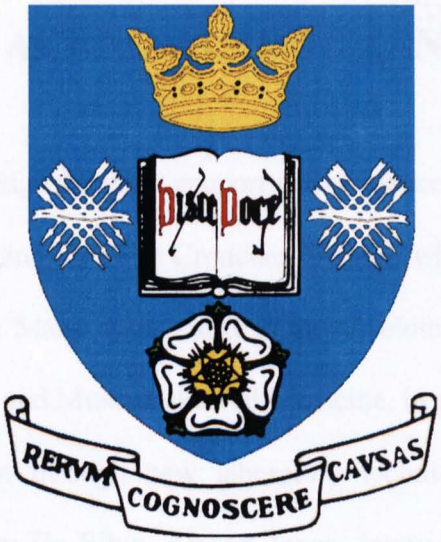


Anti-tumour Effects of Bisphosphonates in Breast Cancer

Thesis submitted to the University of Sheffield for the degree of

Doctor of Philosophy

Satinder P. K. Jagdev

Department of Academic Oncology

University of Sheffield

September 2004

ACKNOWLEDGEMENTS

I would like to acknowledge the great support and guidance provided by my supervisors, Professor Rob Coleman and Dr Peter Croucher, without whom this project would never have got off the ground. Many thanks also to the Myeloma Bone Disease group at the Division of Biochemical and Musculoskeletal Medicine, in particular Claire Shipman, for so patiently guiding me through new laboratory techniques and to Ingunn Holen, Gabrielle Mueller, Nancy Da Silva, Alyson Jones, Jenny Lipton and Colby Eaton for keeping me laughing and sane.

I would like to acknowledge the support of Mrs. Olivia Smith and Dr John Laurie in the flow cytometry analysis of my numerous samples and help with interpretation of the data. Dr Amin Rostami deserves special thanks for the advice on the synergy work and analysis of the data for Chapter 4. I would also like to thank the Research Nurses and Dr Kash Purohit of the Bone Research Team, Cancer Research Centre, Weston Park Hospital, for coordinating the participation of patients in the clinical studies. I am grateful to Yorkshire Cancer Research and Novartis Pharma AG for their generosity in funding this work.

Finally, I am eternally grateful to my family and friends who have been a constant source of encouragement throughout the researching and writing of this thesis, in particular my ever-patient and loving husband, Asif Ramzan, who has cheered and cajoled me on from beginning to end.

TABLE OF CONTENTS

CONTENTS

CHAPTER ONE: INTRODUCTION

CHAPTER TWO: MATERIALS AND METHODS

SUMMARY

Breast cancer very commonly metastasizes to bone, often with devastating consequences, including pain, pathological fractures, hypercalcaemia and nerve compression syndromes. Bisphosphonates are very effective in the management of bone secondaries from breast cancer. Recent clinical studies have also suggested that adjuvant oral clodronate in patients with primary breast cancer may confer a survival benefit thereby raising the possibility that bisphosphonates have an anti-tumour effect. The aim of this PhD was to determine whether bisphosphonates have an anti-tumour effect on human breast cancer cells.

The potent third generation bisphosphonate, zoledronic acid, was found to reduce cell number and increase apoptosis, mediated by inhibition of the mevalonate pathway, in human breast cancer cells *in vitro*. The effects on cell apoptosis were shown to be synergistic when zoledronic acid was combined with a chemotherapeutic agent, paclitaxel. To extend the investigation to the *in vivo* setting, we developed a dual fluorescence labelling technique to isolate apoptotic breast cancer cells from the bone marrow of patients with breast cancer undergoing treatment with intravenous pamidronate or zoledronic acid. The technique was effective at detecting breast cancer cells but studies were inconclusive in determining the effect of intravenous bisphosphonate treatment on breast cancer cell apoptosis *in vivo*. These studies are ongoing. Bisphosphonates may also alter the bone microenvironment by inhibiting

osteoclast-mediated bone resorption and interfering with the release of cytokines and growth factors. To complete these studies, we measured the levels of a panel of cytokines and growth factors in patient serum and bone marrow before and three days after intravenous bisphosphonate treatment and also compared pretreatment levels with those in a baseline group with primary breast cancer. TGFβ–1 was significantly elevated in bone marrow plasma from patients with advanced breast cancer than in patients with primary breast cancer, along with serum IL-6 and sIL-6R. In the advanced breast cancer group, bisphosphonate treatment resulted in significantly lowered levels of serum FGF-2 and bone marrow VEGF.

