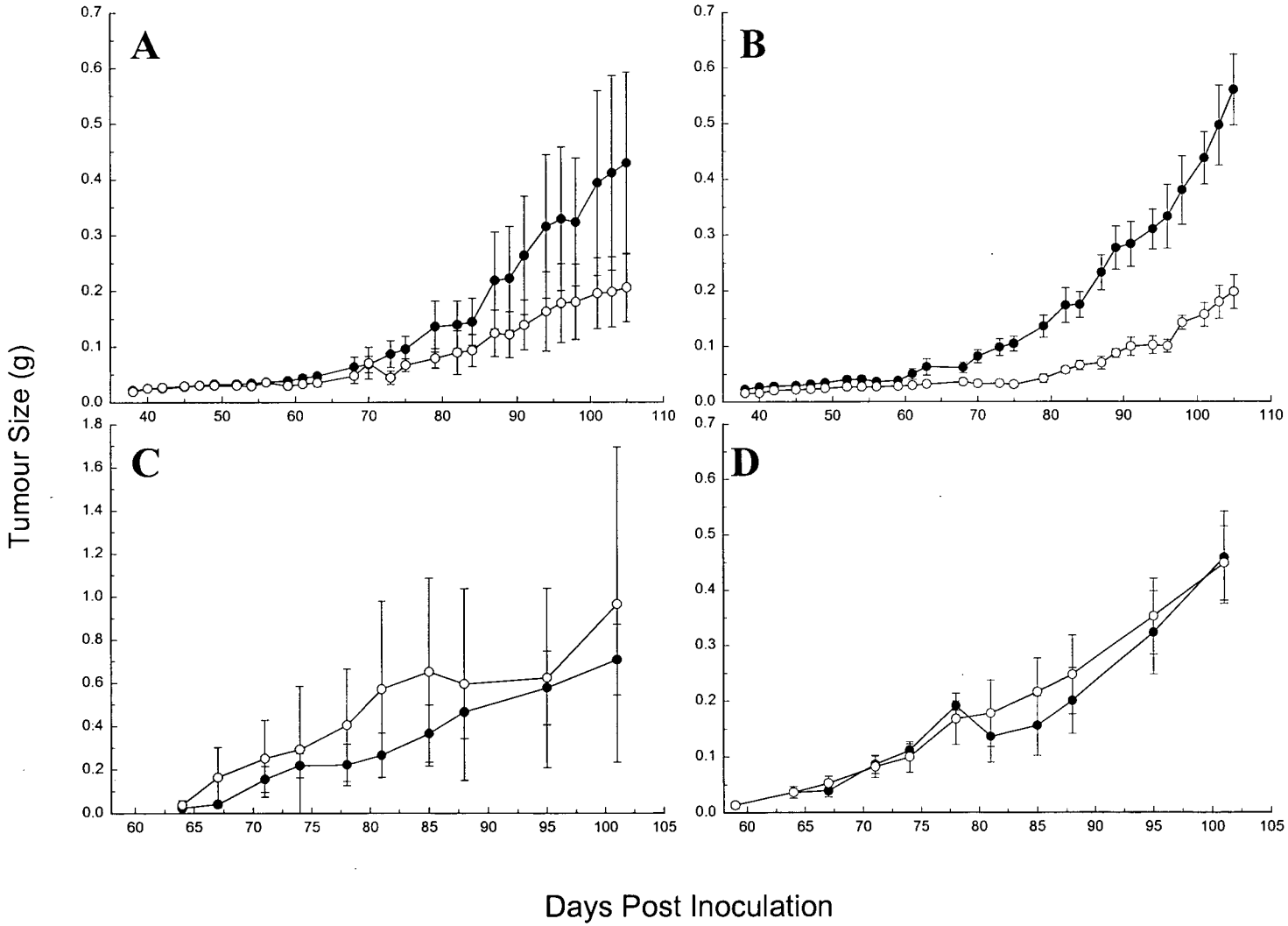

Figure 3.6 Impact of treatment with free doxorubicin in mice bearing MDA-MB-435 (A & C) or MDA-MB-435^{HER2} (B & D) non-established (A & B) or established (C & D) tumours. Data points represent the mean ± standard error of the mean of data from a minimum of 4 animals. (●) Control, no treatment; (○) treatment with 7.5 mg/kg doxorubicin as outlined in text.

of animal models of cancer) for a total of 15 injections had no impact on tumour growth in either MDA-MB-435 or MDA-MB-435^{HER2} (Figure 3.7A & B). H2A-ODN, while ineffective in both non-established and established MDA-MB-435^{HER2} groups (Figure 3.8B & D, respectively) and in the non-established MDA-MB-435 (Figure 3.8A), did show therapeutic activity when administered to established MDA-MB-435 tumours, such that by day 100, the mean size of tumours in the anti-HER-2/neu antisense treated mice were only 34% that of the untreated control mice (Figure 3.8C) (treatment schedules as outlined in Fig. 3.5C & D).

One potential explanation for why the H2A-ODN was not active when given early following tumour cell inoculation is that the region where tumour growth was first initiated did not have an adequate blood supply, and the distribution of ODN at the site where the tumour cells were inoculated was not sufficient to induce an anti-tumour effect. I believe that increases in drug delivery to regions of tumour growth can be achieved through use of methods which decrease the plasma elimination of the active agent. In particular, I have focused on liposomal delivery systems, small (<200 nm) spheres which have an associated active agent surrounded by a bilayer of defined lipid components (see section 1.4).

To assess whether liposomal carriers provided a benefit in terms of enhanced therapeutic activity of the H2A-ODN given to non-established tumours, ODN was encapsulated into liposomes composed of dioleoyl dimethylammonium propane (DODAP), distearoyl phosphatidylcholine, cholesterol and PEG-C_{14}CER in a molar ratio of 2.0:2.5:4.5:1.0 (Figure 2.1). DODAP is an ionisable lipid, and is only in its cationic form at

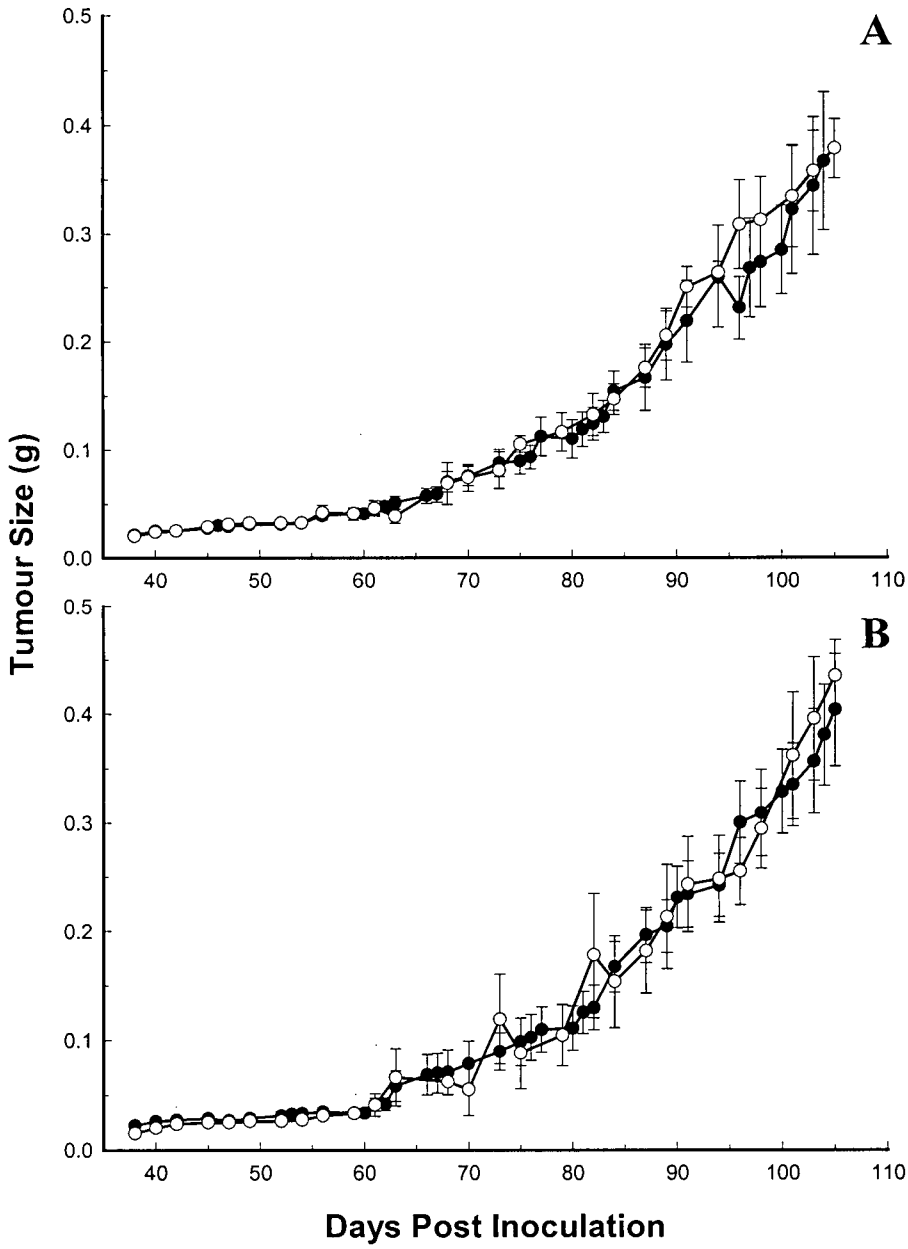

Figure 3.7 Treatment of MDA-MB-435 (A) or MDA-MB-435^{HER2} (B) cell derived xenograft tumours with SCR-H2A-ODN. Solid circles represent tumour growth in control, untreated female SCID/Rag2m mice. Open circles represent tumour growth in mice treated with free SCR-H2A-ODN at a dose of 10 mg/kg i.v., three times per week for a total of 15 injections. Injections were initiated on day 3 post tumour cell inoculation. Data points represent the mean ± standard error of tumour sizes from a minimum of 4 animals.

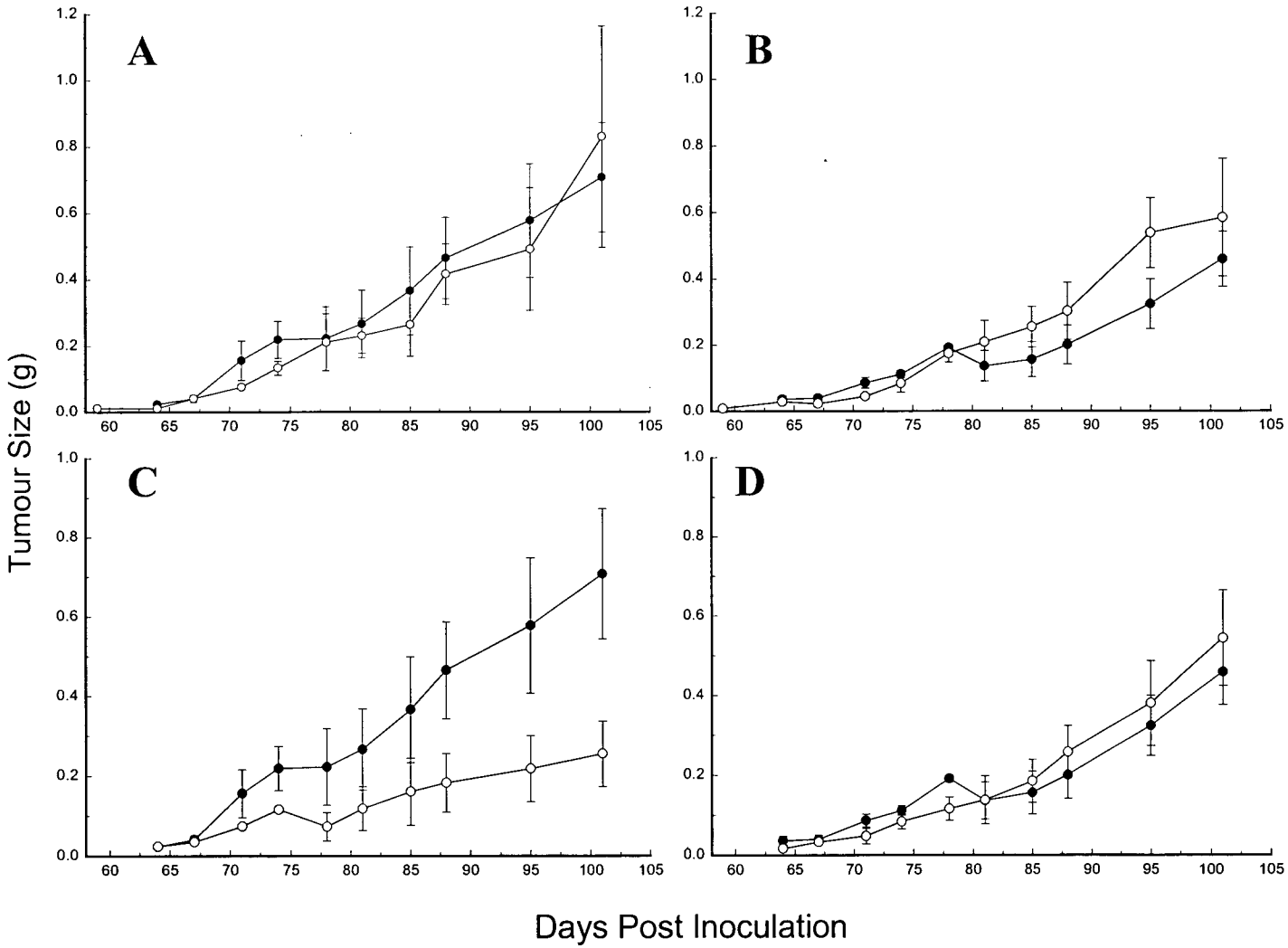

Figure 3.8 Anti-HER-2/neu antisense treatment in MDA-MB-435 (A &C) or MDA-MB-435^{HER2} (B & D) tumour bearing mice. Data points represent the mean ± the standard error of the mean of groups of at least 4 animals. (●) Control, untreated; (○) treatment with 10 mg/kg antisense as outlined in text.

subphysiological pH, it is therefore possible to form liposomes that are differentially charged on the interior relative to the exterior by adjusting the buffers used in the preparation of the liposomes. DSPC and cholesterol were included in the formulation as neutral structural lipids. PEG-C_{14}CER was important in prevention of aggregation during formulation, due to the surface steric barrier it provides. It is important to note that this PEG modified lipid exchanged out of the liposomal membrane *in vivo* within approximately 5 minutes (Semple *et al.*, 1999). This formulation has been shown to protect ODN from the degradative actions of nucleases, while the exchangeable PEG moiety may aid in circumventing potential immunogenicity problems sometimes encountered with repeat injections of formulations containing PEG-conjugated molecules (Semple *et al.*, 1999). Once introduced to the vascular system, these liposomes, with their encapsulated ODN should be able to extravasate at sites of tumour growth due to the leaky nature of the neovasculature of tumours (see Figure 3.9 for diagrammatic representation of liposomal ODN i.v. delivery to tumour site).

3.2.8 Liposomal ODN Characterisation

As ODN were to be administered over a three week period, the stability of the liposomal formulation had to be tested over this length of time. The liposomal antisense formulation used for these studies was characterised with respect to size, encapsulation efficiency and integrity of encapsulated ODN over a period of 22 weeks, stored at 4°C, 21°C or 37°C. Immediately following preparation, the typical mean diameter, as measured by light scattering techniques (see section 2.9.1), was between 120 nm and 150 nm. As may be noted in table 3.2, this size did not change appreciably at any of the three storage

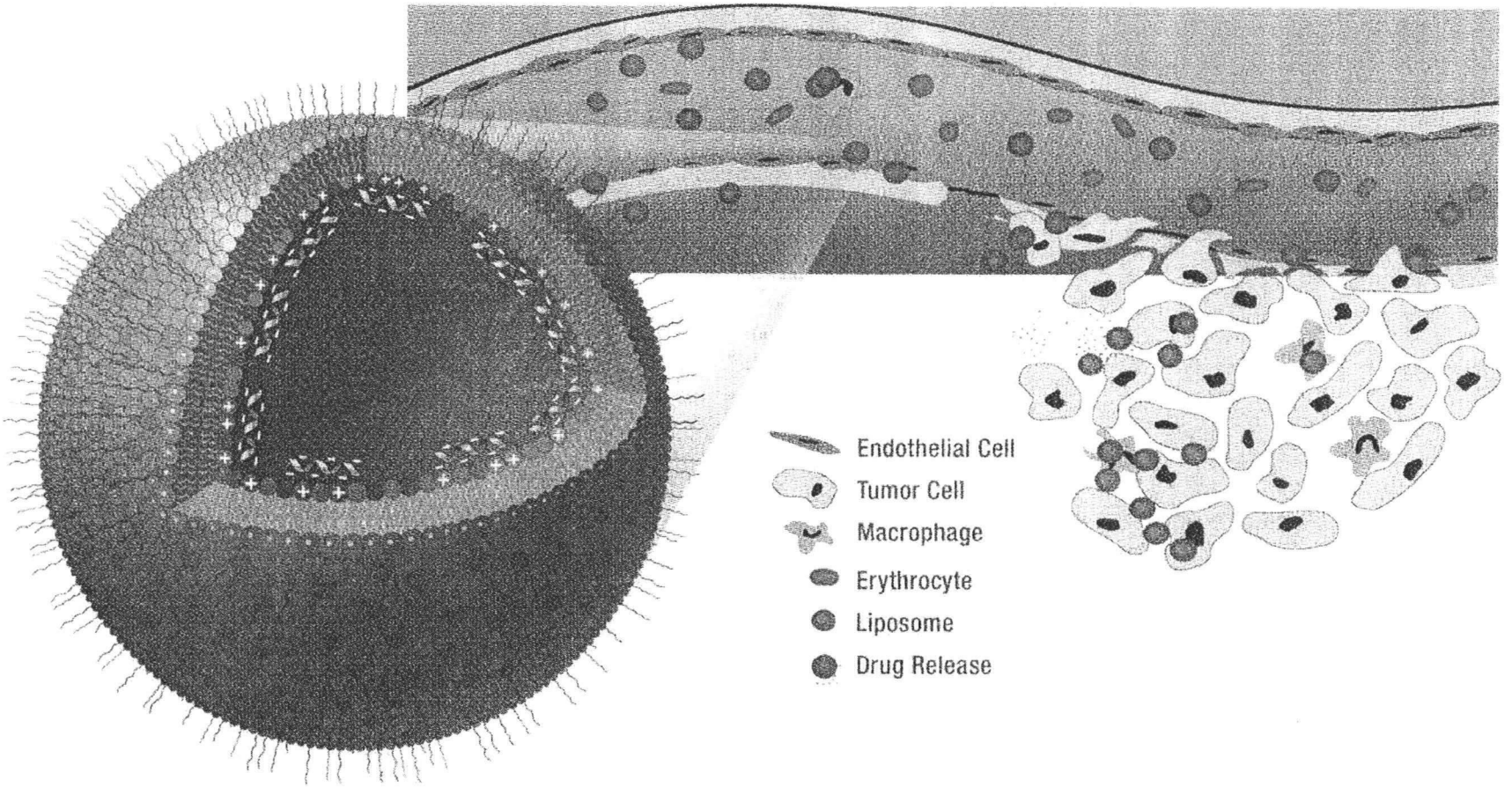

Fig. 3.9 Delivery of liposomal antisense oligonucleotides to tumour site *via* systemic administration. Liposomal ODN is administered either i.v. or i.p. and gains entry to the blood stream. At sites of tumour growth, the liposomes are able to extravasate through the leaky vasculature into the tumour, where the ODN then comes into contact with the tumour cells. (From: Saxon *et al.*, 2000).

temperatures. When prepared in a 0.2:1.0 (w/w) ratio, typical encapsulation was 50%, with a range of 30 – 70% over several experiments. Similar to results seen with size analysis, the encapsulation of antisense was not altered over a 22 week period at any of the storage temperatures, as assessed by pre- and post-column antisense and lipid determinations (Table 3.2). Importantly, when these liposomes were run on polyacrylamide gels and silver stained, the encapsulated antisense was still intact out to the end of the 22 week period, with no detectable degradation products (Fig. 3.10).

3.2.9 Treatment with Liposomal H2A-ODN or SCR-H2A-ODN in Mice Bearing MDA-MB-435 or MDA-MB-435^{HER2} Tumours

Having characterised the liposomal formulation of antisense, this was then taken into the MDA-MB-435 and MDA-MB-435^{HER2} cell derived tumour bearing mice, to assess any enhancement of ODN activity in these models. Tumours were initiated and mice treated with the liposomal formulations of H2A-ODN and SCR-H2A-ODN as for the free antisense (sections 2.11 and 3.2.7).

Active and control sequence antisense ODN, encapsulated in liposomes, were tested for efficacy against both the MDA-MB-435 (Figure 3.11) and MDA-MB-435^{HER2} (Figure 3.12) tumour xenografts, and this efficacy was compared to that observed following doxorubicin treatment. Results from these experiments indicated that liposomal H2A-ODN was able to significantly delay the growth of implanted MDA-MB-435 tumour cells (Fig. 3.11A, open triangles). In these experiments, by 125 days post-tumour cell inoculation, control untreated mice had tumours of a mean size of over 0.70 g. In contrast,

Table 3.2 Liposome size and ODN encapsulation of liposomal antisense oligonucleotides at 4°C, 21°C or 37°C over 22 weeks. Results shown are the mean ± the standard deviation.

Temp.	Test	Initial	Week 2	Week 4	Week 5	Week 13	Week 22
4°C	Size (nm)	179.7 ± 100.8	95.8 ± 99.8	188.6 ±101.3	174.5 ± 95.5	182.4 ± 104.4	174.5 ± 96.8
	Encapsulation (%)	100 ± 4.94	83.54 ± 7.41	81.89 ± 5.35	83.13 ± 1.65	79.01 ± 2.06	76.54 ± 4.12
21°C	Size (nm)	179.7 ± 100.8	185.3 ± 95.8	187.2 ± 108.0	184.1 ± 105.1	132.9 ± 68.0	109.9 ± 52.8
	Encapsulation(%)	100 ± 4.94	90.12 ± 9.88	90.53 ± 1.23	86.01 ± 8.64	87.65 ± 1.65	75.72 ± 1.23
37°C	Size (nm)	179.7 ± 100.8	181.5 ± 107.6	172.7 ± 91.8	155.7 ± 78.9	121.5 ± 45.7	130.4 ± 70.3
	Encapsulation (%)	100 ± 4.94	91.36 ± 6.17	83.54 ± 5.35	76.95 ± 4.94	100.41 ± 13.17	81.07 ± 2.88

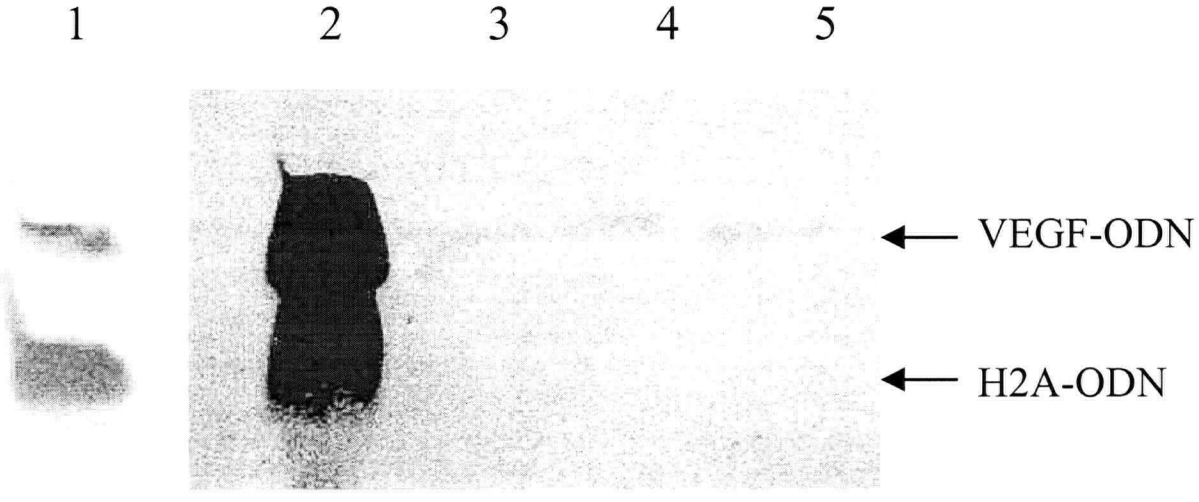

Figure 3.10 Analysis of liposome-formulated VEGF-ODN integrity 12 weeks post preparation by polyacrylamide electrophoresis and visualisation by silver stain. Lanes 1 and 2 are 21-mer and 15-mer ODN standards, respectively. Lanes 3 through 5 are liposomal VEGF-ODN stored at 4°C, 21°C and 37°C, respectively.

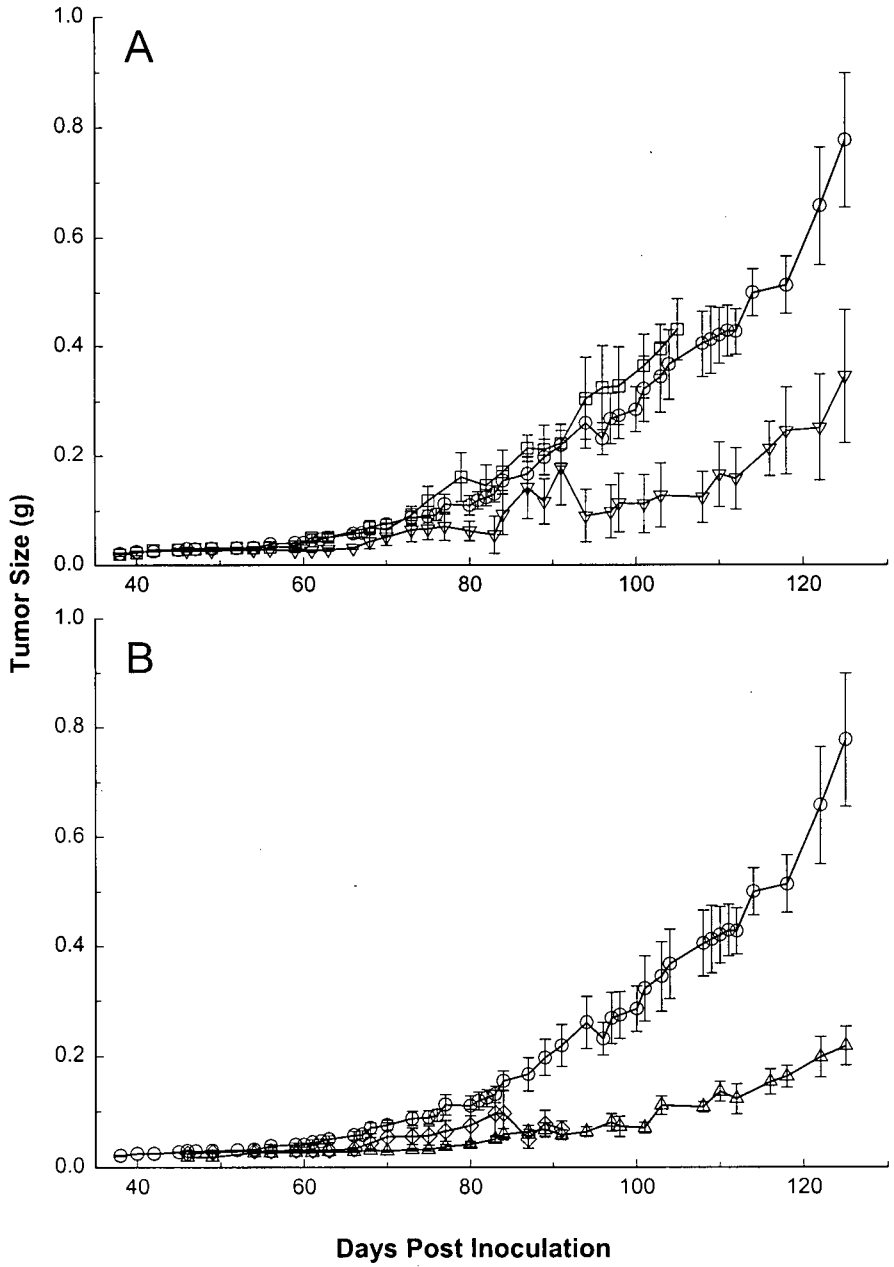

Fig. 3.11 Impact of treatment with liposomal antisense at 10 mg/kg on the growth rate of MDA-MB-435 xenograft tumors (A) or a combination of free doxorubicin at 7.5 mg/kg on days 3, 7 and 11 post tumor cell inoculation, with liposomal antisense at 10 mg/kg (B). SCID/Rag2m mice were injected s.c. with 5 X 10^6 cells on day 0. Treatment was initiated on day 3, with three times weekly i.v. injections of liposomal ODN for a total of 15 injections. (○) Control tumors from untreated mice; (▽) liposomal H2A-ODN; (□) liposomal SCR-H2A-ODN; (◇) free doxorubicin; (△) liposomal H2A-ODN + free doxorubicin. Data points are means of tumor measurements from 4 animals ± standard error of the mean.

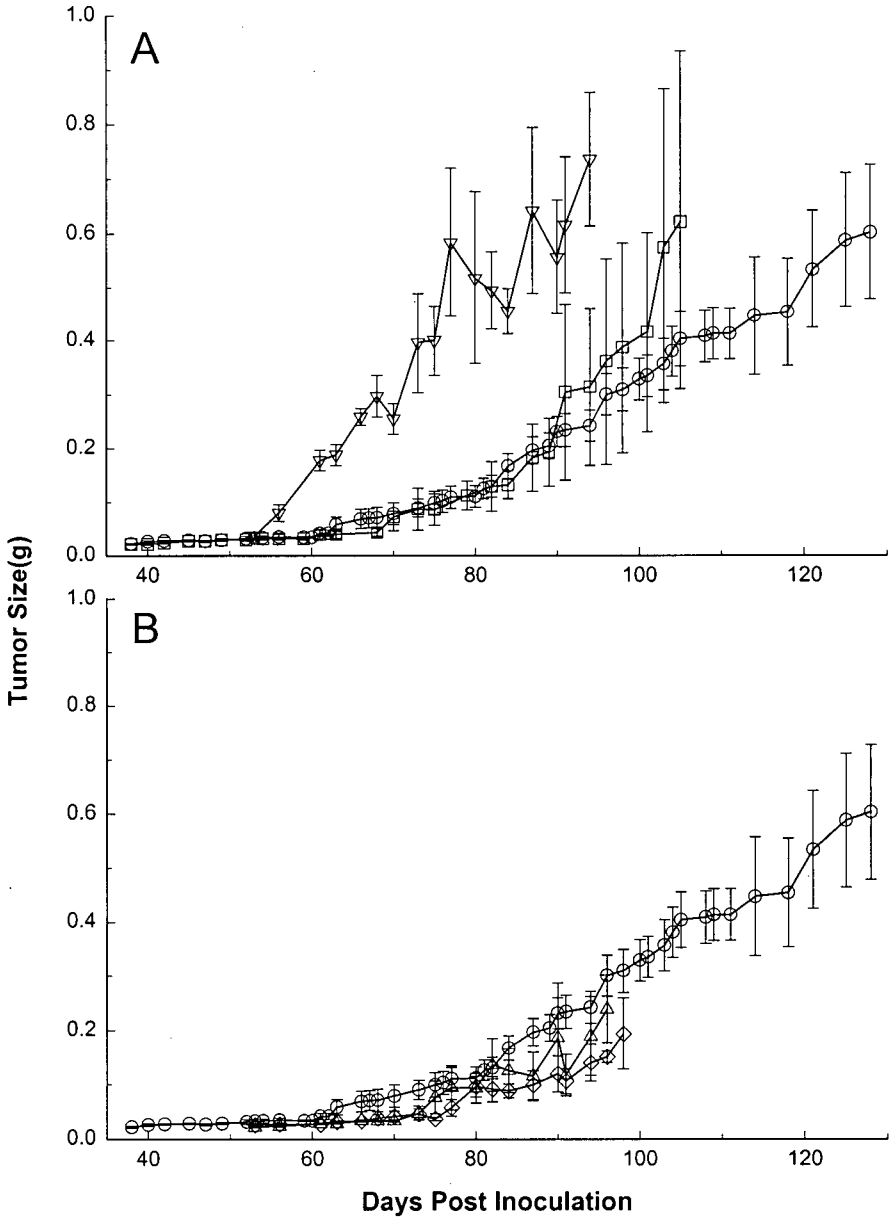

Fig. 3.12 Impact of treatment with liposomal antisense at 10 mg/kg on the growth rate of MDA-MB-435^{HER2} xenograft tumors (A) or a combination of free doxorubicin at 7.5 mg/kg on days 3, 7 and 11 post tumor cell inoculation, with liposomal antisense at 10 mg/kg (B). SCID/Rag2m mice were injected s.c. with 5 X 10^6 cells on day 0. Treatment was initiated on day 3, with three times weekly i.v. injections of liposomal ODN for a total of 15 injections. (○) Control tumors from untreated mice; (▽) liposomal H2A-ODN; (□) liposomal SCR-H2A-ODN; (◇) free doxorubicin; (△) liposomal H2A-ODN + free doxorubicin. Data points are means of tumor measurements from 4 animals ± standard error of the mean.

mice treated with liposomal H2A-ODN beginning on day 3 post-tumour cell inoculation had a mean tumour mass of less than 0.40 g at the same time. The liposomal scrambled control sequence antisense had no effect on tumour growth (Fig. 3.11A, open squares). As with the free antisense, both liposomal formulations were well tolerated in the mice at the dose and schedule used in these studies. MDA-MB-435 tumour bearing mice treated with free doxorubicin at the maximal therapeutic dose (7.5 mg/kg), on days 3, 7 and 11 suffered from delayed (occurring greater than 50 days after the last injection of drug) doxorubicin related toxicities as indicated by weight loss and appearance (scruffiness). These animals had to be terminated on or before day 91 (Figure 3.11B, open diamonds).

Changes in the rate of MDA-MB-435^{HER-2} tumour growth following treatment with encapsulated H2A-ODN (Figure 3.12) were not comparable to those seen with MDA-MB-435. While the SCR-H2A-ODN did not affect tumour growth in the mice bearing MDA-MB-435^{HER2} tumours (Fig. 3.12A, open squares), treatment initiated day 3 post tumour cell inoculation with liposomal active antisense caused a significant ($p < 0.05$) increase in the rate of tumour growth (Fig. 3.12A, open triangles). In this treatment group, tumours were approximately twice the size of control tumours at day 94 post tumour cell inoculation. However, in the group treated with doxorubicin only, mice had to be terminated due to delayed doxorubicin related toxicities.

In effort to determine the effects of treatment with liposomal H2A-ODN on HER-2/neu expression, tumours were removed from mice, formalin fixed, and paraffin sections prepared. Immunohistochemical staining was performed for p185 expression in the

sections of both MDA-MB-435 and MDA-MB-435^{HER-2} tumours, for control (untreated), antisense (free or liposomal), doxorubicin, and combination liposomal antisense plus doxorubicin treated tumours. Tumours derived following s.c. injection of SK-BR-3 breast carcinoma cells, which have high expression of HER-2/neu protein, were used as a positive control for p185 staining (Fig. 3.13A). Fig. 3.13B and 3.13C show the control, untreated tumours for MDA-MB-435 and MDA-MB-435^{HER-2}, respectively. It can be seen that MDA-MB-435 has no apparent staining for p185, while there is a heterogeneous pattern of positive membrane expression present in the MDA-MB-435^{HER-2} tumours. It should be noted here, that although there was no positive p185 staining in the MDA-MB-435 tumour sections, these cells expressed p185 when analysed by Western blot analysis (Fig. 3.14). Figure 3.13D, E and F show the MDA-MB-435^{HER2} tumours derived from mice treated with free H2A-ODN antisense, liposomal SCR-H2A-ODN antisense or free doxorubicin, respectively. As can be seen from these panels, tumours derived from these treatment groups all retained some level of p185 expression. Intriguingly, while the liposomal H2A-ODN-treated MDA-MB-435^{HER2} tumours grew at an increased rate (see Fig. 3.12A), the antisense was clearly effective in decreasing the levels of p185 within these tumours (Fig. 3.13G). Further, although doxorubicin treatment had no effect on p185 expression in these tumours (see panel F), the addition of doxorubicin to liposomal H2A- ODN treatment also demonstrated significant reduction in the levels of p185 within the tumours (Fig. 3.13H).

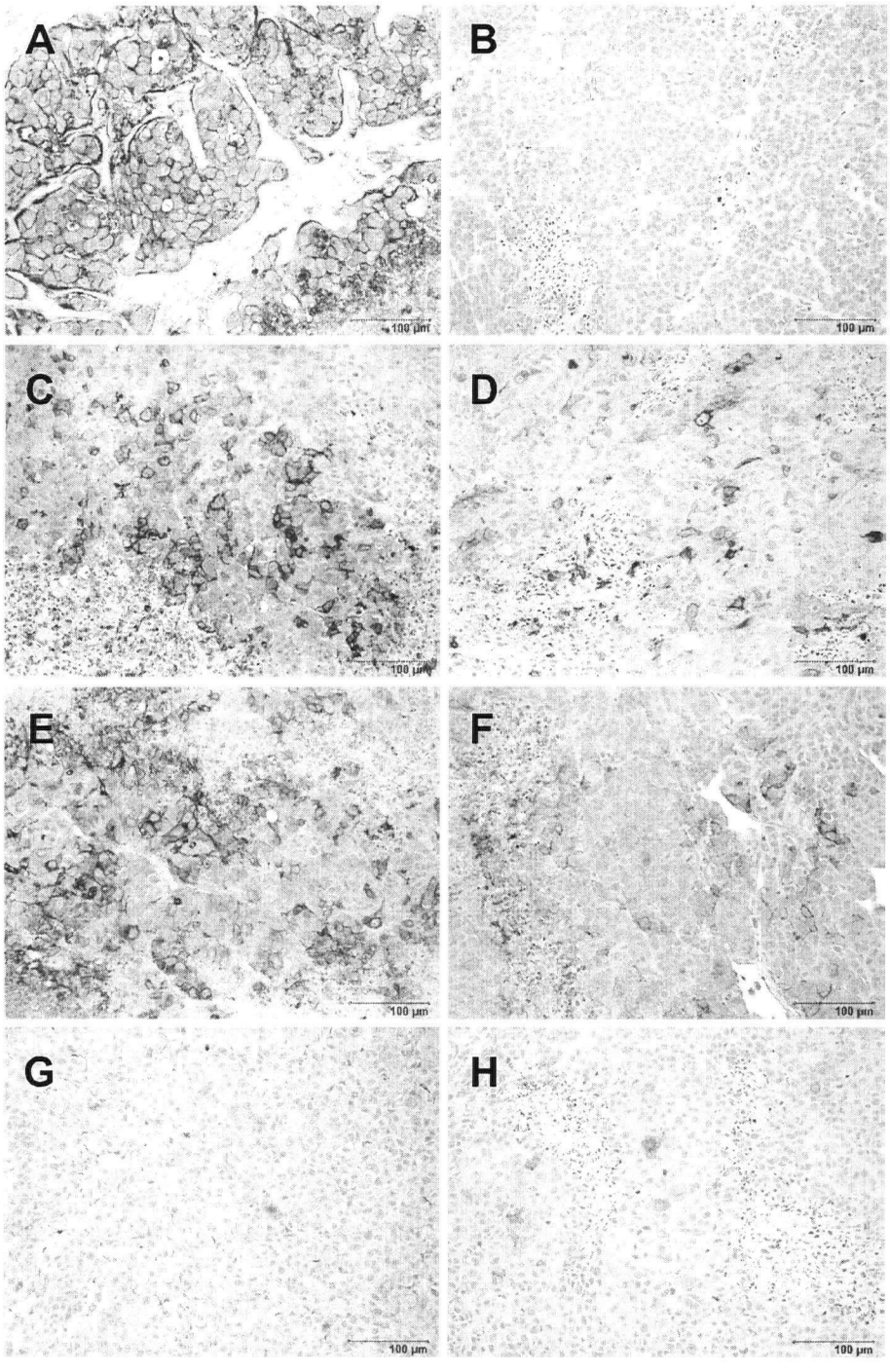

Fig. 3.13 Immunohistochemical assessment of HER-2/neu protein expression in paraffin sections of tumor xenografts, demonstrating effectiveness of H2A-ODN in decreasing p185 expression. (A) SK-BR-3 breast carcinoma positive control; (B) untreated MDA-MB-435; (C) untreated MDA-MB-435^{HER2}; (D) MDA-MB-435^{HER2} treated with free H2A-ODN; (E) MDA-MB-435^{HER2} treated with liposomal SCR-H2A-ODN; (F) MDA-MB-435^{HER2} treated with doxorubicin; (G) MDA-MB-435^{HER2} treated with liposomal H2A-ODN and (H) MDA-MB-435^{HER2} treated with liposomal H2A-ODN and doxorubicin.

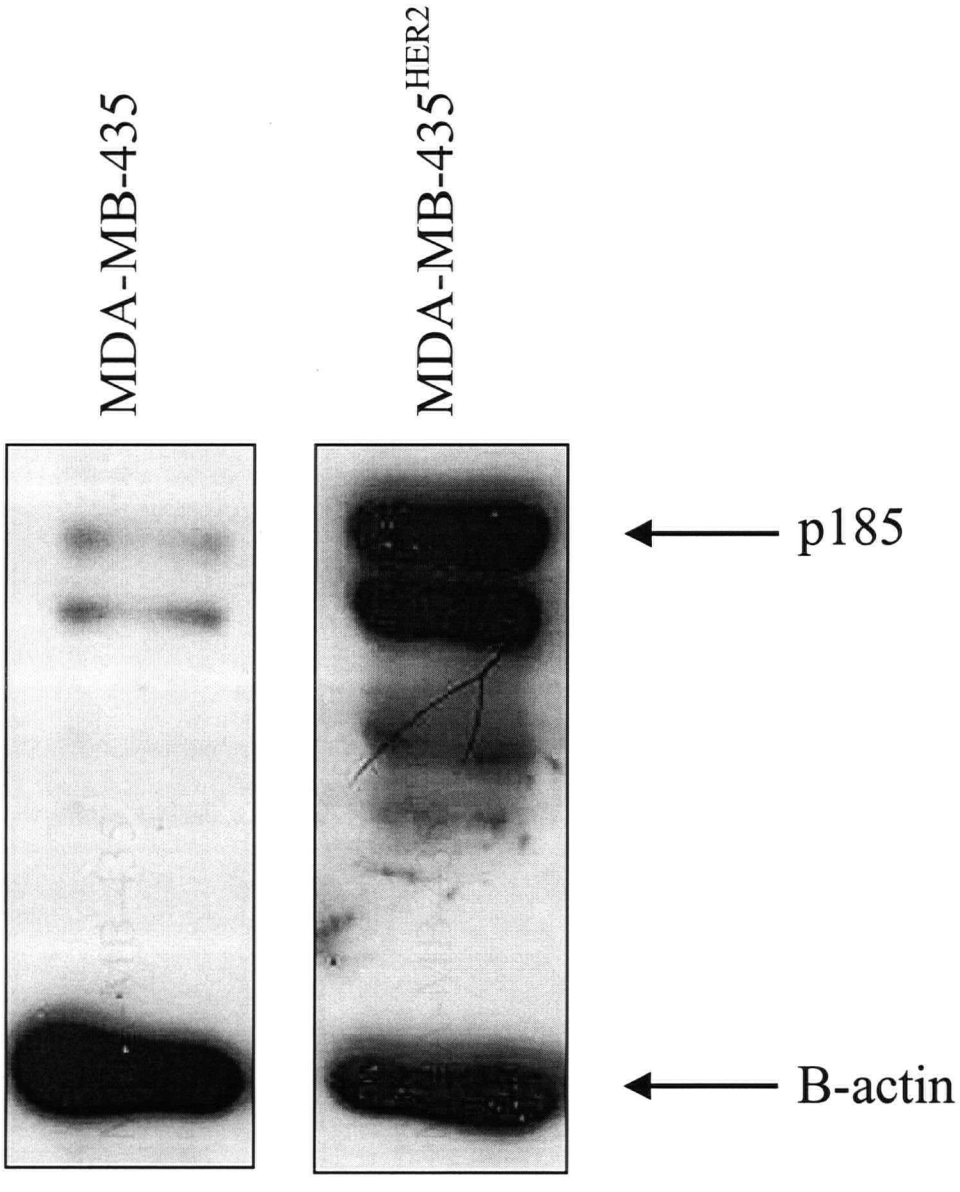

Figure 3.14 Western blot demonstrating HER-2/neu protein expression in MDA-MB-435 and MDA-MB-435^{HER2} cell lysates. β-actin was used as an internal control. Cells were cultured *in vitro* and lysates prepared as outlined in section 2.6.1. Equivalent amounts of protein were loaded on 7.5% polyacrylamide gels and run at 86V for approximately 70 minutes. Proteins were transferred to PVDF membranes and visualised with ECL™ western blotting detection reagents.