The work presented in this thesis demonstrates that the bisphosphonate zoledronic acid does have anti-tumour effects on breast cancer cells *in vitro,* both alone and in synergy with paclitaxel, to induce apoptosis. This raises the possibility of *in vivo* anti-tumour effects in breast cancer. The dual fluorescence labelling technique made it possible to detect breast cancer cells in human bone marrow but the studies regarding the *in vivo* induction of apoptosis in breast cancer cells following intravenous bisphosphonate treatment were inconclusive. There were significant differences in bone-derived growth factors between patients with primary breast cancer and secondary bone metastases. There was also a significant reduction in certain cytokines and growth factors in the bone marrow and serum after intravenous bisphosphonate treatment, suggesting that bisphosphonates may also exert an indirect effect on the bone marrow microenvironment. Further *in vivo* studies in patients with breast cancer are required to confirm both the direct anti-tumour effects and indirect effects.

ABBREVIATIONS

APD	Pamidronate, 3-amino-1-hydroxy-propylidene-1,1-bisphosphonate
ATP	Adenosine triphosphate
b-FGF	Basic Fibroblast Growth Factor (or FGF-2)
BSA	Bovine serum albumin
BSP	Bone Sialoprotein
CL2MBP	Clodronate, dichloromethylene-1,1-bisphosphonate
cm	Centimetre
DMSO	Dimethyl sulphoxide
DAPI	4'-6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dl	Decilitre
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
DCIS	Ductal carcinoma *in situ*
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ER	Oestrogen receptor
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Flouroscein isothiocyanate
FOH	Farnesol
FPP	Farnesyl pyrophosphate
FTase	Farnesyl transferase
G-CSF	Granulocyte-colony stimulating factor
GGOH	Geranylgeraniol
GGtase	Geranylgeranyl transferase
GM-CFU	Granulocyte-macrophage colony forming unit
GTP	Guanosine triphosphate
H+	Hydrogen ions
IC	Inhibitory concentration
ICAM	Intracellular adhesion molecule
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IFNγ	Interferon gamma
IL	Interleukin
IPP	Isopentenyl pyrophosphate
kg	Kilogram
l	Litre
LCIS	Lobular carcinoma *in situ*
µg	Microgram
µl	Microlitre

μm	Micrometre
μM	Micromolar
M	Molar
M-CSF	Macrophage-colony stimulating factor
Mev	Mevastatin
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
MVA	Mevalonate
NF-κB	Nuclear factor-kappa-beta
OCIF	Osteoclastogenesis inhibitory factor
ON	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived Growth Factor
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone related Peptide
RANK	Receptor Activator for NFκb
RANKL	Receptor Activator for NFκb ligand
sIL-6R	Soluble IL-6 receptor
sgp-130	Soluble gp-130
SRE	Skeletal related event
TIMPs	Tissue Inhibitors of Matrix Metalloproteinases
TNFα	Tumour Necrosis Factor alpha
uPA	Urokinase-type Plasminogen Activator
uPAR	Urokinase plasminogen activator receptor
V-CAM	Vascular Cell adhesion Molecule
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

1.1 BREAST CANCER

1.1.1 Introduction

Breast cancer is considered to be one of the "major killers" together with cancers of the intestine, lung and prostate in the industrialised world. Biologically it is a heterogeneous entity and much attention has been focussed by medical researchers on all aspects of the disease, including the aetiology and epidemiology, the development of treatment modalities and the psychosocial impact on patients and carers. Modern diagnostic techniques, greater awareness amongst women and the widespread development of screening programmes have made the diagnosis of early stage breast cancer easier.

It is estimated that during the first decade of the new millennium almost 1 million women will develop breast cancer worldwide. It is the commonest cancer of women in Europe and accounts for 20% of all malignancies. In the UK, it makes up around 15% of new cases per year (Cancer Research UK statistics, 1997). It occurs rarely before the age of 20 years. Up to 50 years of age there is a steady increase in the incidence of breast cancer although the rate of increase slows down in postmenopausal women. Mortality rates from breast cancer in Western Europe and North America are around 15-25/100, 000 women, whilst the incidence is 50-60/100, 000 women.