3.2.10 Combination Therapy: Liposomal ODN with Doxorubicin in the Treatment of MDA-MB-435 and MDA-MB-435^{HER2} Xenograft Tumours

As breast cancer is most often treated with combination, rather than single agent therapy, treatment with liposomal ODN was combined with treatment with doxorubicin (dosage as detailed in section 3.2.6, schedule as in Fig. 3.5). This did not significantly improve the efficacy of liposomal H2A-ODN in MDA-MB-435 tumour-bearing mice (Fig. 3.11B); however this study was not designed appropriately to detect the potential of H2A-ODN/doxorubicin drug combination to provide synergistic effects. However, the addition of doxorubicin to liposomal H2A-ODN treated MDA-MB-435^{HER2} tumour-bearing mice reversed the effect observed with liposomal H2A-ODN alone, bringing the growth rate of these tumours to less than that of control, untreated mice (Fig. 3.12B). Again, these tumours were fixed in formaldehyde for immunohistochemical determination of p185 expression, and, as was the case with MDA-MB-435^{HER2} tumours treated with liposomal H2A-ODN only, a reduction in p185 staining was observed (Fig. 3.13H).

3.3 Discussion

Over the past several years, antisense oligonucleotides have become one of the promising therapeutics in the treatment of several different disease categories. One of the primary steps in the development of a novel antisense agent is to synthesise a number of ODNs targeting different areas of the mRNA of interest, then to screen these sequences for activity. There are several regions of mRNA which are considered to be areas more likely to yield reduction or elimination of protein expression through antisense binding. One of

these is the initiation codon area of the message, or the region directly upstream of the initiation codon. Other areas which have been successfully targeted include the promoter region, the coding region, and the 5 prime cap region. One of the antisense oligonucleotides used in the current study was a 15 nucleotide phosphorothioate backbone molecule targeting the region directly upstream of the initiation codon of the HER-2/neu mRNA (Vaughn *et al.*, 1996). The rationale was that the hybridisation of synthetic DNA to mRNA at this specific site would prevent the formation of the protein assembly apparatus, in addition to destroying the HER-2/neu mRNA through other antisense mediated pathways such as by RNaseH (Crooke, 1999). Despite such elegance in antisense design, it is yet to be clearly established whether ODNs act specifically through mechanisms which involve downregulation of the target protein. While accepting the fact that ODNs can contain sequences which non-specifically stimulate the immune system (Krieg, 1999), the immunohistochemistry results presented in this chapter provide compelling evidence that the selected ODN can inhibit HER-2/neu expression *in vivo*. What is not well understood is how downregulation affected the growth of these model tumours. This discussion will, for this reason, largely focus on ODN-mediated down regulation of HER-2/neu.

I have clearly demonstrated the downregulation of the HER-2/neu protein product in *in vivo* models following administration of liposomal H2A-ODN. This was not observed following administration of the free antisense or free and liposomal control sequences. Similar to arguments that have been developed for liposomal delivery of conventional anticancer drugs (Mayer *et al.*, 1995; Webb *et al.* 1995; 1998a; Tardi *et al.*, 1996; Lim *et al.*, 1997), the enhanced activity of the liposomal formulation is likely due to liposome

mediated increases in ODN delivery and bioavailability (to be discussed further in chapter 5).

The increased tumour growth rate observed in MDA-MB-435^{HER2} tumour bearing mice (Figure 3.1B) was unexpected, particularly when considered with data demonstrating decreased p185 expression. The increased rate of growth in the MDA-MB-435^{HER2} tumours following treatment with liposomal H2A-ODN, could be a consequence of the use of a p185 transfected cell line. It is unclear whether therapeutic responses to HER-2/neu targeted therapies will be the same when using models derived from cell lines which overexpress p185 as a consequence of gene amplification, such as the SK-BR-3 or BT474 breast carcinoma cell lines, or as a consequence of gene transfection.

It is well known that the four members of the class I tyrosine kinase receptor family are able to form homo- and heterodimers and that downregulation of one of the family members may change dimer levels and functions (Siegel & Muller, 1998; Ben-Baruch *et al.*, 1998). It is known, for example, that the different combinations lead to different signal transduction pathways and transcription factor expression profiles, perhaps as a consequence of differential receptor phosphorylation (Olayioye *et al.*, 1998). For example, EGFR is not able to activate Grb2 if it is activated by heterodimerization with HER-3 or HER-4, while it avidly interacts with Grb2 if part of a heterodimer with HER-2 (Nagy *et al.*, 1999). It can be suggested in the MDA-MB-435^{HER2} human xenograft model, treated with a drug that decreases p185 expression, that the cellular machinery has been switched to an alternate pathway for increased growth, possibly involving other members of this

family. In this regard it will be important to gain a better understanding of how p185 downregulation affects other members of the HER family of proteins and some of the associated intracellular kinase activities.

Despite the demonstration of H2A-ODN specific decreases in p185 expression, there remain concerns about the specificity of the antisense approach in therapeutics. The foremost among these is the stimulation of an immune response. It has been demonstrated that antisense sequences containing unmethylated CpG motifs are able to directly stimulate macrophage cytokine release (Sparwasser *et al*, 1997), as well as to significantly enhance cytotoxic T lymphocyte (CTL) response (Davila & Celis, 2000). The active anti-HER-2/neu antisense used in these studies contained a single CpG motif, and so this was an area of concern. B and T cell deficient mice were used in the current studies however, so any immunostimulatory effects will have been isolated to neutrophil/macrophage based tumoricidal activities. As well, to control these effects, the scrambled control sequence antisense also contained not one, but three CpG motifs, therefore any efficacy due to stimulation of an immune response should have been greater with the SCR-H2A-ODN sequence. It has been shown that the flanking sequences are important in consideration of immunostimulatory effects, as both dA and dT sequences enhance the stimulatory capacity of the CpG motif (Mannon *et al.*, 2000). Neither antisense ODN used in these studies had these specific flanking sequences.

Assuming antisense mediated changes in HER-2/neu expression were responsible for the effects observed in the breast tumour models used here, it can be argued that optimal

therapeutic results will only be realised through carefully selected drug and antisense combinations. As indicated above, it is difficult to rationalise ODN combinations without first establishing which key targets may be interacting to regulate the proliferative and metastatic potential of our tumour models and, more importantly, breast cancer in patients with aggressive and unresponsive disease. Part of this effort must consider the use of combinations of ODNs with conventional therapeutic agents, such as doxorubicin and taxol, used to treat breast cancer. Preliminary studies reported here suggest that combinations of doxorubicin and the liposomal formulation of HER-2/neu targeted ODN may be beneficial; however this can only be established through more extensive dosing studies. Since the toxicity concerns associated with antisense administration in humans (Monteith & Levin, 1999) are low due to the specificity afforded by these molecules and to low levels of p185 expression in normal tissues, there should be few additional toxicities associated with this therapeutic strategy when used in conjunction with existing treatment regimens. There are currently studies evaluating, in early phase clinical trials, the use of a bcl-2 targeting ODN sequence in combination with cyclophosphamide and mitoxantrone in the treatment of patients with B-cell lymphoma and prostate cancer, respectively (Chi *et al.*, 2000). I hope that similar efforts will be initiated soon in breast cancer patients.

A drawback to testing the parameters of antisense therapy described above in the MDA-MB-435 tumour models is the extended time frame (>80 days for control groups) over which the tumours grew. For this reason, it was important to establish a different breast cancer xenograft model with a more reasonable growth rate. Thus, the next chapter of this thesis will focus on the characterisation of the MDA435/LCC6 cell line recently described

by Leonessa *et al.* (1996).

In conclusion, I have demonstrated that the H2A-ODN antisense sequence used in this report is effective in the downregulation of the HER-2/neu protein both *in vitro* and *in vivo*, and further, that this downregulation is maintained when combined with doxorubicin treatment. In a model of low p185 expression, a liposomal formulation of this antisense was effective in reducing the rate of tumour growth in SCID/Rag2m mice. I have also shown, however, that in at least one breast tumour model exhibiting high p185 expression, treatment with liposomal anti-HER-2/neu antisense in the absence of doxorubicin treatment resulted in an increased rate of tumour growth, despite significant reductions in HER-2/neu expression as measured by immunohistochemistry.

This result is surprising and somewhat disappointing, however it may be a reflection of the challenges that will be faced when developing any agent selectively targeting a single protein associated with tumour progression. It is likely that multiple proteins must be targeted simultaneously in order to dysregulate the tumour cell's machinery sufficiently to alter its own inherent capacity to survive. Future studies may need to investigate levels of proteins downstream of HER-2/neu as well as proteins where increased expression has been associated with altered HER-2/neu, such as vascular endothelial growth factor (VEGF)(Petit *et al.*, 1997).

Chapter 4

CHARACTERISATION OF THE MDA435/LCC6 HUMAN BREAST CARCINOMA XENOGRAFT TUMOUR MODEL

4.1 Introduction

Many of the approximately 11% of all North American women and 1% of all North American men who will develop breast cancer will also develop metastatic disease. Approximately 35% of those who develop breast cancer will ultimately succumb to their disease. For this reason, there is an obvious need to develop more efficacious treatment strategies. Hand in hand with the need for novel strategies is a requirement for better models in which to test these new ideas, such that they may be more accurately assessed for efficacy in human disease. To be able to develop a good model, researchers must understand the diseases with which they are working, know which pathways or proteins are activated or deactivated, and seek to characterise their models with respect to these pathways and proteins. In this way, the models used will be more relevant to the disease, and results garnered with novel therapeutics will be more easily interpreted as to the mechanism of action. It was with this in mind, that I proceeded to characterise the MDA435/LCC6 model of human breast cancer for general *in vitro* and *in vivo* growth characteristics, sensitivity to anti-cancer drugs and expression of several proteins relevant to breast cancer.

Research has identified several specific prognostic and predictive factors to identify patients at high risk for more aggressive breast disease, metastasis and for recurrence of disease in order to combat the high mortality associated with these factors. Several studies have demonstrated, for example, that an increase in cytosolic levels of vascular endothelial growth factor (VEGF) in tumour tissue samples is indicative of poorer prognosis for patients with node-negative breast carcinoma (Anan *et al.*, 1998; Gasparini, 1997). If a tumour is to exceed 1 - 2 mm in diameter, it must be able to initiate the formation of an adequate blood supply to receive sufficient oxygen and nutrients. The process by which this neovascularisation occurs is known as angiogenesis (Folkman, 1992), and the extent of tumour angiogenesis is now widely accepted as a prognostic factor in several cancer types (Araya *et al.*, 1997; Axelsson *et al.*, 1995; Fox, 1997; Heimann *et al.*, 1996; Weidner *et al.*, 1992), with higher levels being indicative of more aggressive disease.

A possible link between VEGF and another marker of poor prognosis in both node positive and node negative breast cancers has also been postulated, namely, the overexpression of the protein product of the HER-2/neu oncogene (Petit *et al.*, 1997). Research into a mechanism for HER-2/neu action has shown that overexpression of HER-2/neu in cell lines increases cell migration, an important step in metastasis formation (Verbeek *et al.*, 1998). It has also been suggested that there may be a link between HER-2/neu expression and the up-regulation of anti-apoptotic Bcl-2 and Bcl-XL proteins, and suppression of tamoxifen-induced apoptosis (Kumar *et al.*, 1996). More recent research however, has linked the absence of bcl-2 expression with a poorer overall survival (Bhatavdekar *et al.*, 2000) or conversely, that increased bcl-2 conferred decreased risk (Jager *et al.*, 2000). In light of

these observations, I felt it was important to characterise the human breast cancer xenograft model, MDA435/LCC6 to determine if it expressed the above markers.

The final protein assessed in this study was Cathepsin D. This protein has been proposed to play an important role in breast cancer invasion and metastasis (Han *et al.*, 1997). Because of this, patients with elevated cathepsin D levels, defined as those tumours with clearly detectable cathepsin D levels (greater than or equal to 10% positive cells)(Gonzalez-Vela *et al.*, 1999) have an adverse prognosis (Solomayer *et al.*, 1998). As a protease, cathepsin D is likely to be involved in the degradation of extracellular matrix and therefore, tumour cell spread in circulatory and lymphatic systems. This same mechanism may also contribute to distant metastases. There is a trend of increased cathepsin D in oestrogen receptor positive breast cancer, but there is disagreement in the significance of this tumour cell associated cathepsin D. Some researchers dispute the relation to other clinicopathologic parameters (Gonzalez-Vela *et al.*, 1999), while others find significant correlation between Cathepsin D positive staining in tumour cells, and higher frequency of recurrence and shorter disease free survival (Lah *et al.*, 2000).

The discovery of new prognostic and predictive markers for breast cancer is exciting, and has opened the way for a new generation of targeted therapeutics for treating this disease. In order to exploit these markers, as stated above, researchers require predictive *in vivo* models that can be used to i) establish the role of these markers in breast cancer progression and ii) screen agents directed against these targets for therapeutic activity. With regard to genetic targets, those cell lines used to establish models must also express relevant targets

and or pathways. The development of a human xenograft ascites model fulfilling the first two of these requirements has been achieved in the MDA435/LCC6 model (Leonessa *et al.*, 1996). This model has a reproducible growth curve in mice bearing these tumours, and responses to cytotoxic drugs are similar to those seen in many human breast cancer patients (Leonessa *et al.*, 1996). An additional feature that supports the use of this cell line is its increased growth rate as compared to the parental line, which necessitated *in vivo* experiments extending past 100 days (section 3.2.2). The cells may also be easily maintained *in vitro*, thus allowing rapid assessment of drugs both *in vitro* and *in vivo*.

The herein described characterisation of MDA435/LCC6 demonstrated that *in vitro*, these cells display a three-fold increase in VEGF expression over parental MDA-MB-435 cells. This pattern is also readily apparent *in vivo* as determined by immunohistochemical staining. The model also shows readily detectable levels of both bcl-2 and cathepsin D expression, therefore making the MDA435/LCC6 a useful model for therapies that target these proteins.

4.2 Results

4.2.1 *In Vitro* and *In Vivo* Growth Characteristics

The purpose of these studies was to characterise the MDA 435/LCC6 tumour cell line to determine its value as a model of human breast cancer. Part of this assessment included the *in vitro* growth characteristics of MDA 435/LCC6 as compared to the parental MDA-MB-435 line. As seen in Figure 4.1A, MDA 435/LCC6 was a more rapidly growing cell line *in*

vitro, with the growth rate from day three onward being more rapid than MDA-MB-435. The doubling time for LCC6 cells from day 3 onward was approximately 20 hours, while that of MDA-MB-435 was 72 hours. This suggests that the LCC6 variant may be intrinsically more aggressive (as defined by proliferation rate) than the parental line.

Having confirmed a more aggressively growing phenotype *in vitro*, I sought to determine whether this was also the case *in vivo* (Figure 4.1B, C & D). When grown as a subcutaneous tumour on the back of female SCID/RAG2m mice, the *in vivo* doubling time of the LCC6 variant was roughly 3 fold faster than the parental MDA-MB-435 cell line, a result that is consistent with the data in Figure 4.1A. There are two features of this *in vivo* growth curve worth noting. First, the LCC6 cell line tumour growth becomes grossly apparent on day 20 to 25 post inoculation, while the parental growth is not apparent until day 60 to 70. Second, once growth is initiated, the tumour doubling time from 0.2 to 0.4 grams is approximately 12 days for tumours arising from the LCC6 cell line and 15.5 days for tumours arising from the parental cell line. MDA435/LCC6 also grows as a subcutaneous tumour in the mammary fat pad. Tumour growth curves from tumours grown in this area in female SCID/Rag2m mice are shown in Figure 4.1C. These tumours were followed out to day 35 post-tumour cell inoculation, by which time they had reached approximately 0.2 g. This form of inoculation was not pursued further due to the frequency with which tumours in this location became ulcerated.

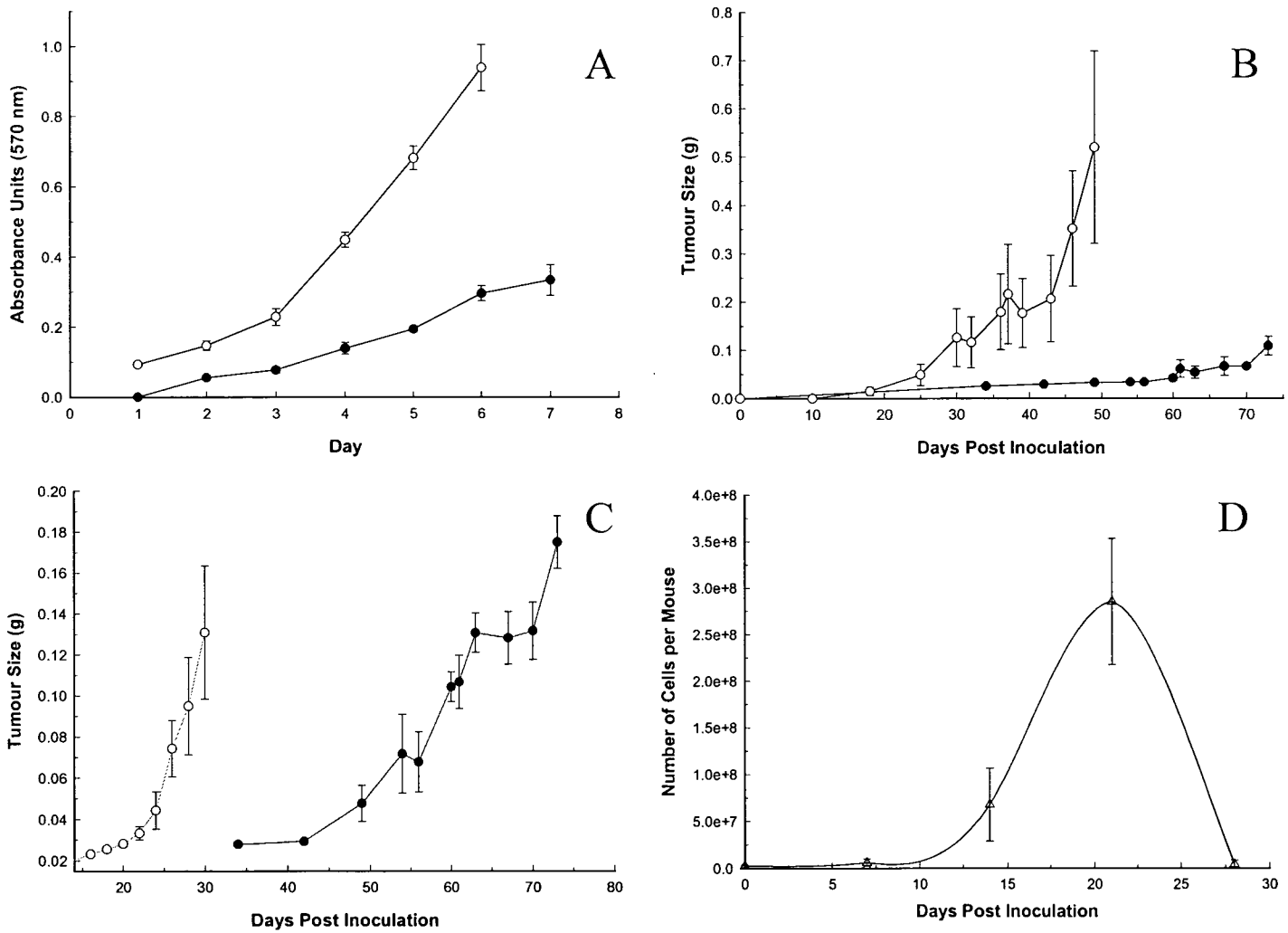

Figure 4.1 Growth characteristics of MDA435/LCC6 cells *in vitro* (A) and *in vivo* (B – D). (B) Grown as solid tumour on back; (C) solid tumour in mammary fat pad; (D) ascites tumour. Inoculums were 2 X 10^6 cells per mouse. Data points are mean ± standard error of the mean of data from a minimum of 4 animals (6 replicates for the data shown in Fig. 4.1A). (●) MDA-MB-435; (○, △) MDA435/LCC6.

4.2.2 Cytotoxicity of Taxol, Vincristine and Doxorubicin Against MDA435/LCC6

To further the characterisation of the LCC6 variant, I tested the susceptibility of both the parental and variant lines to several commonly used anticancer agents, namely taxol, doxorubicin and vincristine. Results from these *in vitro* tests are shown in Figure 4.2A and B, and are compared to the cytotoxicity profile exhibited by the parental MDA-MB-435. It was important to demonstrate that this newly derived breast cancer cell line maintained a response to common cytotoxic drugs as is demonstrated by MDA-MB-435. As is highlighted in Figure 4.2, the IC_{50} values for the two lines are not substantially different, indicating neither increased resistance nor sensitivity to these drugs.