1.1.2 Risk Factors for the Development of Breast Cancer

Several risk factors have been identified as being associated with an increased risk of breast cancer. These include genetic and familial factors, hormonal, dietary and environmental factors as well as benign breast disease. However, 50% of women who

develop breast cancer have no identifiable risk factors other than being female and their age (Madigan M, 1995).

1.1.3 Pathology

- ***Non-invasive breast cancer***

Two patterns of non-invasive breast cancer are well recognised. Lobular carcinoma *in situ* (LCIS) is a proliferative lesion limited to one or more mammary lobules and is composed of cells which morphologically have malignant characteristics but do not breach the basement membrane. Many cases (50-70%) are multicentric and occur mainly in premenopausal women. Lobular carcinoma *in situ* carries with it a risk of 10-15% of developing invasive cancer in the same breast at 10 years if not treated with mastectomy. Ductal carcinoma *in situ* (DCIS) is characterised by the proliferation of malignant cells within the ducts but without invading the surrounding stroma. It is multifocal in around 30% of cases. These lesions are divided into comedo and non-comedo types. Comedo DCIS is characterised by the presence of necrosis at the centre of the ducts and has a higher risk of recurrence, especially after breast conserving management.

- ***Invasive breast cancer***

The most common type of breast cancer is invasive ductal carcinoma, comprising approximately 70% of all cases. Invasive lobular carcinoma represents 10-20% of all breast cancers. The remaining 10% of cases are accounted for by several other types of invasive cancers, including medullary carcinoma, mucoid carcinoma, papillary carcinoma and inflammatory carcinoma.

1.1.4 Prognostic factors

Certain histopathological and biological features of breast tumours can give some indication of prognosis and may predict response to anti-neoplastic therapy. Prognostic factors, related to the patient and disease at presentation, may also be important in defining the best therapy available and may aid the estimation of the costs and benefits of a particular treatment programme. These are outlined in Table 1 below.

Patient and Clinical Features	**Tumour Related Features**
Age	Nodal status
Menopausal status	Pathological tumour size
Estimated duration of tumour growth	Multicentricity
Clinical presentation	Involvement of resection margins
Imaging presentation (multicentricity)	Histopathogical features (histological type, grade, vascular invasion, intraductal component)
Liver, renal and bone marrow function	Oestrogen (and progesterone receptor) status
Baseline marker	Marker of proliferation (thymidine incorporation, Ki-67, flow cytometry)
	Growth factor receptors (epidermal growth factor receptor, HER2/*neu*, Insulin-like growth factor receptor)

Table 1 Important features with prognostic relevance which influence treatment choice. Adapted from Goldhirsch, 1997 (Goldhirsch, 1997).

1.1.5 Treatment of primary breast cancer.

There have been many changes in the management of invasive carcinoma of the breast confined to the mammary gland and ipsilateral axillary lymph nodes. The major treatment modalities and their indications are discussed briefly below.

- ***Surgery***

Breast surgery is primarily indicated for tumours presenting as T-stage <4 (i.e. no involvement of the skin or chest wall) and nodal N-stage <2 (mobile ipsilateral axillary nodes). In T3 tumours, i.e. that are larger than 5cm, primary systemic cytotoxic therapy should be considered. Multicentre randomised clinical trials have demonstrated that breast conservation and total mastectomy with axillary clearance produce equivalent survivals (Harris JR, 1992). Good surgical technique significantly influences the ultimate cosmetic outcome.

- ***Radiotherapy***

Radiotherapy retains a central role in the management of breast cancer. In the case of breast conserving surgery, radiotherapy to the conserved breast has been found to significantly reduce the incidence of relapse in the ipsilateral breast (Scnitt, 1993). Several factors, including multicentricity, total tumour excision, the presence of extensive intraductal component and lymphatic invasion, influence how much radiotherapy will help to control disease in the conserved breast.

- ***Adjuvant Systemic therapy***

Many breast cancer patients who remain disease free after local and regional treatment eventually relapse and die as result of overt metastases. The aim of adjuvant systemic therapy is to treat micrometastatic disease present at the time of initial surgery and it is estimated that it may prevent relapse in about one third of patients who would have relapsed without it (Goldhirsch, 1997). Adjuvant systemic therapies include chemotherapy, endocrine therapy, combined chemo-endocrine therapy and ovarian ablation.

1.1.6 Treatment of metastatic breast cancer

The development of overt metastastic breast cancer results in chronic incurable disease with a survival time that may vary from a few weeks to many years. Overall, the median duration of survival is 2 years. Thus, the treatment choice in this setting should be guided by the extent of the disease, its related symptoms and an estimation of survival. The aim of local and systemic therapies in this setting is to palliate symptoms foremost. The choice of systemic treatments includes chemotherapy and hormone therapy.

In a substantial number of cases hormone therapy is considered as first line treatment due to its relatively low toxicity. Certain clinical features, such as long disease-free interval and non-visceral disease (Brufman, 1993), along with high expression of oestrogen or progesterone receptors (Brooks S, 1980; Dao T, 1980; Degenshein Q, 1980) are predictive of higher rate of response. Endocrine therapies can be classified as ablative (eg oophorectomy, radiotherapy ablation or chemical suppression with eg goserelin) or

additive (eg megesterol acetate/ aromatase inhibitors). Ablative therapies are aimed at ovarian suppression in pre-menopausal women whereas additive therapies are aimed at antagonising the production or peripheral effects of oestrogens, ie; the anti-oestrogens. Pure anti-oestrogens are steroidal analogues of oestradiol and bind to the oestrogen receptor leading to disruption of transcriptional activity. These include tamoxifen, which has been in use for the management of breast cancer since the 1970s and is well established in the adjuvant and metastatic settings. Newer steroidal anti-oestrogen agents include toremifene and faslodex. The other class of anti-oestrogens are the aromatase inhibitors. In postmenopausal women, the major source of oestrogen is from the conversion of adrenal and ovarian androgens (androstenedione and testosterone) to oestrogens in peripheral tissues, including breast, bone, peripheral vasculature and brain. Aromatase is the enzyme involved in the conversion of androgens to oestrogen and this is the target of the aromatase inhibitors. First generation agents, such as aminoglutethemide, had relatively low efficacy and a poor side effect profile. Second generation agents overcame the side effect problems somewhat but it is the third generation aromatase inhibitors, such as anstrazole, letrozole and exemestane, that have real benefits in terms of potency and reduced toxicity. Randomised controlled trials in advanced breast cancer have demonstrated the benefits of aromatase inhibitors over tamoxifen (Bonneterre J, 2000; Nabholtz JM, 2000; Mouridsen H, 2001) and, for post-menopausal women who are eligible for hormone therapy, these agents are becoming widely used for first line metastatic therapy.

Chemotherapy interventions are generally more toxic and are often used in hormone-refractory disease. However, these may be offered as first line therapy if the patient has rapidly progressive disease or extensive visceral metastases. As chemotherapy is not a curative measure, a careful balance must be struck between optimum palliation of symptoms, disease response and the patient's quality of life. The toxicity may be considerable. Chemotherapeutic agents are often given in combination and the optimum duration is unclear. There is little evidence to suggest that continuing treatment beyond a partial or complete response is beneficial. Indeed, there is clinical evidence suggesting that discontinuing treatment at response and restarting at further progression gives an equivalent survival to continuous therapy but with less toxicity (Muss H, 1991). Once progression occurs, the patient may be treated successfully with other regimens but the chances of response are diminished with each subsequent regimen. New biological therapies, such as monoclonal antibodies to the growth factor receptor Her 2, have also emerged as effective agents in combination with chemotherapy.

Local treatments for symptomatic metastases include radiotherapy and surgery. The management of bone metastases in particular is discussed below.

1.2 BONE METASTASES IN BREAST CANCER

1.2.1 Clinical features

Bone metastases are extraordinarily common in patients with breast cancer. Breast cancer, together with prostate cancer, comprises over 80% of cases of metastatic bone disease (Rubens, 1991). A study of 587 women dying of breast cancer revealed that 69%

had radiological evidence of skeletal disease before death compared with 27% with lung and liver metastases (Coleman RE, 1987). Interestingly, the site of first recurrence of breast cancer appears to significantly impact on subsequent survival. Patients with first relapse of breast cancer confined to the skeleton had a median survival of 20 months compared with 3 months for those with first recurrence in the liver (Coleman RE, 1987). The disease in bone therefore follows a protracted course and the associated morbidity forms a significant clinical problem. A study of 367 patients with metastatic breast cancer revealed that the development of secondaries at extraskeletal sites also influences the probability of survival from bone metastases (Coleman RE, 1988a). Patients with bone metastases and additional soft tissue secondaries had a median survival time of 1.6 years compared with 2.1 years for those with bone only disease. Other factors predicting prognosis included tumour histological grade and type, oestrogen receptor status and menopausal status.