The growth of LCC6 as an ascites tumour, with total number of viable cells excluding any red blood cells in the peritoneum, plotted over time is shown in Figure 4.1D. The typical doubling time when grown as an ascites tumour is less than four days once the tumour becomes established (after day 14). Between days 21 and 28, the peritoneal cavity becomes so crowded with cells that the tumour cells start to die due to nutrient depravation, such that by day 28, the bulk of the tumour cells are non-viable.

4.2.3 Markers of Aggressive Breast Cancer

Having demonstrated a more rapidly and aggressively growing tumour both *in vitro* and *in vivo*, an assessment of the levels of several proteins that have been associated with more aggressive breast was completed. I screened the two cell lines for VEGF, HER-

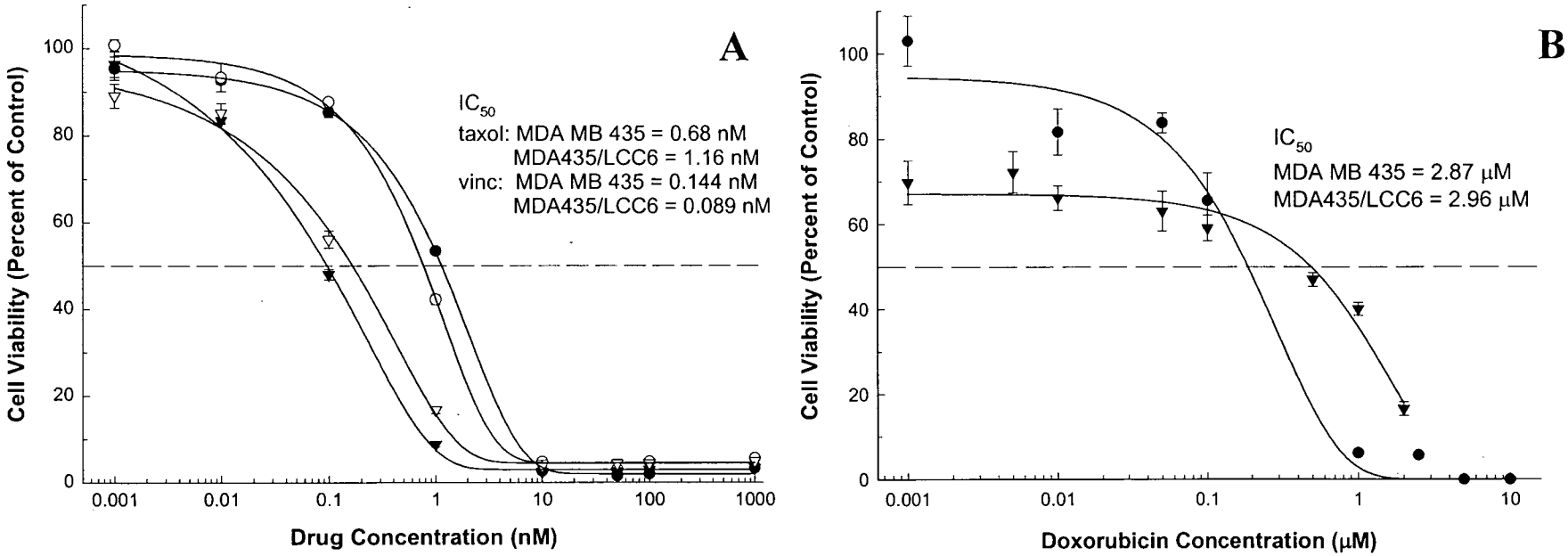

Figure 4.2 Cytotoxicity of (A) vincristine, taxol and (B) doxorubicin against MDA-MB-435 and MDA435/LCC6 cells. Cells were plated at 3000 cells per well in 96 well plates. Vincristine or taxol was added to wells in concentrations ranging from 0.001 nM to 1000 nM, doxorubicin from 0.001 μM to 10 μM. Plates were incubated at 37°C for 4 hours. An MTT assay was performed to determine cell viability. Data points represent the mean ± standard error of 6 replicate wells. A: (▼): vincristine, MDA435/LCC6; (▽): vincristine, MDA-MB-435; (●): taxol, MDA435/LCC6; (○): taxol, MDA-MB-435. B: (▼): MDA435/LCC6; (●): MDA-MB-435.

2/neu and bcl-2. Cathepsin D expression in 5 μm paraffin embedded sections of subcutaneous tumours was also determined. Initial assessment of VEGF was in supernatants collected from confluent cells. These data, shown in Figure 4.3A, indicate that there was almost a 3-fold increase in VEGF levels of MDA435/LCC6 cell supernatant when compared to levels produced by the parental line.

The VEGF levels in the ascites fluid collected from mice bearing ascitic MDA435/LCC6 tumours (Figure 4.3B) as measured by ELISA, increased as the tumour progressed. Between day 21 and day 28 there was a decline in the number of viable tumour cells in the peritoneum and this corresponded with the decline in peritoneal VEGF levels. As indicated in the insert to Figure 4.3B, serum VEGF levels in these mice steadily increased over the course of tumour progression with a more dramatic increase after day 21. This may have been due, in part, to the progressive breakdown of vascular beds in the peritoneum as judged by a significant increase in red cell contamination in the peritoneal cavity. It should be noted that plasma VEGF was not detected in animals bearing solid tumours derived from the LCC6 cell line. To determine if the increased VEGF level was also seen in solid tumours, I immunohistochemically stained tumour sections taken from mice bearing MDA-MB-435 and MDA435/LCC6 tumours for VEGF. The expression patterns for VEGF in the two tumours is shown in Figure 4.4, where sections derived from A431 tumours were used as a positive control for VEGF expression (panel A). VEGF protein staining of LCC6 tumour (panel E) was less than that observed for A431, but greater than the parental MDA-MB-435 (panel C).

HER-2/neu expression was also determined using several methods. Western analysis of

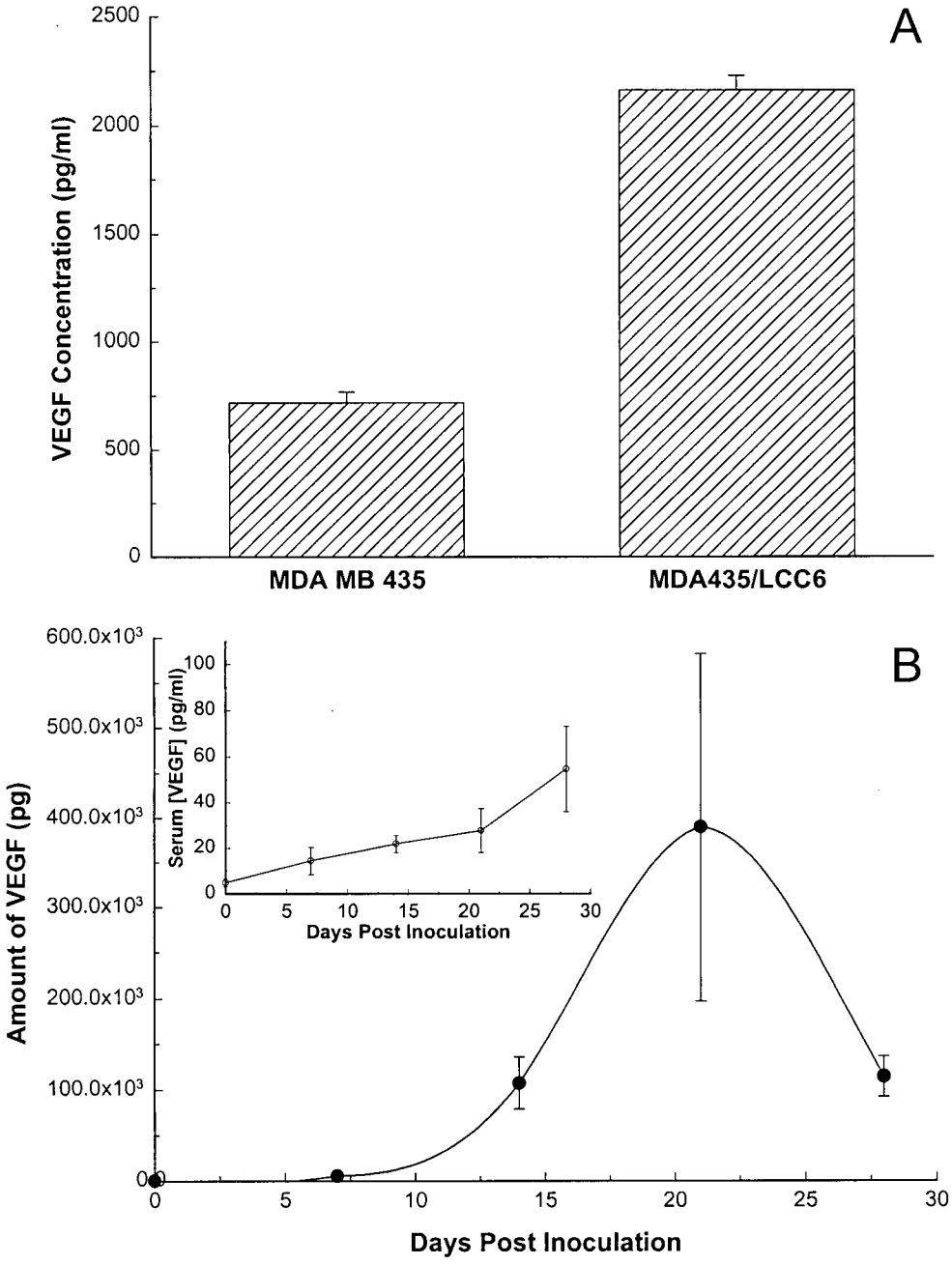

Figure 4.3 (A) In vitro VEGF levels in supernatant collected from confluent MDA-MB-435 and MDA435/LCC6 cells, measured by ELISA. Results in pg/ml ± standard error of the mean, and (B) in vivo VEGF levels from peritoneal fluid of MDA435/LCC6 derived ascites tumours, collected 0, 7, 14, 21 or 28 days post tumour cell inoculation. Inset shows serum VEGF concentration from these same animals. Ascites and serum were assayed by ELISA; results show pg and pg/ml, respectively, ± standard error of the mean.

parental and MDA435/LCC6 was completed using the highly overexpressing SK-BR-3 as a positive control. These data suggested that there was a 1.3 fold increase in the level of HER-2/neu protein in the LCC6, as compared to parental.

However, MDA-MB-435 HER-2/neu expression was 0.7% that of SKBR-3, and LCC6 HER-2/neu expression was 1 % that of SK-BR-3 as determined by flow cytometric analysis. These cell lines may be considered low expressers of p185. As may be seen in panels D (MDA-MB-435) and F (MDA435/LCC6) of Figure 4.4, when grown as a solid tumour neither cell type expressed sufficient levels of p185 for detection by immunohistochemistry.

Analysis of bcl-2 expression levels by flow cytometry (Figure 4.5, panels A MDA-MB-435, and B MDA435/LCC6) shows that these cells express equivalent levels of bcl-2. Western blot analysis (Figure 4.5, panel C), with the strongly bcl-2 expressing MCF-7 as a positive control, indicated an approximate two-fold increase in bcl-2 levels in MDA435/LCC6 as compared to parental as determined by densitometry. Immunohistochemical staining of solid tumour sections (Fig. 4.6) indicated that both MDA-MB-435 and MDA435/LCC6 have readily detectable levels of bcl-2.

Tumours with increased expression of the oestrogen regulated lysosomal protease cathepsin D have been correlated with increased invasion and metastasis of breast cancer. It was apparent from immunohistochemical staining (Figure 4.7) that MDA-MB-435 and MDA435/LCC6 express significant and approximately equivalent quantities of cathepsin D. How this relates to aggressiveness, likelihood for recurrence or metastatic potential has

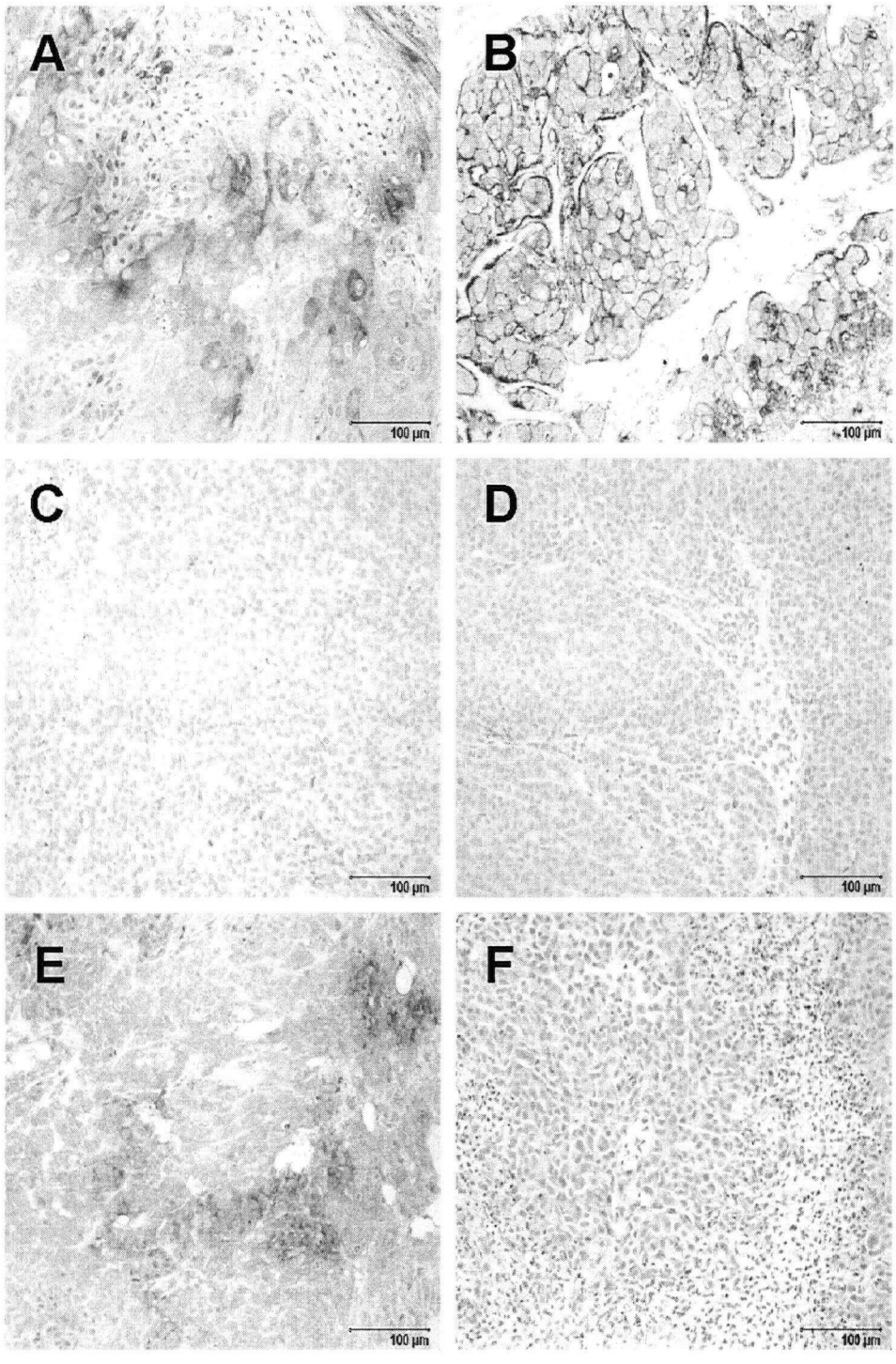

Figure 4.4 Immunohistochemical assessment of in vivo VEGF and HER-2/neu levels. 5 X 10^6 A431 (A), SK-BR-3 (B), MDA-MB-435 (C & D) or MDA435/LCC6 (E & F) were inoculated s.c. into female SCID/Rag2m mice. Tumours were collected in formalin before reaching 1 gram in size, paraffin embedded and stained immunohistochemically for VEGF (panels A, C and E) or HER-2/neu (panels B, D and F). Panels (A) and (B) are positive controls for VEGF and HER-2/neu staining, respectively. Visualisation was with diaminobenzidine, where brown areas indicate positive staining. Tissues were counterstained with Gill's hematoxylin.

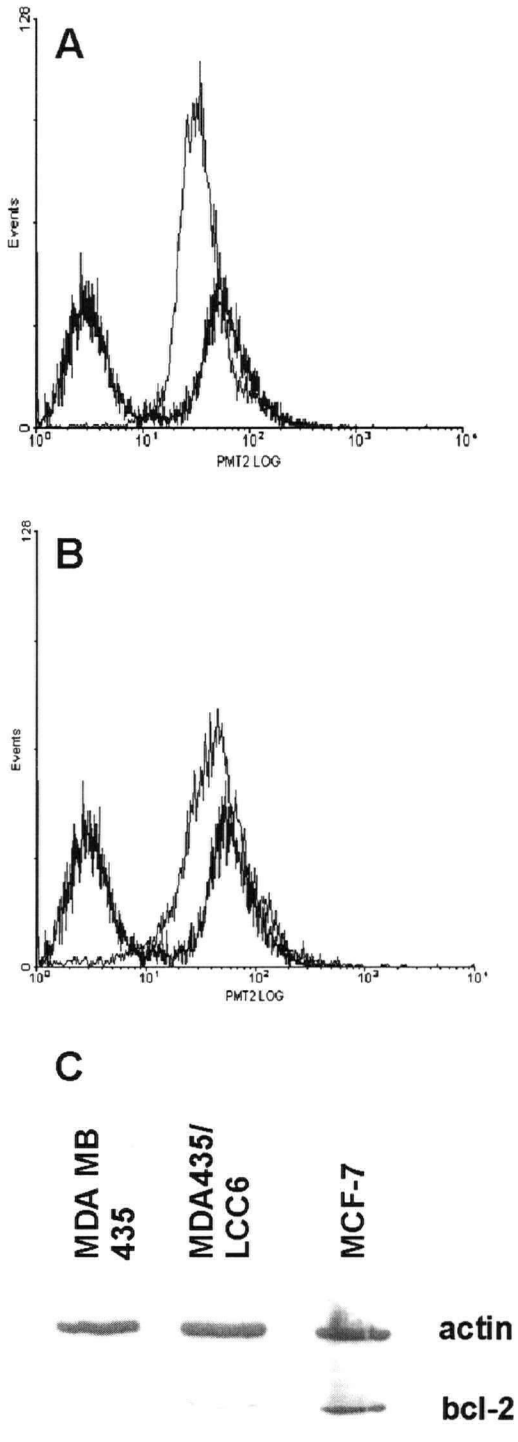

Figure 4.5 Bcl-2 protein levels in MDA-MB-435 and MDA435/LCC6 tumour cells. (A) flow cytometric representation of bcl-2 protein levels in MDA-MB-435 cells; (B) flow cytometric representation of bcl-2 protein levels in MDA435/LCC6 cells; (C) western blot of bcl-2 protein levels in MDA-MB-435, MDA435/LCC6 and MCF7 (positive control) cells.

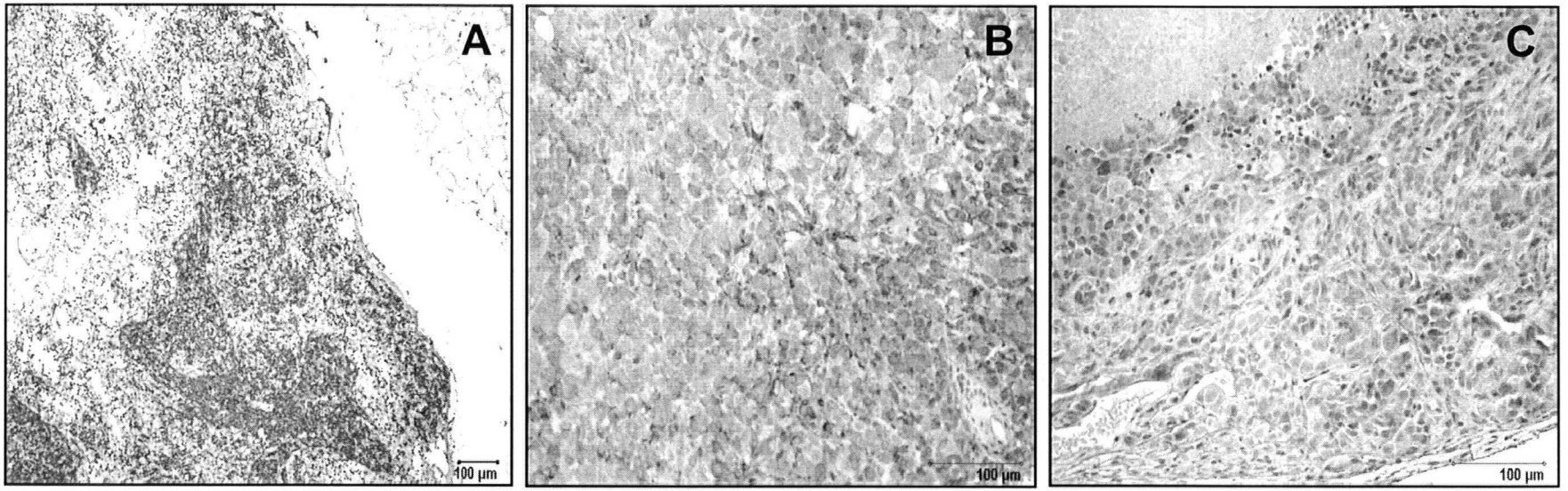

Figure 4.6 Bcl-2 staining on MDA-MB-435 (B) and MDA435/LCC6 (C) solid tumour sections. Panel A is a positive control for bcl-2 staining (lymphoma). Tumours were fixed in formalin, embedded in paraffin, and 5 µm sections prepared. Staining was as outlined in text. Visualisation was with DAB, where positive staining is indicated by brown areas in sections.

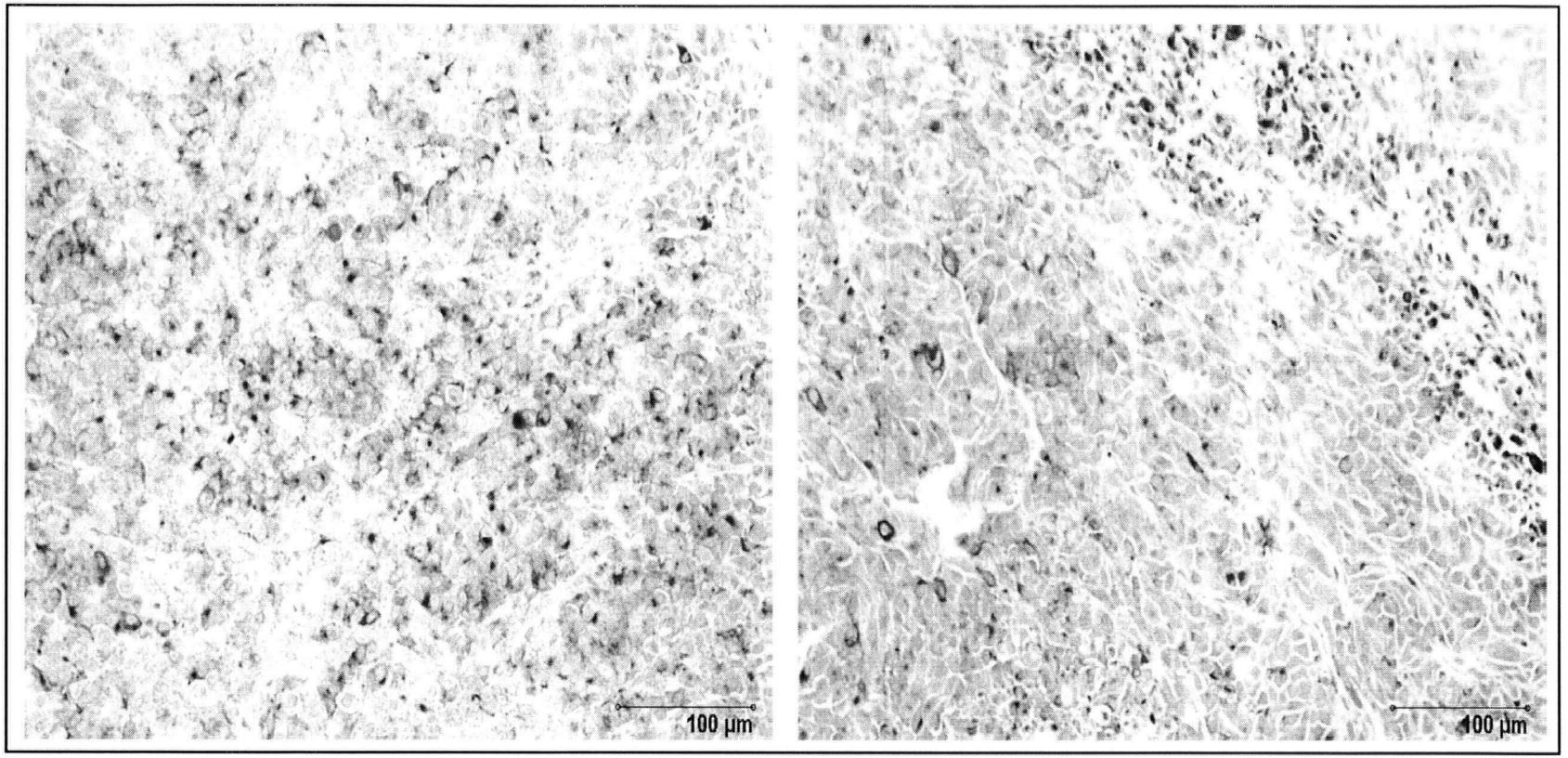

Figure 4.7 Cathepsin D staining on MDA-MB-435 (A) and MDA435/LCC6 (B) solid tumour sections. Tumours were fixed in formalin and embedded in paraffin. Sections (5µm) were affixed to slides and processed for cathepsin D staining as outlined in the text. Visualisation was with DAB, where brown areas represent points of positive staining for protein.

not been addressed in this study. The MDA-MB-435 model is a much slower growing tumour compared to the MDA435/LCC6 model, thus the cathepsin D is not an indicator of aggression as measured by growth rate; however it may reflect the potential of these cells to metastasise. This may be demonstrated by the fact that the original MDA-MB-435 cell line was derived from a pleural effusion (Callieau *et al.*, 1974) and the MDA435/LCC6 line is a metastatic variant of this line.

4.3 Discussion

Assessment of the potential clinical suitability of novel anticancer therapeutics typically includes the determination of anti-tumour activity in preclinical animal models of cancer, most of which are mouse based. Ideally, the anti-tumour response to the agent being tested should assist in predicting activity when used in a clinical setting. An associated correlate to this ideal is that the model used should also mimic the human disease state or condition. Because of this, researchers continue to strive for improved models of human disease. Historically, commonly used models for rapid screening and efficacy tests of novel anti-cancer agents have been the murine ascites models, L1210 and P388. These models provided the benefits of reproducibility, speed and certain similarities of response to chemotherapy observed for the human disease. One of the predominant drawbacks to these models is that they are murine in origin. In addition, the molecular defects associated with these murine malignancies may not be of relevance when considering events in human disease. Consequently, when considering the development of drugs for the treatment of aggressive breast cancer, it would be ideal if the model used expressed some or all of the attributes of an aggressive breast cancer in humans.

The MDA435/LCC6 ascites tumour model has been previously reported to be a fast growing and reproducible xenograft model of human cancer. This cell line is a direct derivative of the oestrogen receptor negative, invasive and metastatic MDA-MB-435 cell line (Cailleau *et al.*, 1974; Price *et al.*, 1990), and has been characterised as such by isotype and karyotype analysis (Leonessa *et al.*, 1996). The cells were isolated from a spontaneous ascites that developed in a NCr nu/nu athymic nude mouse bearing a MDA-MB-435 tumour in the mammary fat pad. In this study, further characterisation of the MDA435/LCC6 cell line has been presented, focusing on markers associated with aggressive breast cancer in humans. Protein expression was characterised for VEGF, HER-2/neu, bcl-2 and cathepsin D, and correlating the parameters with tumour growth properties and response to chemotherapy.

Many tumour cells express and secrete VEGF at high levels, with the effect of inducing the formation of new vasculature to promote tumour growth. VEGF is able to induce angiogenesis in two ways. Primarily, it does so by means of specific mitogenic activity on endothelial cells, but it also indirectly contributes to angiogenesis by its ability to induce vascular permeability with a potency some 50 000 times that of histamine (Collins *et al.*, 1993), thereby influencing the egress of plasma proteins that are able to stimulate angiogenesis. The LCC6 variant has been shown here to have VEGF expression some three times higher than the parental MDA-MB-435. Therefore tumours derived from this cell line may have an increased ability to promote tumour vascularisation, with associated accelerated growth. This may be a contributing factor in MDA435/LCC6's intrinsically

more aggressive behaviour, as reflected in both *in vitro* and *in vivo* growth rates (Figure 4.1). Importantly, when combined with the ease of growth of this cell line as an ascitic model, a factor which may be attributable to overexpression of VEGF (Luo *et al.*, 1998), the LCC6 variant may be seen as a powerful model for studies of tumour angiogenesis and anti-angiogenic agents.

It has been suggested that the co-expression of molecular markers in neoplastic transformation functions as a significant prognostic indicator for recurrence, and that the number of co-expressed markers is directly related to this significance (Han *et al.*, 1997). I examined the expression of four markers of aggressive breast disease in this study; p185, bcl-2, VEGF and cathepsin D. Neither the MDA-MB-435 or the MDA435/LCC6 tumours have detectable p185 expression in the solid tumours, as determined by immunohistochemistry, although western blot analysis data did indicate that these cell lines each express low levels of the protein. Although they are not strong overexpressers of bcl-2, both MDA-MB-435 and MDA435/LCC6 do express this protein, and therefore may be used as representative models for therapies targeting either the protein itself, or a component of the apoptotic pathway mediated by bcl-2.

Although perhaps not significant when expressed singly, when the expression of cathepsin D is combined with p53, nm23 (not assessed) and HER-2/neu expression, there is an 80% agreement between positive expression of all of these markers, and recurrence of disease (increased from 47.1% when cathepsin D is not included) (Han *et al.*, 1997). Both the parental MDA-MB-435 and the LCC6 variant have strong expression of this protein as

determined by immunohistochemistry.

The LCC6 variant is an extremely versatile tumour model in that it grows more rapidly both *in vitro* and *in vivo* than the widely used parental MDA-MB-435 cell line and can also be employed as an ascites tumour model. In comparison, the parental line can only be grown as a solid tumour and expresses decreased levels of VEGF compared to the LCC6 line. The ease of growth as an ascitic tumour for MDA435/LCC6 cells lends the added benefit that cells can be easily removed from the peritoneum and assayed by various means, such as western blot or flow cytometric analysis. For the above reasons, the MDA435/LCC6 model of human breast cancer has the potential to be a powerful tumour model in the analysis of human response to breast cancer therapies.

Chapter 5

PHARMACODYNAMIC BEHAVIOUR OF LIPOSOMAL ANTISENSE OLIGONUCLEOTIDES TARGETING HER-2/NEU AND VASCULAR ENDOTHELIAL GROWTH FACTOR AS MEASURED IN THE ASCITIC MDA435/LCC6 HUMAN BREAST CANCER MODEL

5.1 Introduction

The previous chapter of this thesis dealt with the characterisation of the MDA435/LCC6 cell line as a xenograft model of aggressive human breast cancer. It was shown that these cells grow well both *in vitro* and *in vivo* (Fig. 4.1), and importantly, will grow as both solid and ascitic tumours. Based upon the finding that the MDA435/LCC6 tumours express increased amounts of vascular endothelial growth factor (Figs. 4.3 & 4.4), it was reasonable to proceed to test antisense oligonucleotides targeted against this protein in the model. In addition, ODN targeted against the HER-2/neu protein were shown to be efficacious in the low HER-2/neu expressing tumour model, MDA-MB-435. It followed then, that this treatment modality may be effective in another model with low HER-2/neu expression, namely the MDA435/LCC6 model.

Although significant efficacy, as judged by delays in tumour growth or increases in the percentage of long term survivors, is the ultimate goal to consider when developing new therapeutics, it is imperative to gain an understanding of the pharmacokinetic and pharmacodistribution behaviour of the agent being tested. With this in mind, the MDA435/LCC6 model was used to assess the pharmacodynamic behaviour of the two

antisense oligonucleotide sequences designed to inhibit expression of VEGF and HER-2/neu. ODNs are designed to be far more specific than traditional chemotherapeutics, yet they can be designed to have chemical and pharmaceutical attributes that make applications of this form gene therapy similar to conventional therapeutic agents. ODNs have been extensively researched as pharmaceutical agents, however even with the chemical modification of the phosphate backbone to a more stable phosphorothioate bond, they are rapidly cleared from the circulation, primarily by the kidneys (Levin, 1999). Although ODNs are efficacious when given *in vivo*, activity is often dependent on use of aggressive dose scheduling, involving daily injections for time periods in excess of two weeks, or use of micro-infusion pumps (Akhtar and Agrawal, 1997). There is therefore, a push to develop formulations which demonstrate enhanced circulation longevity, as well as increased delivery of the ODN to sites where the target cells are localised. A well established method to achieve this involves the use of lipid based carriers (see section 1.4.1), leading to the experiments detailed in this chapter, wherein lipid carriers were utilised for delivery of antisense oligonucleotides to tumour cells *in vivo*. The two antisense oligonucleotides utilised in these studies were a 21-mer with a phosphorothioate backbone, targeted against human VEGF with demonstrated activity against production of this protein in an *in vitro* model (Masood *et al.*, 1997), and a 15-mer phosphorothioate ODN specific for the area of HER-2/neu mRNA immediately upstream of the initiation codon (Vaughn *et al.*, 1996; Saxon *et al.*, 2000). These antisense sequences were encapsulated in a novel liposomal formulation, recently described by Semple *et al.* (2001) (Section 2.8), and their pharmacodynamic behaviour, delivered in free or liposomal form was determined using the MDA435/LCC6 ascites model.

5.2 Results

5.2.1 Plasma Elimination and Tissue Distribution of i.v. or i.p. Injected Free or Liposomal Antisense

The plasma elimination of antisense, free or liposomal, was determined after i.v. and i.p. administration. These data have been summarised in Figure 5.1, which incorporates plasma antisense levels as measured using tracer levels of [^{3}H]-H2A-ODN and plasma liposomal lipid levels as determined by a [^{14}C] labelled CHE. It is important to note that the antisense levels measured may not represent the intact sequence and may be due to a radiolabelled metabolite. The plasma antisense levels obtained following i.v. (Fig. 5.1A) and i.p. (Fig. 5.1B) injection of free (open circles) or liposome encapsulated (open triangles) H2A-ODN at an antisense dose of 10 mg/kg, clearly illustrate the primary benefit, enhanced plasma AUC attributable to use of liposomal carrier systems. Free antisense oligonucleotides were eliminated rapidly following i.v. injection, with greater than 95% of the injected dose eliminated within the first time point (1 hr). When free ODN was given by i.p. injection, the blood levels were slightly higher at the first time point and this is reflected by a slight increase in mean $AUC_{0\text{-}24h}$ obtained when the drug was given i.p. (see Table 5.1). In contrast, when the liposomal formulation was given i.v., there were significant increases in circulating blood levels of ODNs at every time point evaluated and this was reflected by the substantial increase in plasma $AUC_{0\text{-}24h}$ when comparing the liposomal formulation to the free ODN (Table 5.1). Similar increases in plasma mean

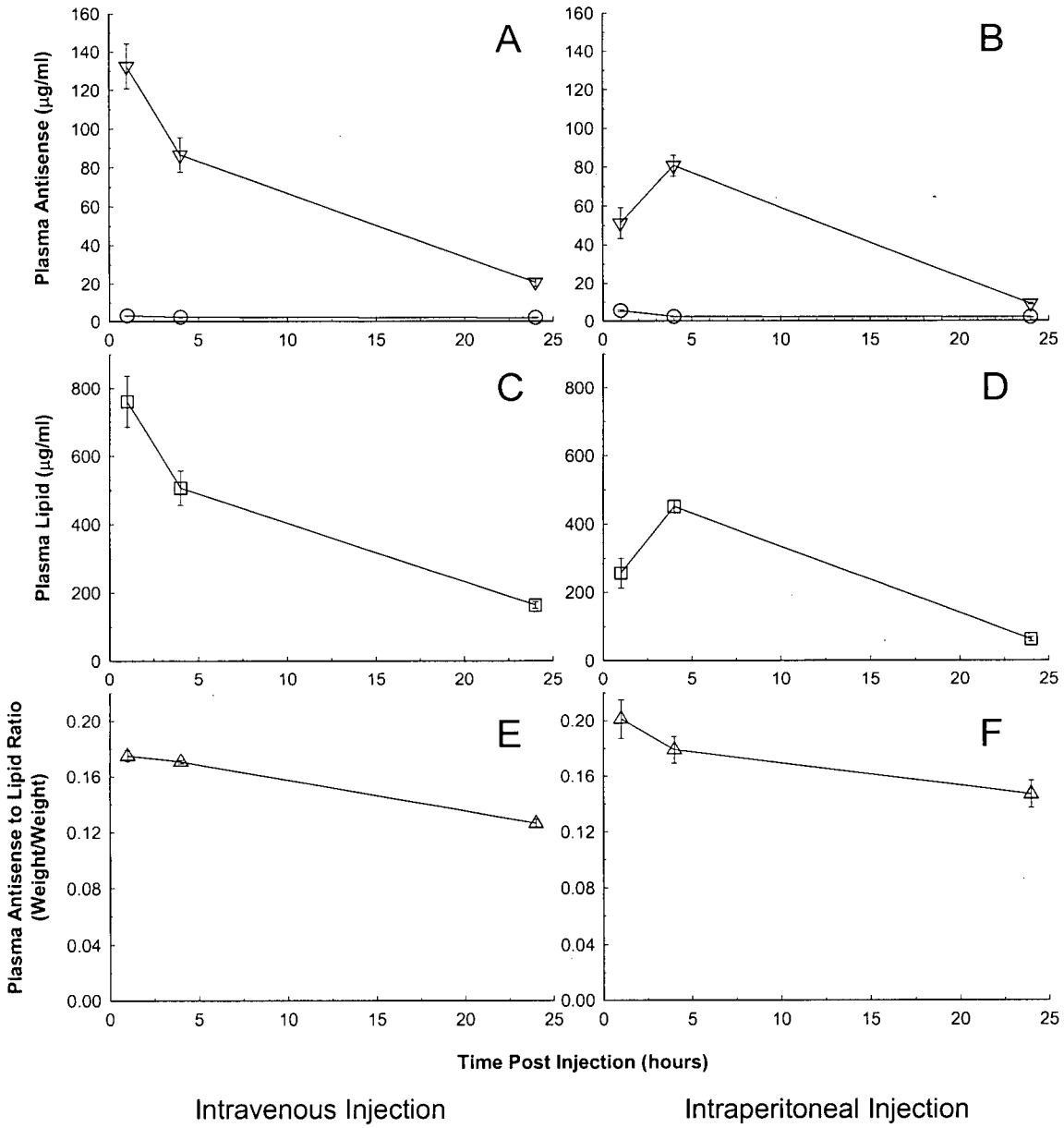

Fig. 5.1. Plasma antisense, lipid and antisense to lipid ratios following i.v. or i.p. injection of free or liposomal H2A-ODN at an antisense dose of 10 mg/kg into tumour-free female SCID/Rag2m mice. (A & B) free and liposomal antisense plasma levels following i.v. or i.p. injection, respectively. (C & D) plasma lipid levels following i.v. or i.p. injections, respectively. (E & F) plasma antisense to lipid ratios following i.v. or i.p. injections, respectively, of liposomal antisense. (▽) liposomal ODN; (○) free ODN; (□) liposomal lipid. Results are the mean of 4 ± standard error of the mean.

AUC_{0-24h} were observed when the liposomal formulation was given by i.p. injection (Fig. 5.1B). As expected however, the levels in the plasma at the 1h time point following i.p injection were significantly ($p < 0.005$) lower than those obtained following i.v. administration. At 4h, the level of ODN in the plasma was equivalent for both the i.p. and i.v. route of administration and this is consistent with the observations that liposomes given i.p. rapidly enter the blood via the lymphatics (M. Bally, unpublished observation).

Table 5.1 Mean AUC from 0 – 24 hours for free and liposomal ODN given both intraperitoneally and intravascularly. Values are derived by the standard trapezoidal formula and are from the mean of a minimum of 4 animals.

Free ODN i.v. (μg·h/ml.)	Free ODN i.p. (μg·h/ml.)	Liposomal ODN i.v. (μg·h/ml.)	Liposomal ODN i.p. (μg·h/ml.)
148.9	153.8	1565.7	1219.7

The plasma liposomal lipid levels (Figure 5.1 C and D) indicate that this liposomal formulation, when given i.v., exhibits long circulation lifetimes. As much as 20% of the injected lipid dose was still present in the plasma compartment 24h after injection. The plasma levels of lipid obtained after i.p. injection were lower and less than 10% of the injected lipid dose could be found in the plasma compartment at 24h. Figure 5.1 E and F includes calculated data based on the ratio of measured plasma ODN concentration and measured plasma liposomal lipid concentration. This ratio is used as an indicator of the stability of the liposomal formulation in the plasma compartment. A decrease in the ODN to lipid ratio is indicative of antisense release (dissociation) from the liposomes. The results suggest that over the 24h time course there was a steady decrease in this ratio indicating that the antisense sequence can be released from the liposomes within the plasma

compartment. It is assumed that the majority of ODN within the plasma compartment is associated with the liposomal carrier.

Similar plasma elimination studies were conducted in MDA435/LCC6 tumour bearing SCID mice, where mice were injected i.p. with 2 X 10^6 MDA435/LCC6 cells and 17 days later they were injected i.v. with either free or liposomal antisense at an antisense dose of 10 mg/kg. The plasma ODN levels have been summarised in Figure 5.2 for both the H2A-ODN (Fig. 5.2A) and the VEGF-ODN (Fig. 5.2B). The data in Figure 5.2A is very comparable to the plasma elimination data obtained in tumour free mice, suggesting that the presence of an established tumour has little impact on the elimination of either the free or liposomal ODN. In addition, the data in Figure 5.2 suggests that the plasma elimination behaviour of both ODN sequences are also comparable, even though the H2A-ODN is a 15 nucleotide sequence and the VEGF-ODN is a 21 nucleotide sequence. As was the case for non-tumour-bearing mice, the encapsulated antisense exhibited a much greater plasma residence time for each of these oligonucleotides, with 4 hour levels being approximately 50-fold higher for liposomal formulation as compared to free. Mean AUC_{0-24} analysis of these data demonstrated that the liposomal formulation provides for a 10-fold increase in mean plasma AUC_{0-24} when compared to the free ODN (see Table 5.1).