- ***Morbidity***

Bone metastases from breast cancer cause significant morbidity and form a sizeable clinical problem because of the protracted nature of the disease when confined to the skeleton. The problems encountered include bone pain, impaired mobility, hypercalcaemia, pathological fractures, nerve root and spinal cord compression syndromes and bone marrow infiltration. In one study, patients with breast cancer bone metastases experienced a skeletal related event, such as a pathological fracture, every 3-4 months (Hortobagyi GN *et al.*, 1996). Coleman et al found in a study of 498 patients with first recurrence from breast cancer in bone that 29% (145) developed one or more major

complications of bone destruction. 17% (86) developed hypercalcaemia, whilst pathological long bone fractures occurred in 16% (78) and spinal cord compression in 3% (13) (Coleman RE, 1987).

Hypercalcaemia is a common complication of malignancy and is often associated with squamous cell carcinomas of the lung, renal cell carcinoma and adenocarcinomas of the breast (Guise T, 1998). It results in a number of symptoms involving the kidneys, gastrointestinal tract and central nervous system. If left untreated, it may result alterations in conscious level along with potentially fatal acute renal failure and cardiac arrythmias. Hypercalcaemia is very often an indication of advanced disease.

The bone destruction caused by metastatic disease results in disruption of the bone architecture. This, in turn, may compromise its weight-bearing capacity and ultimately lead to loss of bone integrity and pathological fracture. Pathological fractures commonly occur in the axial skeleton, although fractures of the long bones may cause the most disability. Radiographic assessment of the size of lesions and the extent of bone destruction provides valuable information on which patients may benefit from prophylactic surgery (Coleman, 1998). The presence of back pain along with an abnormality on plain radiograph of the spine may herald impending spinal cord compression. Prompt management and early referral for radiotherapy or decompression and spinal stabilisation are crucial to ensure maximum recovery of neurological function. Bone pain is a significant problem and often difficult to control. It may be associated with pathological fractures or nerve entrapment syndromes and treatment modalities include

analgesic medication, radiotherapy, nerve blocks, surgical stabilisation and bisphosphonates.

Radiologically, breast cancer is commonly characterised by the development of predominantly osteolytic skeletal lesions, although osteosclerotic and mixed lytic/sclerotic lesions are also seen. The development of bone disease is associated with an increase in osteoclast activity leading to an uncoupling of bone resorption and formation which results in excessive bone destruction. In order to understand the development of bone metastases in breast cancer, it is necessary first to review the process of normal bone remodelling.

1.2.2 Bone Remodelling

- ***Cells involved in bone remodelling***

Osteoclasts are the cells responsible for bone breakdown or resorption. They are large multinucleated cells which are derived from the granulocyte-macrophage colony forming unit (GM-CFU) of the haemopoietic lineage (Mundy, 1996). They are located either on the surface of bone or in resorption pits termed Howship's lacunae. The osteoclast has several specialist features to enable it to resorb bone. A peripheral actin ring forms a sealed environment mediated by integrins on the cell membrane which recognise certain proteins in the bone matrix. In this zone, a specialised part of the cell membrane, the ruffled border, secretes two products which will lead to bone breakdown. The first step is dissolution of the bone mineral by H+ ions (secreted via a proton ATPase) under the

action of carbonic anhydrase. Lyososomal enzymes, such as cathepsin K and collagenases, are then secreted via the ruffled border to degrade the matrix components. Bone matrix is synthesised by osteoblasts, cells which derive from mesenchymal progenitors (Fleisch, 2000). They secrete the osseous organic matrix, or osteoid, which forms a layer under the cuboidal osteoblast cells. This osteoid is subsequently mineralised to form the calcified bone matrix. The osteoid layer is widened in pathological conditions which result in absence of mineralization, such as osteomalacia. When osteoblasts are not involved in bone formation they lie flat and are termed resting osteoblasts or lining cells.

Eventually osteoblasts become embedded in bone and are located in lacunae. They are then termed osteocytes (Puzas, 1996). These cells are connected to each other and osteoblasts and lining cells by long cytoplasmic processes contained in canaliculi. These also contain extracellular fluid and osteocytes may influence calcium homeostasis by altering its composition. Osteocytes may also be involved in the response and adaptation of bone to mechanical stimuli.