The tissue distribution of ODN and liposomal lipid following i.v. administration of the VEGF-ODN was also determined and the results have been summarised in Table 5.2 for both free and liposomal ODN. There are two important points that can be made on the basis of the data in Table 5.2. First, the highest tissue levels of ODN obtained after

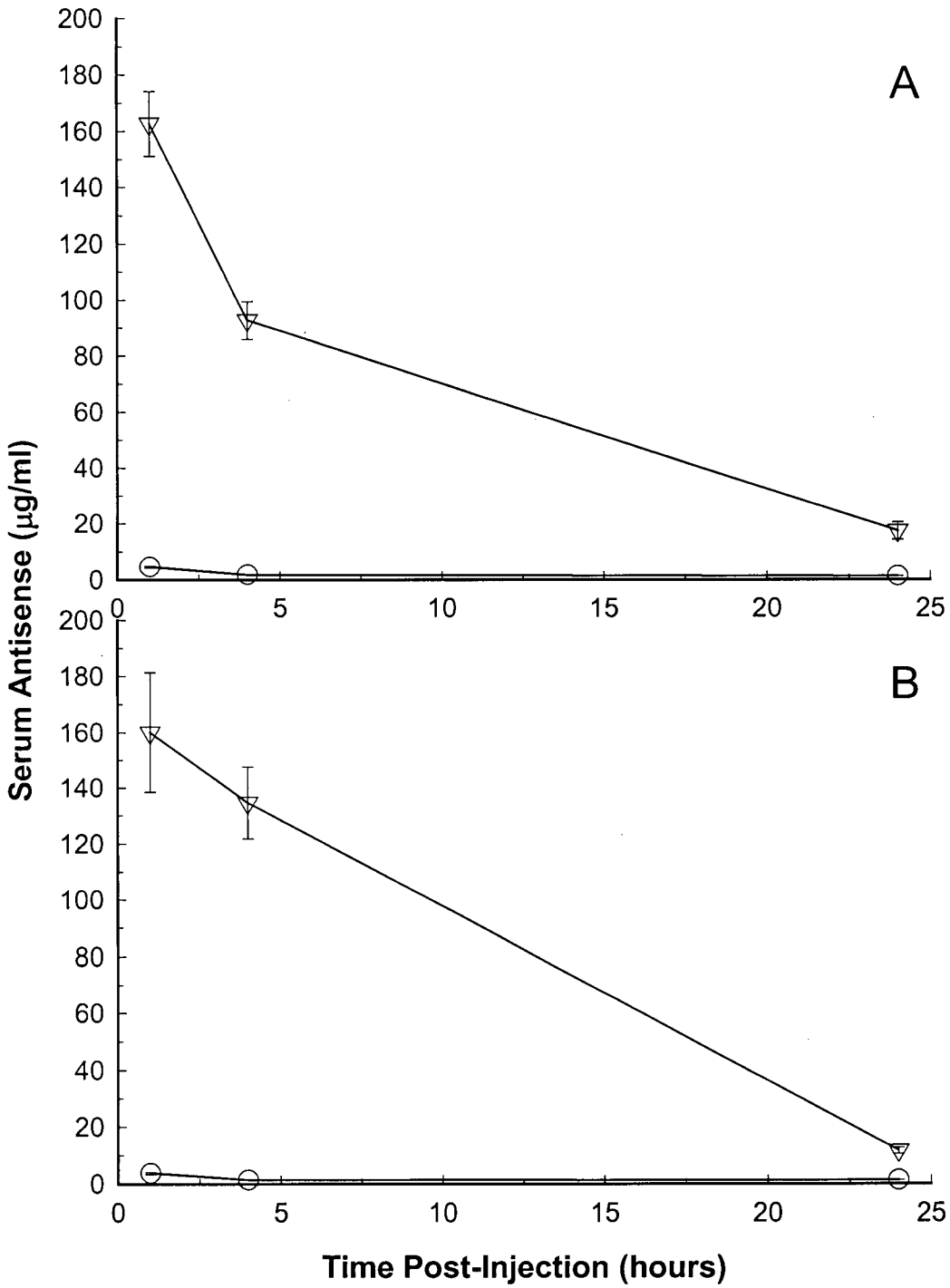

Fig. 5.2 Serum antisense levels in MDA435/LCC6 ascites tumour-bearing female SCID/Rag2m mice following injection of free or liposomal (A) H2A-ODN or (B) VEGF-ODN. Antisense was administered on day 17 post tumour cell inoculation. (▽) liposomal ODN; (○) free ODN. Results are the mean of 4 ± standard error of the mean.

administration of the unencapsulated form was in the kidney. This is consistent with previous studies (Levin, 1999) and is indicative of rapid renal clearance of this agent. Second, the levels of ODN in the spleen and liver were equivalent on a weight basis and the levels within these tissues were reasonably constant over the 24h time course. Liver accumulation of ODN accounted for less than 10% of the injected ODN dose, while the spleen accumulation accounted for less than 1% of the injected dose. When comparing these data to the liposomal formulation (Table 5.2) there are several notable differences. First, liver and spleen ODN levels were substantially higher when the ODN was given in liposomal form. Liver levels were almost 3-fold greater in animals given liposomal ODN and spleen levels were 7 to 10-fold greater, depending on the time point. This increase in spleen and liver delivery is a consequence of the natural tendency of liposomes to accumulate in these organs. Second, the level of ODN in the kidney was at least 5-fold lower when the ODN was given in the liposomal form, an observation that is consistent with the fact that liposomes do not readily accumulate in organs such as the kidney (liposomal lipid levels were below detectable limits at the 1 and 4 hour time points). Similarly, the muscle ODN and lipid levels were below detectable limits, a consequence of little or no liposome accumulation in muscle tissue. It can be concluded from the data in Table 5.2 that the distribution of ODN, when given in the liposomal formulation is dictated by the biodistribution behaviour of the liposomes. It is worth noting that ODN to lipid ratios, estimated on the basis of the biodistribution data in Table 5.2, were lower than those measured in the plasma compartment (see Figure 5.1E) and suggest that the ODN was lost from the liposomes both during and after tissue localisation. Interestingly, the ODN to

Table 5.2 Tissue antisense and lipid levels expressed as μg per g tissue, following i.v. injection of free or liposome encapsulated VEGF-ODN at an antisense dose of 10 mg/kg (mean ± standard error of the mean) in MDA435/LCC6 ascites tumour-bearing female SCID/Rag2m mice. Antisense was administered day 17 post tumour cell inoculation.

		Antisense			**Lipid**		
	Tissue	1 hour	4 hours	24 hours	1 hour	4 hours	24 hours
Free ODN	Liver	14.29±1.27	18.72±0.79	19.38±0.93			
	Lung	3.18±0.54	2.36±0.10	1.10±0.07			
	Spleen	13.37±1.79	14.43±1.35	12.07±1.12			
	Kidney	50.35±9.11	61.58±1.64	64.05±5.98			
	Muscle	1.02±0.33	0.94±0	0.84±0.17			
Liposomal ODN	Liver	41.4±1.3	48.8±4.3	54.3±11	557.9±36.5	610.8±115.7	805.1±240.2
	Lung	N.D.[a]	2.4±1.3	2.3±0.6	N.D.	30.3±41.0	31.6±24.7
	Spleen	92.2±9.4	148.5±17.2	138.7±29.2	884.7±182.9	755.9±184.3	1454±225.9
	Kidney	5.5±1.0	9.3±0.6	14.0±1.9	N.D.	N.D.	36.24±13.50
	Muscle	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a]N.D. Below detectable limits which were set at a dpm value 2 times greater than background

lipid ratio in the kidney, (estimated at the 24h time point to be 0.4), was almost 3-fold higher than that measured in the plasma compartment. This is consistent with the fact that the kidney is the primary organ for ODN removal, once the ODN has been released from the liposomes.

One of the benefits of utilising a tumour model where the cells grow as an ascitic tumour is that it provides easy access to the cell populations residing within the peritoneal cavity and this, in turn, means that drug delivery and associated cellular effects can be readily measured. To assess antisense delivery to the peritoneal cavity, MDA435/LCC6 tumour bearing animals (prepared as described for the studies shown in Figure 5.2) were given an i.v. injection of free or liposomal VEGF-ODN on day 17 post-tumour cell inoculation. At 1, 4 and 24 hours post antisense injection, peritoneal fluid and associated cells were recovered by peritoneal lavage, as described in sections 2.14.4 and 2.14.5. The results from this study are summarised in Fig. 5.3. The levels of ODN in the cell-free peritoneal fluid were comparable when the ODN was given as a free or liposomal formulation. This result was surprising considering that there was a substantial increase in the blood levels and circulation lifetime for the liposomal formulation. For conventional small molecule anticancer drugs, delivery in a liposomal carrier typically results in substantial increases in peritoneal cavity delivery, both in ascites tumour bearing and tumour free animals (Bally *et al.*, 1994). It should be noted that the level of delivery is quite low, with as little as 1% of the injected dose accessing the peritoneal cavity at 24h. Another important conclusion made regarding the data in Figure 5.3 concerns the increase in peritoneal cell delivery achieved when using the liposomal formulation. Although the error in this analysis is large,

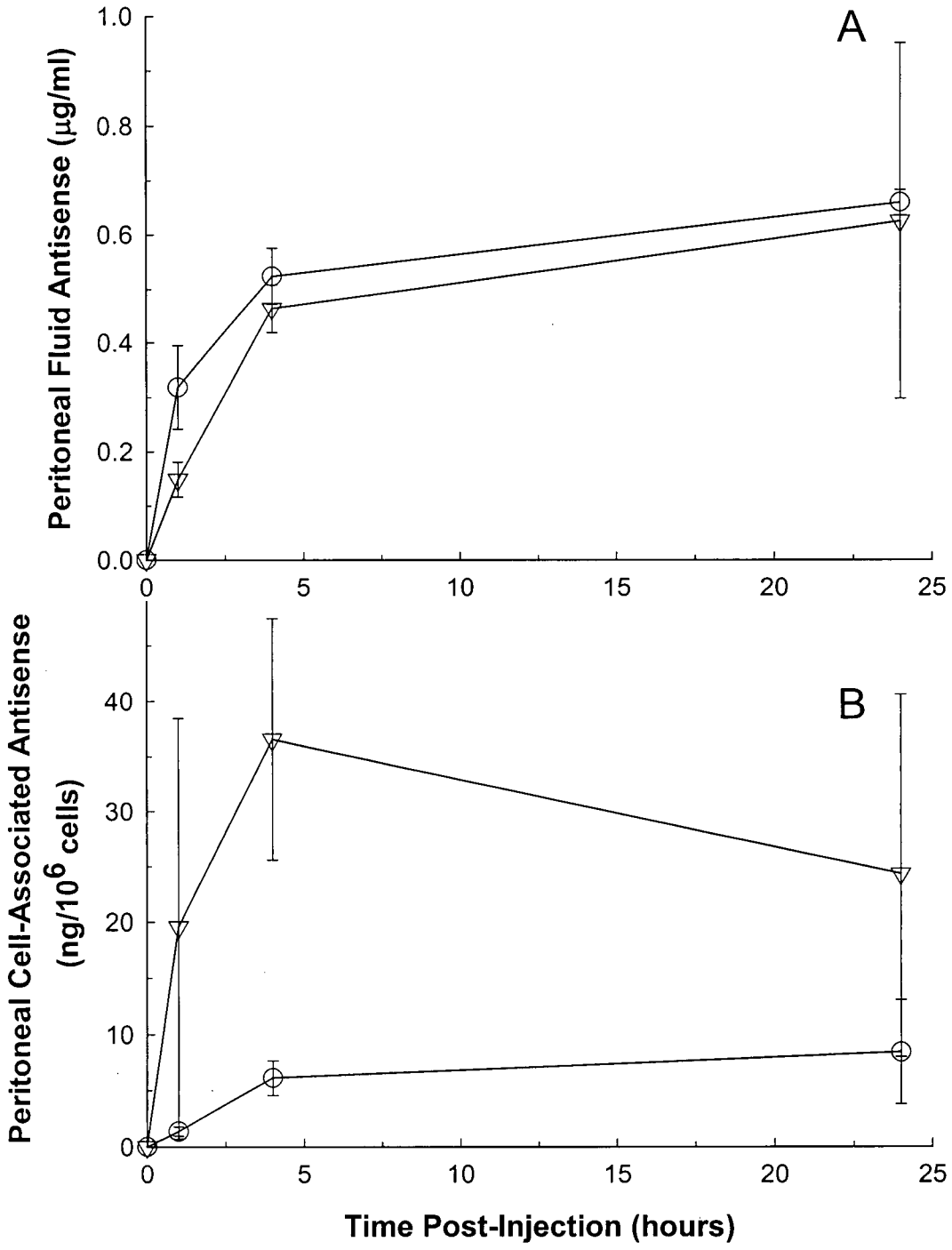

Fig. 5.3 Peritoneal fluid (A) and peritoneal cell-associated (B) antisense levels following administration of free or liposomal VEGF-ODN at an antisense dose of 10 mg/kg into female SCID/Rag2m MDA435/LCC6 ascites tumour-bearing mice. Antisense was administered day 17 post tumour cell inoculation. (∇) liposomal ODN; (O) free ODN. Results are the mean of 4 ± standard error of the mean.

and significant differences were only notable at the 4h time point ($p<0.05$), it is evident that there was a substantial increase in cell delivery. As much as 3 to 5 times greater peritoneal cell associated ODN was found following liposomal ODN administration than that achieved when the free ODN was given.

5.3 Discussion

Liposomes are advantageous for the delivery of many therapeutics as they are able to extend the circulation lifetime as well as engendering significant increases in the plasma concentration of the associated agent (see section 1.4.1). The increased circulation lifetime and plasma concentration have been shown to effect increases in drug accumulation at sites of disease, particularly those diseases which cause damage to the surrounding blood vessels. This is particularly true for sites of tumour growth where there is induction of neovasculature and the release of factors, such as VEGF, which cause increases in blood vessel permeability to circulating macromolecules (Nagy *et al*, 1989; Bally *et al.*, 1994). Studies demonstrating the therapeutic activity of ODNs *in vivo* have had to rely on prolonged administration of the drug in free form, therefore it was anticipated that liposomal formulations of ODN should increase delivery of the intact sequence to sites of disease and reduce the frequency of drug administration required with the free form.

The pharmacokinetic benefits of administering ODNs in a liposomal formulation have been illustrated well by the data presented in this chapter, where the mean plasma $AUC_{0\text{-}24h}$ was more than 10-fold greater than that achieved following i.v. administration of free ODN (see

Table 5.1) and the biodistribution of the ODN was dictated by the biodistribution of the liposomal carrier (see Table 5.2).

Importantly, the work presented in this chapter demonstrated the feasibility of systemic ODN delivery to a tumour site using a liposomal carrier formulation, and moreover, that a greater amount of ODN was delivered to tumour cells when encapsulated within the liposomes. Also of note is that the size of the ODN molecule did not alter the pharmacodynamics of the formulation (Fig. 5.2), with the plasma elimination of both the 15 and the 21 base ODN being equivalent.

Having established enhanced delivery of encapsulated ODN to tumour cells in an ascites model of human breast cancer expressing increased levels of vascular endothelial growth factor, the logical progression of experiments was to proceed with efficacy studies using the liposomal VEGF-ODN as well as the liposomal H2A-ODN in this model for the reasons outlined in section 5.1.

Chapter 6

EFFICACY OF LIPOSOMAL ANTISENSE OLIGONUCLEOTIDES IN THE MDA435/LCC6 HUMAN BREAST CANCER XENOGRAFT MODEL

6.1 Introduction

The humanised monoclonal anti-HER-2/neu antibody trastuzumab is available for treatment of those patients with a strongly positive (3+ category as defined by the FDA approved HercepTest) level of expression of HER-2/neu, which is also corroborated with HER-2/neu gene amplification. By the very nature of treatment acceptance criteria, there are many breast cancer patients who do have some degree of HER-2/neu overexpression, but who do not qualify for trastuzumab treatment. In addition, there are those patients whose tumours are not classified as being overexpressers of HER-2/neu, but who may benefit from HER-2/neu targeted therapy as evidenced by the data presented in chapter 3 of this thesis, in which it was demonstrated that antisense ODN directed against HER-2/neu mRNA was efficacious in the treatment of mice bearing human xenograft tumours with low expression of HER-2/neu.

The use of antisense oligonucleotides in these settings is attractive due to some of the drawbacks of trastuzumab therapy. Some of these have been mentioned in section 1.3.3, however in addition, it is known that the external portion of the p185 molecule can be shed into the circulation (Pupa *et al.*, 1993). This shedding of the receptor has two main implications from a therapeutic perspective. Patients with high levels of circulating shed

HER-2/neu may be resistant to the antiproliferative effects of trastuzumab by virtue of antibody plasma sequestration by binding to the circulating shed receptor, thus limiting the number of receptors in the plasma membranes that are available to bind to the antibody. In addition, it may be possible that the truncated fragment of the receptor resulting from the cleavage process is more oncogenic than the full receptor itself. Each of these possibilities suggest that a treatment modality other than antibodies which bind to the extracellular portion of the p185 molecule might be preferable. Antisense oligonucleotides fit this requirement, and may in addition be beneficial to a larger subset of patients than is trastuzumab by being beneficial to those patients with low p185 expression.

Experiments with liposomally encapsulated H2A-ODN in a model of low p185 expression have shown some degree of efficacy of this molecule (MDA-MB-435; chapter 3). As was discussed in chapter 4 however, the MDA-MB-435 model was inferior to MDA435/LCC6 in several regards, including time to experimental conclusion (see Fig. 4.1) and versatility of tumour growth (i.e., solid and ascites in the case of MDA435/LCC6). These features, and its low HER-2/neu expression combine to make the MDA435/LCC6 model an attractive and obvious choice for continuation of efficacy experiments with H2A-ODN. It was also important to verify the positive results obtained in the MDA-MB-435 model following treatment with liposomal H2A-ODN (Fig 3.12) by using another model with similar low levels of p185 expression.

Her-2/neu is one oncogene implicated in aggressive and metastatic breast cancer. As discussed earlier, there are several proteins and/or pathways which may be involved in

tumorigenesis, tumour progression and metastatic spread. One of these, as detailed in section 4.1, is vascular endothelial growth factor. The MDA435/LCC6 model has a VEGF expression level approximately three times that of the parental MDA-MB-435, thus providing an ideal model for testing the efficacy of antisense targeted against VEGF. VEGF is also an ideal target as there have been several studies linking the expression and/or stimulation of HER-2/neu with that of VEGF (Petit *et al*, 1997; Xiong *et al.*, 2001).

Preliminary experiments with liposomally encapsulated antisense oligonucleotides (ODN) targeted toward the mRNA of the HER-2/neu proto-oncogene provided conflicting results, as detailed in chapter 3. It is probable that cancer cells do not rely upon a single protein or pathway in order to outgrow their surroundings and spread to other locations in the body. Rather, they are more likely to have in place redundant systems, such that if one pathway is shut down, they are still able to proliferate and spread by relying on another. This theory is supported in part by the well known fact that in many cases, a combination of drugs is more effective than a single chemotherapeutic agent in combating cancer. A mixture of different drugs will target different pathways within the cell, for example, mitotic spindle assembly or DNA replication, thus limiting the repertoire of the cancer cell, and improving the chances for total cancer cell kill.

This reasoning led to the experiments described within this chapter, in which two different antisense oligonucleotides have been tested for efficacy in the MDA435/LCC6 model, namely H2A-ODN and VEGF-ODN. It is hoped that once all parameters of single ODN therapy have been established, it will be possible to combine two or more ODN in the

treatment of aggressive breast cancer. Prior to this happening however, a complete understanding of the effects and downstream events of therapy with a single ODN must be firmly established.

6.2 Results

6.2.1 Treatment of MDA435/LCC6 Ascites Tumour Bearing Mice with H2A-ODN

Female mice were inoculated with MDA435/LCC6 cells as described in section 2.13.2 for formation of ascites tumours. On day 3 post inoculation, 5 times weekly treatment with either free or liposomal H2A-ODN was initiated. Following a total of 15 ODN injections at an antisense dose of 10 mg/kg, peritoneal fluid and cells were harvested by peritoneal lavage such that both tumour burden and p185 expression could be evaluated. Flow cytometric analysis of HER-2/neu expression in peritoneal tumour cells (Fig. 6.1) indicated a decrease in expression of HER-2/neu such that tumour cells removed from free H2A-ODN-treated mice had a HER-2/neu expression equivalent to 60% that of control untreated mice. This benefit was further enhanced following treatment with liposomal H2A-ODN, where the expression level of HER-2/neu in the treated mice was equivalent to 21% that of HER-2/neu expression in tumour cells harvested from untreated mice.

The total number of cells recovered from the peritoneal cavities of those mice treated with liposomal H2A-ODN was significantly less than the control, untreated mice (figure 6.2a). In addition, when cells were spun down onto slides and stained (modified Wright – Giemsa stain), those cells which were present in the peritoneal cavities of liposomal H2A-ODN

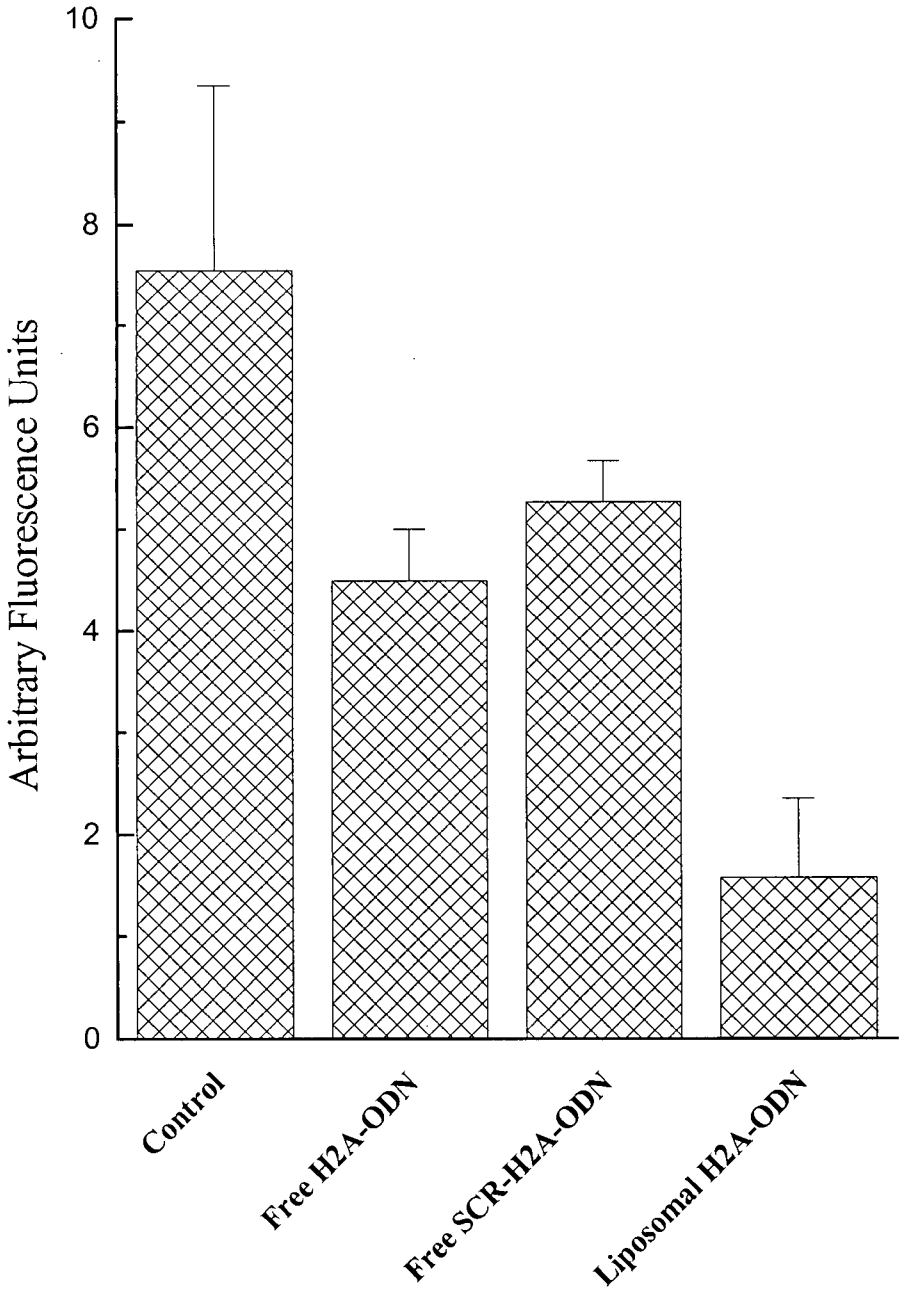

Figure 6.1 HER-2/neu expression in peritoneal tumour cells taken from mice bearing MDA435/LCC6 ascites tumours following treatment with saline, free H2A-ODN or free SCR-H2A-ODN. Results represent the mean ± standard error of the mean of flow cytometric data derived from equivalent numbers of cells from a minimum of 4 female SCID/Rag2m mice.

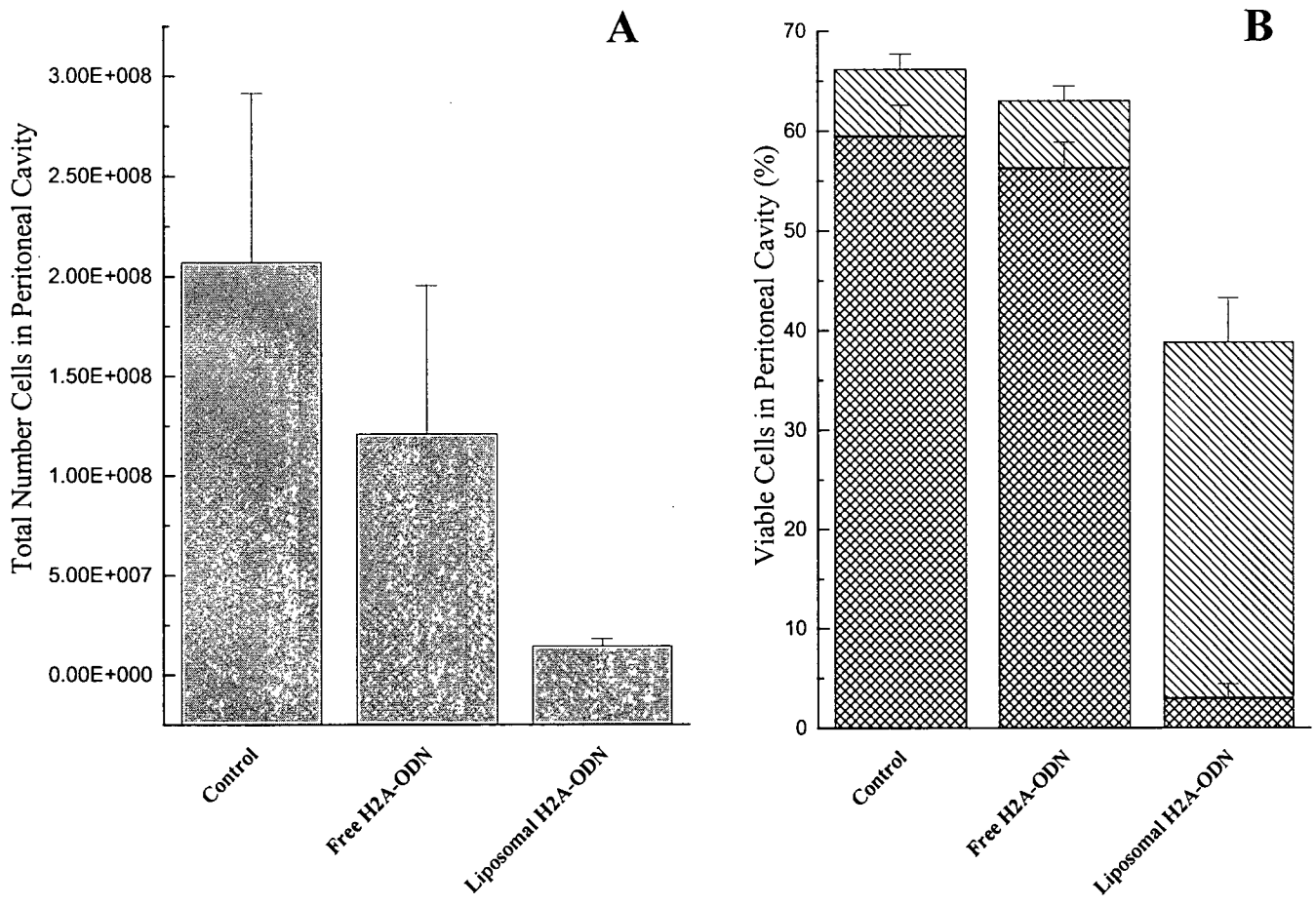

Figure 6.2 Tumour burden (A) and percent murine(slashed)/human (hatched) cells (B) in MDA435/LCC6 tumour bearing female SCID/Rag2m mice following treatment with free H2A-ODN or liposomal H2A-ODN. Results are the mean ± standard error of the mean of a minimum of 4 animals.

treated mice were predominantly mouse reactive cells, such as neutrophils, macrophages, and possibly natural killer cells (Figure 6.3), as determined by staining with the anti-CD90 antibody, Thy-1. In fact, when the total number of viable human cells from the peritoneal cavities of these mice was estimated (Figure 6.2b), those mice treated with liposomal H2A-ODN had only 5% the number of tumour cells as compared to untreated controls ($p<0.05$). This dramatic reduction in the number of peritoneal tumour cells was not observed in mice treated with free H2A-ODN, signifying the utility of the liposomal carrier employed in these studies.

6.2.2 Treatment of MDA435/LCC6 Ascites Tumour Bearing Mice with Trastuzumab

It was not possible to correctly interpret the above results without the inclusion of an empty liposome control. As well, it was important to include a trastuzumab treatment group for a reference. Trastuzumab is known to be efficacious in the treatment of solid tumours with high levels of p185 expression (T. Denyssevych, personal communication), but it was not known how tumours with low expression of p185 would respond. As such, it was important to be able to directly compare the effects of the two anti-HER-2/neu therapies in this new model of human breast cancer.

In solid tumour models of high p185 expression (MCF-7^{HER2}), trastuzumab has been demonstrated to be efficacious at doses as low as 0.3 mg/kg on an every other day schedule for a total of 15 doses. This experiment used a dose of 10 mg/kg, a dose which, in the aforementioned model, completely halted tumour growth. The results from this experiment are shown in figure 6.4, in which it may be seen that trastuzumab was not effective in

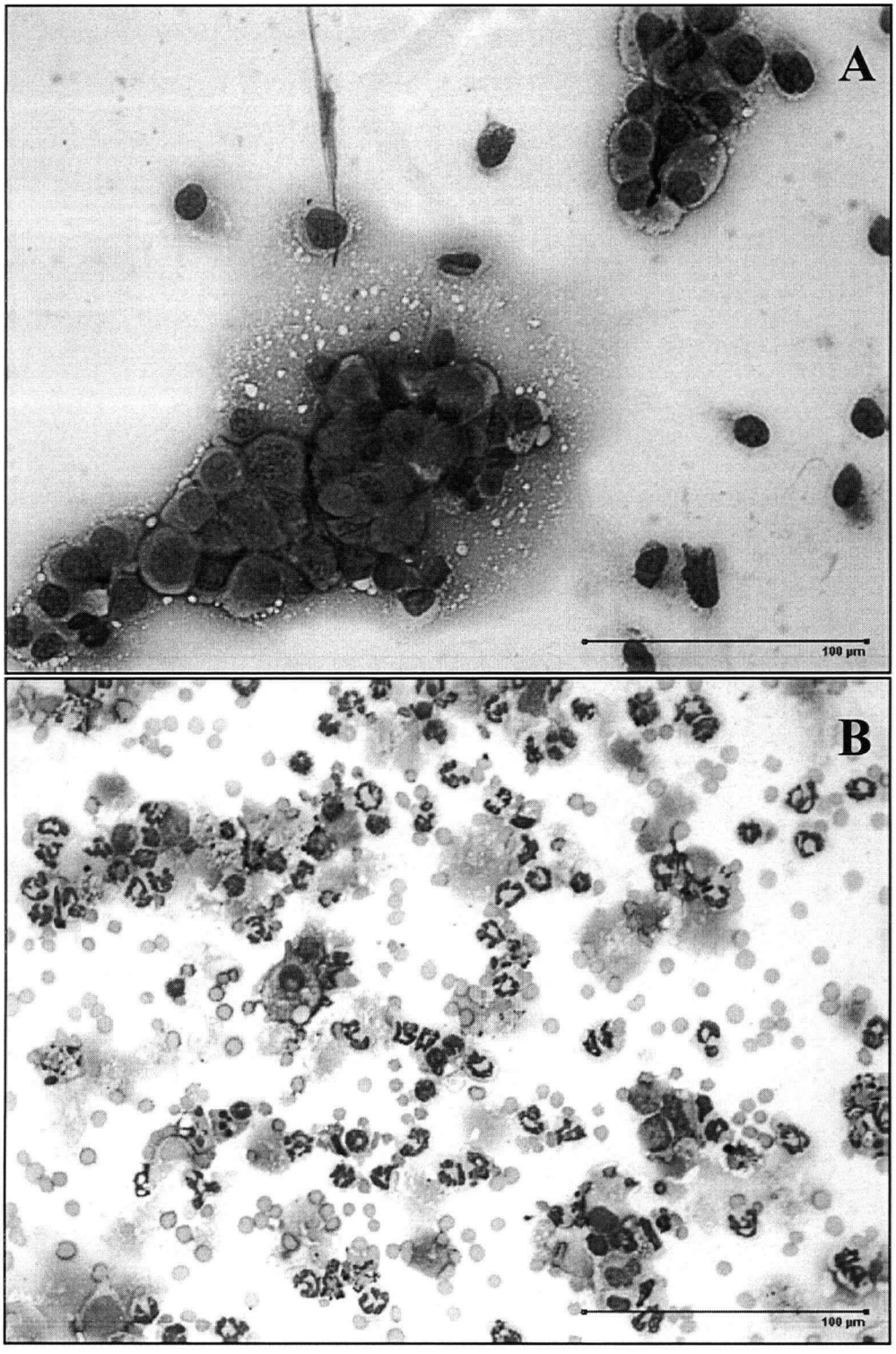

Figure 6.3 Modified Wright-Giemsa stain of (A) MDA435/LCC6 tumour cells harvested from peritoneal cavity of an untreated mouse, and (B) cells harvested from peritoneal cavity of a mouse inoculated with MDA435/LCC6 cells and treated with liposomal H2A-ODN as outlined in the text.

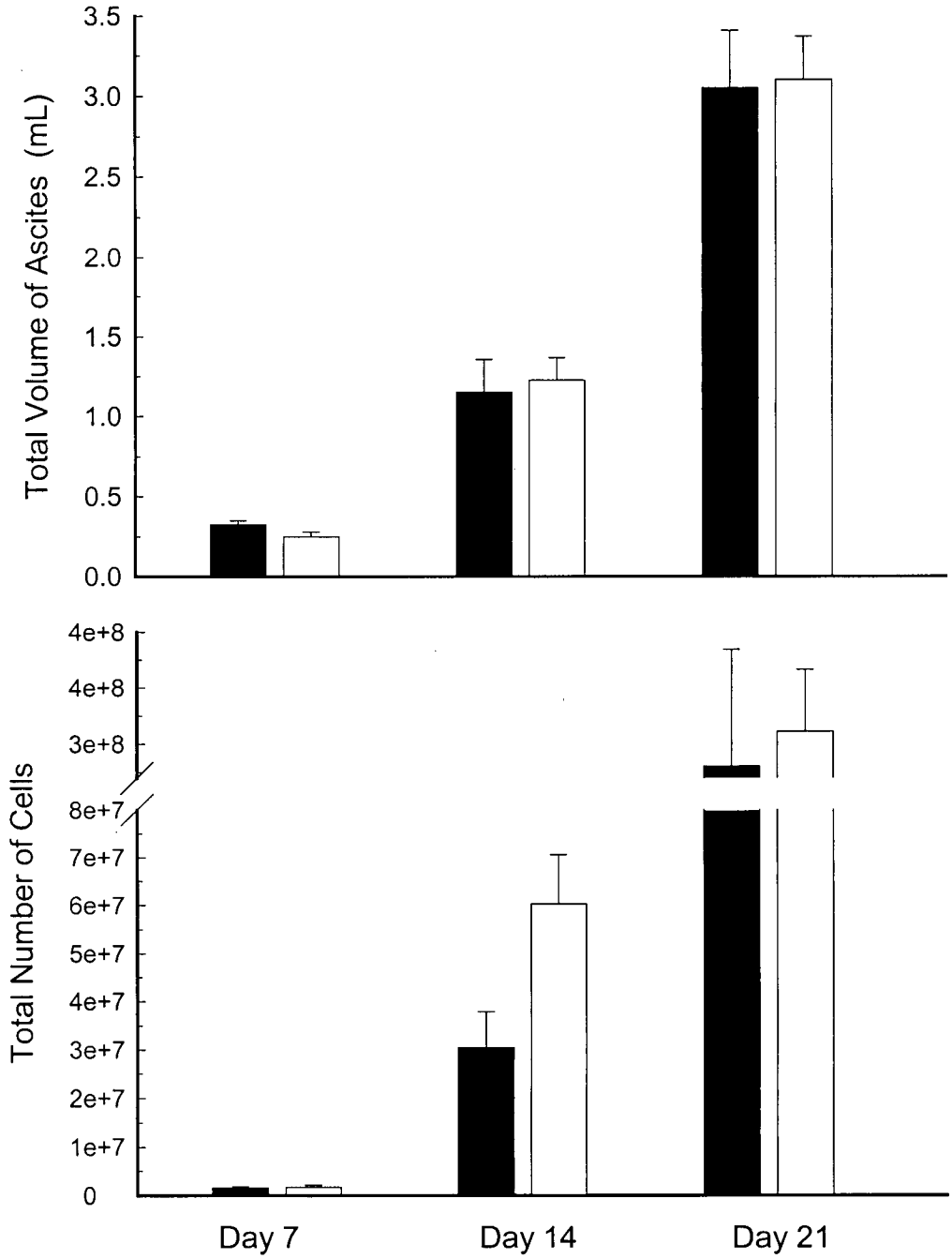

Figure 6.4 Total volume (A) and total number of peritoneal cells (B) harvested from mice inoculated with MDA435/LCC6 cells and treated with saline (black bars) or trastuzumab (clear bars). Results are the mean ± standard error of the mean of data from a minimum of four animals.

delaying tumour growth in these mice.

While preliminary *in vitro* testing with VEGF-ODN did not indicate significant benefit to the use of the drug in terms of VEGF downregulation, the ODN was taken into *in vivo* experiments in a limited nature, simply due to the lack of complete understanding of the precise mechanism in which antisense oligonucleotides exert their actions, and the necessary cellular environments for optimisation of this action.

6.2.3 Treatment of MDA435/LCC6 Ascites Tumour Bearing Mice with VEGF-ODN

Antisense efficacy experiments were initiated with VEGF-ODN, as the MDA435/LCC6 cells produced increased amounts of VEGF in comparison to the parental cell line. Ascites tumours were produced as before. Free VEGF-ODN was administered dissolved in sterile water at an antisense dose of 10 mg/kg three times weekly, for a total of 10 treatments, starting on day 3 post-tumour cell inoculation. The mice were terminated one day following the final antisense injection. When the total tumour burden in both volume and absolute peritoneal tumour cell numbers was compared between the free VEGF-ODN-treated, and untreated groups, there were no significant differences noted. This was also the case for VEGF protein levels measured in both the peritoneal fluid and in the plasma of each of these two groups. To assess the efficacy of a VEGF targeted antisense in the MDA435/LCC6 tumour model, groups of SCID/Rag2m mice were inoculated on day 0 with 2 X 10^6 MDA435/LCC6 cells, then beginning on day 3 post tumour cell inoculation, mice were injected daily for 10 days with liposomal VEGF-ODN at an antisense dose of 10 mg/kg. As noted in Table 6.1, approximately 2 X 10^8 cells were isolated from the

peritoneal cavity under the conditions employed in these studies and this represented a 20-fold increase in cellularity when compared to non-tumour bearing mice (the average peritoneal cell yield from 19 non tumour-bearing mice was $4.0 \times 10^6 \pm 2.8 \times 10^5$). Under conditions where the tumour bearing animals were treated with saline (control treatment groups), almost 40% of the cells isolated from the peritoneal cavity were murine (i.e. non-human) as judged by flow cytometric analysis. Importantly less than 10% of these murine cells could be identified as peritoneal macrophages. Also the influx of murine cells, primarily neutrophils, into the peritoneal cavity was probably a consequence of tumour growth and perhaps the associated production of VEGF (see Figure 4.3).

Table 6.1 Tumour burden (total peritoneal fluid volume and tumour cell count), VEGF levels per million peritoneal tumour cells, and serum VEGF (pg/ml) in control, untreated mice, and mice treated with 10 – three times weekly 10 mg/kg VEGF-ODN injections. Tumours were initiated on day 0 with i.p. injections of 2×10^6 MDA435/LCC6 cells. Treatment was started on day 3 post tumour cell inoculation. Numbers represent the mean of 8 ± standard error of the mean.

	Control	VEGF-ODN
Tumour Burden (cells)	$1.66 \pm .42 \times 10^8$	$2.02 \pm .59 \times 10^8$
VEGF (pg/10^6 cells)	285.34 ± 43.00	311.26 ± 52.32
VEGF (pg/ml serum)	54.52 ± 18.46	65.89 ± 18.77

As may be noted from the data in Table 6.1, the treatment of LDA435/LCC6 ascites tumour-bearing mice with liposomal VEGF-ODN did not cause any reduction in tumour burden in these mice, nor did the levels of VEGF measured in the serum decrease. Due to the lack of activity seen with VEGF-ODN in free form in both *in vitro* and *in vivo* tests, I chose not to pursue the liposomal formulation or combinatorial studies with VEGF-ODN at that time.

6.3 Discussion

Although the MDA435/LCC6 model does overproduce vascular endothelial growth factor, I was unable to achieve a reduction in tumour burden, or decrease in VEGF production with the chosen anti-VEGF antisense sequence. I feel it is important to note that there are many parameters in the use of therapeutic antisense oligonucleotides which have not been resolved satisfactorily. These include i) determination of the most efficacious ODN sequence, ii) determination of the most adequate delivery method and schedule, and iii) assessment of sequence independent non-specific host antisense reactions. Further work in these areas is required, with a focus on identification of whether VEGF is significant in tumour vascularisation, ascites growth and/or metastases of MDA435/LCC6 tumours. With these factors in mind, I believe this model will be beneficial for studies involving anti-angiogenic compounds, including those targeting VEGF.

In the studies presented above, the efficacy of liposomally encapsulated anti-HER-2/neu ODN in the treatment of MDA435/LCC6 derived ascitic tumours was presented (Fig.6.2). This supported earlier observations that H2A-ODN is effective in delaying the growth of tumours with low expression of HER-2/neu protein (sections 3.2.7 and 3.2.10), (Saxon *et al.*, 1999). This is an important observation given the number of breast cancer patients whose tumours express the HER-2/neu protein at levels lower than those currently set as the level for inclusion in a trastuzumab containing regimen.

In addition, it was shown that when encapsulated in liposomes, the H2A-ODN was more effective in the downregulation of the HER-2/neu protein than when administered in free

form. This is not surprising considering the results shown in section 5.2 (Fig 5.3), where it was demonstrated that more antisense was delivered to the tumour cell population within the peritoneal cavity with the encapsulated form than with free. This work has set the groundwork for many future studies in the MDA435/LCC6 model, including further experiments targeting HER-2/neu, as well as those targeting other proteins associated with aggressive breast cancer in humans.

Chapter 7

CONCLUSIONS

The diagnosis of breast cancer is one of the most dreaded events in the lives of most women. Nearly all North American women have known either a close friend or relative who have experienced this devastating disease, and have borne witness to both the emotional and physical challenges it brings. Of the approximately 11% of women who develop breast cancer, fully one quarter will die of their disease.

It has long been known that some breast cancers are more aggressive than others. While certain gross and histopathological features such as tumour type (e.g., comedo, lobular, ductal) and mitotic index have been identifiable for some time to aid in prognosis, scientists are now turning with ever increasing frequency to the use of molecular markers. It has been found that increased (or in some cases, decreased) expression of certain proteins, whether singly or in combination with other molecular factors, can be indicative of the nature and course of a specific breast cancer, and can therefore aid in selection of treatment options on an individual patient basis.