- ***The remodelling process***

The skeleton is a dynamic structure, undergoing constant structural remodelling to maintain its integrity. This process occurs in discreet units termed "bone remodelling units" by Frost (Frost, 1964) who initially described the sequence of events involved. The sequence begins with bone resorption by osteoclasts followed by new bone formation by osteoblasts to produce a "bone structural unit". The process of bone remodelling is outlined in Figure 1.1 below.

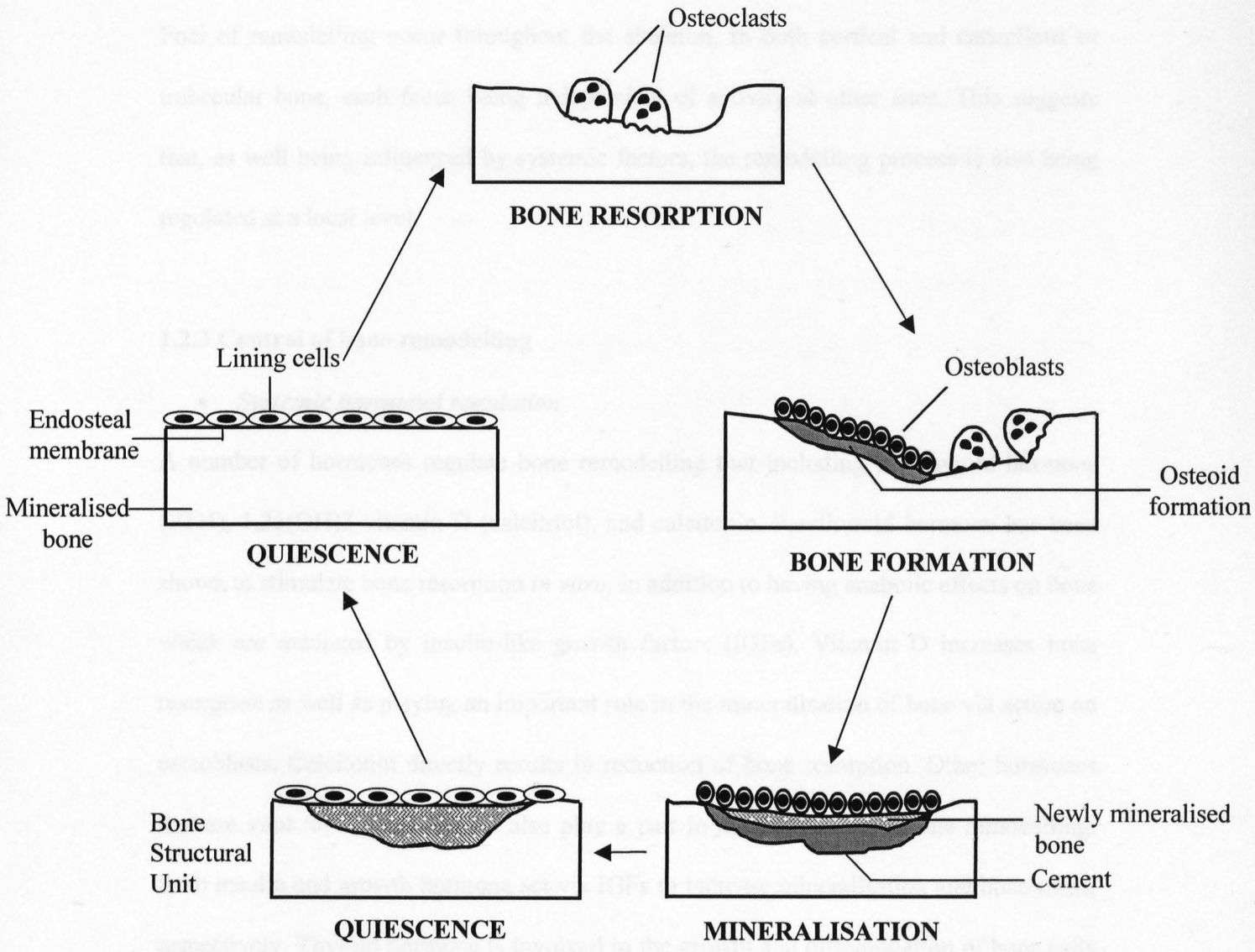


Figure 1.1 Bone Remodelling. Biochemical and mechanical stimuli result in the retraction of bone lining cells and exposure of the bone surface to begin bone remodelling. Multinucleated osteoclasts form by fusion of their precursors and are activated to commence the formation of a resorption cavity. Once the cavity is smoothed by mononuclear cells, osteoblasts are recruited and a highly mineralised cement line is laid at the base of the resorption cavity. This is the reversal phase. Osteoblasts are then recruited to this site and produce an uncalcified osteoid matrix which subsequently undergoes mineralization. During the final step, quiescence, osteoblastic activity ceases. The newly formed bone is termed a bone structural unit. Adapted from Compston,

Foci of remodelling occur throughout the skeleton, in both cortical and cancellous or trabecular bone, each focus being independent of activity at other sites. This suggests that, as well being influenced by systemic factors, the remodelling process is also being regulated at a local level.

1.2.3 Control of bone remodelling

- ***Systemic hormonal regulation***

A number of hormones regulate bone remodelling that including parathyroid hormone (PTH), 1,25(OH)2 vitamin D (calcitriol), and calcitonin. Parathyroid hormone has been shown to stimulate bone resorption *in vitro,* in addition to having anabolic effects on bone which are mediated by insulin-like growth factors (IGFs). Vitamin D increases bone resorption as well as playing an important role in the mineralisation of bone via action on osteoblasts. Calcitonin directly results in reduction of bone resorption. Other hormones that are vital for normal growth also play a part in the regulation of bone remodelling. Both insulin and growth hormone act via IGFs to increase mineralisation and bone mass, respectively. Thyroid hormone is involved in the growth and differentiation of bone cells and oestrogen and testosterone both inhibit bone resorption. Glucocorticoids are also known to directly inhibit osteoblast activity, resulting in decreased bone formation, as well as stimulating increased bone turnover.