A marker which falls into the category of one which has prognostic significance when combined with expression of other markers, is the protease Cathepsin D (section 1.2.2.1). While overexpression of this protein alone is not indicative of more aggressive disease, when it is co-expressed with other proteins such as p53, the significance of its

overexpression is clearly linked to adverse disease outcome (Han *et al.*, 1997; Solomayer *et al.*, 1998).

Vascular endothelial growth factor (section 1.2.2.2) is another protein identified in many types of cancer, including breast cancer (Gasparini *et al.*, 1997; Anan *et al.*, 1998). When overexpressed by tumour cells, the tumour is able to induce the formation of a rich blood supply, thereby ensuring adequate nourishment for further growth. VEGF has also been implicated in the process of lymphangiogenesis in tumour growth. This protein is an example of one in which overexpression of a single protein may be linked to more aggressive disease and increased metastatic events. Downregulation of VEGF has been shown to halt the progression of tumour cell growth both *in vitro* and *in vivo* (Masood *et al.*, 2001). This type of therapy would be ideal in combination with a cytotoxic therapeutic, as a two-pronged approach to cancer care, in which further growth of the tumour is halted by the anti-VEGF agent, while cell kill of existing tumour cells is accomplished by the conventional drug.

The protein with which the bulk of this thesis has been focused upon is the protein product of the HER-2/neu oncogene, namely p185 (section 1.2.2.3). This member of the epidermal growth factor receptor family has been linked to more aggressive cancer, increased metastatic potential (Allred *et al.*, 1992; Press *et al.*, 1997; Pich *et al.*, 2000), short time to relapse (Slamon *et al.*, 1987) and resistance to certain types of therapy (Piccart *et al.*, 1988; Mourali *et al.*, 1993). The overexpression of p185 has been noted in many types of cancer, however this discussion will centre around breast cancer both due to the content of this

thesis, and because the bulk of research directed against HER-2/neu has been conducted in either breast cancer models or breast cancer patients.

In normal adult breast tissue, typical, non-malignant epithelial cells will have relatively few (less than 100) p185 receptors on their surface. In some breast cancer cells this number can be increased to 2000 or more receptors per cell (V. Dragowska, personal communication). This huge increase in receptor number leads to increased interaction of the HER-2/neu receptor with other members of class I RTKs, as well as homodimerisation, and subsequently increased signalling events and increased transcription of nuclear factors, culminating in increased tumour cell growth and spread.

With the greater understanding of tumour biology that is now being realised, including the molecular features linked to rapid growth, invasiveness, metastasis, chemo- and radiation-resistance, there will likely be improvements in treatment options. These new therapies will link gene discovery, proteonomics and drug discovery to define exquisitely specific drugs that target molecular defects inside diseased cells. It is critical that the drugs emerging from these efforts are used in a manner consistent with our enhanced understanding of the disease.

Cancer may be considered as a process of dynamic derangement of both growth and death pathways, with malignancy arising from a progressive accumulation of genetic defects that allow for the cancer cell to escape normal regulatory controls and develop malignant potential. The implication of the above is that we can attempt to re-impose biologic control

over the malignant cells and restore a normal growth pattern, however treatment will likely involve more than one molecular target or pathway in order to ensure eradication of a malignant clone. Due to the complexity and heterogeneity of cancer, it is likely that many of the new molecularly targeted therapeutic agents may not have sufficient activity as a single agent, but rather that their value will be realised through identification, in pre-clinical animal models, of effective combination regimens.

There are many currently available therapeutic options in the treatment of metastatic breast cancer. One of the foremost drugs in use is doxorubicin, typically in combination with one or more other cytotoxic drugs, such as cyclophosphamide and 5-fluorouracil. In addition to the conventional drugs, the arsenal of weaponry in the fight against this disease also contains agents such as anti-oestrogens, aromatase inhibitors and anti-progestins, as well as the newer strategies of monoclonal antibodies.

The first approved therapy targeting p185 is trastuzumab, a humanised monoclonal antibody targeting the extracellular portion of the p185 molecule. While this therapy has shown impressive results in a small number of breast cancer patients with overexpression of p185, it has been ineffective in many more, with an overall response rate of only 21% following use as a single agent (Cobleigh *et al.*, 1998). While it is true that the location of p185 on the cell surface makes it an ideal target for an antibody based therapy, what is not clear, is why this therapy appears to be selectively effective in breast cancer patients. It may be possible that in those patients where trastuzumab is non-effective, there remains another, as of yet unidentified protein which is able to function in the absence of HER-

2/neu overexpression to maintain the aggressive features of the tumour. It is also possible that tumours from different individuals react in differing ways to the anti-p185 antibody. It has been demonstrated in nude mice that treatment with some p185 targeted antibodies actually caused an increased rate of tumour growth, perhaps by stimulating increased phosphorylation of the HER-2/neu receptor (Stancovski *et al.*, 1991). These same researchers demonstrated that all antibodies tested, accelerated, albeit to differing degrees, the rate of receptor turnover (endocytosis upon antibody binding). They surmised that the different end results of antibody binding (either reduced, increased or unchanged tumour growth rate) was due to the different extracellular epitopes of the p185 molecule to which these antibodies bound. They concluded that the positive tumour growth resulted from stimulation of the tyrosine kinase function of p185.

The newcomer to the battle against cancer is the strategy of gene therapy (discussed in section 1.3.4), with its several variations, including that of antisense oligonucleotides. This modality brings to the field an extreme specificity for its target, rivalled only by the use of antibody therapy. These drugs, as discussed, may be targeted against a specific protein product of a gene, or at a point downstream in the signalling pathway. For the specific example of p185 directed ODN, it is hoped that this will render the tumour cells less aggressive and more amenable to eradication by either the p185 targeting therapy itself, or a conventional cytotoxic agent.

Chapter three of this thesis addressed the treatment of MDA-MB-435 and HER-2/neu transfected MDA-B-435 human xenograft tumours grown in female SCID/Rag2m mice,

with antisense oligonucleotides targeted against the mRNA for the HER-2/neu protein. It was thought to be important at this stage to use cell lines with differing HER-2/neu expression levels (low and high) that had a minimum of additional differing factors, which may have confounded any results obtained. By minimising differences other than HER-2/neu expression, the results would be rendered less ambiguous, and more easily linked to direct outcome of expression. As was demonstrated in section 3.2.1, the HER-2/neu transfected cells grew more rapidly *in vitro* than the parental cell line. This was consistent with other observations that overexpression of HER-2/neu conferred a growth advantage to cells (Liu *et al.*, 1995). However, increased rate of tumour growth was not observed *in vivo*. It is possible that the similarity in tumour size may not be reflective of the content of live cells within the tumour mass. Rather, it is possible that there is a greater percentage of viable cells within the MDA-MB-435^{HER2} cell-derived tumours as compared to the parental cell-derived tumours. This has indeed been found to be true for other cell lines with direct comparisons between parental and HER-2/neu transfected derivatives (V. Dragowska, personal communication). It is also possible that a greater degree of disparity in the growth rates of the two tumour types may have been noted had the tumours been permitted to grow further, as while not significant, there is a slight separation between the growth curves of the two tumour types, with MDA-MB-435^{HER2} being higher, by day 104 post tumour cell inoculation (Fig. 3.1).

It is important to note that the commonly used cytotoxic agent, doxorubicin, was efficacious in both the MDA-MB-435 and MDA-MB-435^{HER2} tumour models, thereby demonstrating that these cells respond in an expected fashion to drugs used in the treatment

of aggressive breast cancer. Also importantly, neither of the two tumour types responded in any fashion to the scrambled control sequence of anti-HER-2/neu antisense, whether free (Fig. 3.7) or liposomal (Figs. 3.11 & 3.12). Of note, the free, active antisense, H2A-ODN, was efficacious in the established MDA-MB-435 cell-derived tumours, but not in the HER-2/neu transfected counterpart (Fig 3.8). It was anticipated that, given the extended circulation longevity typical of liposomal drug preparations (Boman *et al.*, 1994; Webb *et al.*, 1998c), there would be more potential for response in non-established tumours if the ODN were administered in liposomal form, rather than in free form. This was indeed the case, as was demonstrated in Figure 3.11, clearly defining the benefit of having an encapsulated form of anti-HER-2/neu antisense.

While the free H2A-ODN had no impact on tumour growth in either established or non-established MDA-MB-435^{HER2} tumours, it was a surprising result to see an increased rate of tumour growth in the non-established MDA-MB-435^{HER2} cell derived tumours (Fig. 3.12) following liposomal H2A-ODN treatment. Clearly, when one considers the difference in the free *versus* liposomal antisense in the tumour growth rate of the parental line, one can understand that it is probable that an increased amount of antisense has been delivered to the cells of the tumour mass. This was substantiated by immunohistochemical studies wherein visible expression of p185 was reduced to levels of the parental cells (Fig. 3.13). This presented a challenging set of results for interpretation. While it was apparent that the H2A-ODN had resulted in the desired effect of reduced p185 protein expression, it was unclear as to why this would consummate in an increased rate of tumour growth. To expand on the earlier theory that perhaps the MDA-MB-435^{HER2} cell-derived tumours have

a higher percentage of viable cells, there exists the possibility that by bringing the p185 levels down in these cells, they are now able to enter into an alternate pathway for enhanced growth, and as a result of high viability, the growth rate is able to increase substantially.

An intriguing set of results has been reported by Koscielny *et al.* (1998, 1999), which have been independently confirmed by several researchers (Dittadi *et al.*, 1997; Pawlaowski *et al.*, 1999). These results demonstrated that tumour HER-2/neu levels *lower* than those found in normal breast tissue, resulted in a negative prognostic outcome, as compared to tumours with normal HER-2/neu expression. They suggest that this observation may rely on the interaction between several oncogenes, for which the lack of expression of one may be counterbalanced by a concomitant overexpression of another, e.g., EGFR. They further suggest that perhaps it is not the overexpression or low HER-2/neu values that should be focused upon, but rather the deviation from normality in expression, in assessing prognosis in breast cancer patients. It would be of great interest in any future experiments, to include measurements of all the HER family members when downregulating HER-2/neu, as it may be possible that by having decreased HER-2/neu protein levels to lower than normal levels, the cell counterbalances by increasing expression of other proteins, as discussed by these researchers.

Finally, it is possible that results presented here using HER-2/neu transfected MDA-MB-435 cells may differ from cells which have endogenous expression of the gene. This is because the HER-2/neu proximal promoter may not contain all the regulatory sequences for

the gene and other important control elements may be found both upstream and downstream of the sequence inserted into the transfection vector. For this reason, it is important to include tumours with endogenous overexpression of p185 in the experiments. Historically, these tumours, for example, SK-BR-3 and BT-474, have proven to be very difficult to establish in mice, and therefore were not included in these studies. There are however, new cell lines being established on an ongoing basis, and it is hoped that such a line will be available soon, as research into HER-2/neu is dependent upon it. A possible contender in this category is the newly described cell line, KPL-4, a human breast carcinoma line with endogenous overexpression of HER-2/neu (kurebayashi *et al.*, 1999).

In the experiments presented above, I believe that the most plausible explanation for enhanced tumour growth in the face of reduced HER-2/neu protein expression, follows from an enhanced number of viable cells in those tumours with overexpression of HER-2/neu, as compared to tumours with baseline HER-2/neu expression. Subsequent to the downregulation of p185 in these tumour cells, I believe that an alternate pathway of enhanced cellular growth was entered into, plausibly involving HER-1, VEGF, a combination of these, or some other growth signalling pathway.

To expand upon the requirements in breast cancer research, new, more relevant models, are required. Since the beginnings of what we consider as biomedical research, millions of laboratory animals have been experimented on and sacrificed for the purposes of research and education; their role purportedly the betterment of human health and welfare. There is however, a strong movement in most industrialised countries toward finding alternatives to

these animal experiments or towards the reduction in the numbers of animals required to achieve a particular aim. The rationale for this effort is based on scientific, economic and ethical considerations.

Alternatives are any procedures which can completely replace the need for animal experiments (replacement), reduce the number of animals required (reduction), or diminish the amount of pain or distress suffered by the animals in meeting the essential needs of man and other animals (refinement). In the replacement of animal procedures, researchers have turned to methods such as cell culture, mathematical modelling, computer simulations and other such techniques.

If experiments performed in cell lines derived from non-human sources cannot be assumed to model human disease accurately, neither can human-derived cells, growing in non-human hosts. There are a host of interactions between tumour cells and the environment in which they are growing, many of which may be governed by species specific proteins and pathways. Hence, since we are by necessity, limited to using non-human hosts in the modelling of human cancer, any results garnered in these models must be interpreted with extreme caution before moving on to designing clinical trials. This makes it imperative that any models we do use, are exquisitely carefully selected on the basis of their relevance to the specific disease, and that responses to established chemotherapeutic agents in these models mimic those responses seen in the clinic. Only in this way will the models chosen serve to reduce the overall burden of animal suffering.

Important aspects in the development of murine models of human cancer, specifically those models surrounding the development of therapeutics targeting HER-2/neu, include a more complete understanding of the complexity of signal transduction which commences following activation of the HER-2/neu receptor. We do not yet have a full understanding of the different environments in which the different heterodimers of the HER-2 family are formed, i.e., why in certain physiological conditions, certain dimers may be preferred over others. As well, further research needs to be conducted into the multiplicity of different signalling molecules which occur downstream in the signal transduction pathways, and how these molecules interact with each other, and why some pathways may take preference over others, again, triggered by specific physiological conditions. Until these parameters are met, we cannot hope to completely understand the interaction of any novel pharmaceutical agent, whether it be a small molecule, peptide, antibody or DNA based therapeutic, with the varying components of the HER-2/neu signal transduction pathway. Although some of these relationships can be studied *in vitro*, it is impossible to develop this understanding without considering *in vivo* parameters.

The work presented in this thesis outlines quite clearly, the requirement for this understanding, as it has been demonstrated that antisense oligonucleotides targeted against the HER-2/neu mRNA, are in fact, quite capable of downregulating the absolute amount of p185 expression on the cell surface, but at the same time, this reduction in expression may lead to increased tumour growth rates. It is not clear whether the apparent anomaly is due to the interaction of human tumour cells with the murine environment, or if this response is

intrinsic to the therapeutic used. It will be impossible to test this in the current state of human tumour modelling.

With the above in mind, chapter four of this thesis was concerned with the development and characterisation of a new model for human breast cancer, the MDA435/LCC6 xenograft model. As the main thrust of this research was the development of therapeutics for breast cancer with overexpression of certain molecular markers which rendered that cancer more aggressive and more likely to metastasise, it was these targets which were initially characterised, along with such parameters as tumour growth rate and responsiveness to chemotherapeutic drugs.

As was shown in section 4.2.2, the MDA435/LCC6 cell line was responsive to the drugs taxol, vincristine and doxorubicin in degrees comparable to the parental cell line, MDA-MB-435, and as would be anticipated for aggressive human breast disease (Fig. 4.2). This line was shown to be higher in VEGF expression than the parental line (Figs. 4.3 and 4.4), while retaining similar levels of HER-2/neu, bcl-2, and Cathepsin D expression (Figs. 4.4, 4.6 and 4.7, respectively), features which made it a good model for VEGF targeting therapeutics. Although considered a low expressor of HER-2/neu, this cell line also provided an opportunity to confirm results obtained when treating the parental MDA-MB-435 cell line with the liposome formulation of anti-HER-2/neu antisense.

The MDA435/LCC6 tumour model was found to grow reproducibly as an ascites model (Fig. 4.1), making it amenable for rapid screening of anti-cancer drugs such as antisense

oligonucleotides, either singly, or in combination with other anti-cancer agents. In summary, having characterised this model as discussed, it was found to be a rapidly growing, reproducible and relevant model, suitable for further testing of the agents with which this thesis is concerned.

Having the MDA435/LCC6 model in place, and having characterised the liposomal formulations of ODN, I was then able to turn to a more thorough examination of the pharmacodynamic properties of the antisense oligonucleotides, both free and liposomal. It was anticipated that by virtue of encapsulating antisense oligonucleotides into liposomal carriers, that greater circulation longevity would be achieved, and hence enhanced tumour cell delivery. This was indeed found to be the case, as was seen in Figures 5.1 and 5.2 and Table 5.1. Importantly, the enhanced longevity was similar for either the 15 or the 21-mer ODNs, indicating that the length of the molecule did not affect the circulation time, but rather, that it was the nature of the liposomal formulation which engendered this enhancement. Plasma elimination studies of free ODN administered either intraperitoneally or intravenously, showed essentially no difference in the plasma AUCs over 24 hours (Fig. 5.1), therefore allowing alternate routes of administration of the free ODN. This was an important consideration given the extended nature of the treatment schedule with either free or liposomal ODN, with typically over ten injections being administered over a short course of time.

It was essential to show at this point that the increased circulation longevity afforded the ODN by encapsulation was translated to enhanced tumour cell delivery. This was

demonstrated in the MDA435/LCC6 ascites tumour model by measuring both the peritoneal fluid ODN levels as well as the peritoneal tumour-cell associated ODN. As was anticipated, those mice which received liposomal ODN rather than free, had a greater amount of tumour cell associated ODN (Fig. 5.3), although the total level of ODN delivery to the peritoneal cavity was comparable in animals given free and liposomal ODN. This was unexpected given previous studies demonstrating enhanced peritoneal delivery of liposome associated drugs, such as doxorubicin or vincristine.

It is well known that liposomal delivery of drugs often results in increased uptake of both lipid and encapsulated drug in the organs of the MPS. With this in mind, increased levels of ODN were expected to be seen in the spleens and livers of those mice receiving the liposomal drug. This was indeed the case, as was shown in Tables 5.2 and 5.3 for MDA435/LCC6 ascites tumour bearing mice. It is important to note at this point that there was no toxicity associated with these increased uptake levels, as would be evidenced by general appearance or behaviour of the animals, or by gross pathological observations. This was not expected, given the relatively low toxicity of phosphorothioate ODN and the level of dosing used in these studies (Levin, 1999). Enhanced levels of ODN in the kidneys was likely a consequence of enhanced levels of ODN in the animals given the liposomal formulations and the slow dissociation of the ODN from lipid carriers in the plasma compartment or tissues. Renal function was not assessed in these studies.

With the MDA435/LCC6 model in place and characterised with respect to tumour growth parameters, protein expression, and pharmacodynamic behaviour of the injected drugs, it

was possible to re-address the question of efficacy of the H2A-ODN in a model with low expression of the HER-2/neu protein. The initial observation of efficacy in the MDA-MB-435 model was entirely unexpected, and yet was thought provoking and exciting. There are many breast cancer patients whose HER-2/neu expression levels are too low to allow them to qualify for treatment with trastuzumab, the monoclonal anti-HER-2/neu antibody, and yet who may indeed benefit from a therapy targeting this protein.

The lack of reduction in tumour burden associated with treatment of MDA435/LCC6 ascites tumour bearing mice with free VEGF-ODN was perhaps not an entirely unanticipated result, as VEGF levels in both the ascites fluid and plasma also were not impacted. It may of worth to remember as well, that ascites tumours are by their nature, bathed in fluid in the peritoneal cavity, and those clumps which are present, are small and attached at points along the peritoneal wall. Due to the dispersed nature of these cells, they would not be expected to rely heavily upon angiogenesis for supply of oxygen and nutrients. This is work which would be important for future studies of VEGF targeted therapy. In all, the experiments presented in chapter 6 have laid groundwork for future studies in which the combination of two or more strategies could be assessed.

I believe that the combination of strategies targeting HER-2/neu and VEGF could have major impact on the growth inhibition of tumours, but that before this can be accomplished, there needs to be a careful study into the optimal method for selection of antisense sequences such that maximal protein downregulation is achieved. The field of antisense oligonucleotide therapy is relatively young, and advances in this area are being made on a

constant basis. One has also to be aware of some sequence independent toxicity properties of antisense oligonucleotides that are related to the chemical and physical properties of the molecules. However, these toxicities, primarily transient activation of the complement cascade as well as an inhibition of the clotting cascades, are only observed when ODN are administered at very high levels (LD_{50} = 750 mg/kg in primates) (Levin, 1999). It is hoped that in the very near future there will be in place a strategy for ODN sequence selection that fulfils the above requirements.

To return to the objectives of this thesis, I have successfully developed and characterised the MDA435/LCC6 xenograft model of aggressive human breast cancer, and used that model to assess the actions of an ODN sequence, targeting HER-2/neu, both in free and in liposomal form. In addition, the combination of antisense oligonucleotide drugs with conventional cytotoxic agents (doxorubicin) was explored. I believe these combinations will maximise the efficacy achievable in the treatment of aggressive disease, while minimising morbidity, and are the direction that future cancer therapy must take in order to make gains in both extension and quality of life. I have also shown that the encapsulation of ODN into a liposomal carrier is an effective means of extending the circulation lifetime of the drug, as well as maximising tumour cell exposure, and hence increasing efficacy.

As a concluding comment, I believe it is important to remember that the evaluation of the primary target (e.g., p185) may be insufficient to account for the overall effect of molecular targeted therapies on the growth of a tumour. It becomes essential to examine other, downstream effects of these therapies, and to examine redundant pathways within the cells,

as many signalling pathways interact at various points and appear to be multifactorial (Nieves-Neira and Pommier, 1999). With the advent of more powerful techniques for assessing multiple points within a pathway, and indeed, multiple pathways, and as our understanding of the diversity and interactions of these pathways increases, it will be possible in the not too distant future, to screen individual patients and tailor therapy regimens to best suit their individual disease. This work may aid in the identification of more relevant targets, for example, perhaps the ideal strategy for targeting HER-2/neu overexpressing tumours is not to target the mRNA for the protein itself, but to target instead, the HER-2/neu promoter binding factor, an action which would serve to halt the transcription of the gene itself. This remains to be experimentally determined. I anticipate that these regimens will consist of both targeted agents, such as the HER-2/neu or VEGF ODN or agents such as tyrosine kinase or farnesyl transferase inhibitors in combination with cytotoxic agents with known effects. In this manner, we will be able to make further advances in the care and treatment of cancer patients, both those with aggressive breast cancer and those with other, equally devastating forms of this disease.

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