- ***Local Regulation***

Factors local to the bone microenvironment also modulate the rate of bone turnover. The bone matrix is a storehouse of growth factors, such as TGFβ and IGFs, that are known to stimulate bone formation and reduce bone resorption. Other factors, including fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs) influence both bone resorption and formation to increase bone turnover. Certain cytokines that are produced by bone cells and stromal cells are also known to be important. Il-1 and IL-6 stimulate osteoclast formation and bone resorption (de la Mata J, 1995; Ishimi Y, 1990; Pfeilschifter J, 1989) as well as macrophage colony-stimulating factor (M-CSF) and prostoglandins amongst others (Fleisch, 2000).

A recently discovered factor which inhibits osteoclast formation and activity is osteoprotogerin (OPG, also known as osteoclastogenesis inhibitory factor, OCIF) (Anderson DM, 1997; Simonet WS, 1997). This is a glycoprotein of the TNF receptor family, which is produced by stromal cells, osteoblast lineage cells and pre-B cells. It acts as a decoy receptor and binds to an osteoclastic differentiation factor called receptor activator of NF-κB ligand (RANKL) which is located on the membrane of osteoblast lineage cells. RANKL binds to its transmembrane receptor RANK (Nakagawa N, 1998) on osteoclast precursors to stimulate osteoclast recruitment, differentiation and activation (Fuller K, 1998; Lacey DL, 1998; Yasuda H, 1998) [Burgess TL, 1999 May 3; #308]. Therefore the action of OPG as a decoy receptor is to inhibit osteoclast maturation and activation, thereby inhibiting bone resorption (Yasuda H, 1998). Studies in mice bearing a defective RANKL gene show that these animals suffer severe osteopetrosis as

osteoblasts are unable to stimulate osteoclastgenesis (Kong YY, 1999) suggesting that the RANK, via the actions of RANKL, is a key receptor for the activation and survival of osteoclasts.

1.2.4 Pathogenesis of bone metastases

- ***General mechanisms of metastasis***

Once tumour cells at the primary site have established, there are multiple steps involved in the growth and spread of the tumour to bone (Figure 1.2). In order to invade the host tissue and metastasise, the tumour cells must possess an aggressive phenotype, which enables them to promote angiogenesis and cell migration, adhere selectively to other cells, basement membranes and extracellular matrices and produce proteolytic enzymes to complete the invasive process (Aslakson C, 1992; Folkman, 1984; Mundy, 1997).

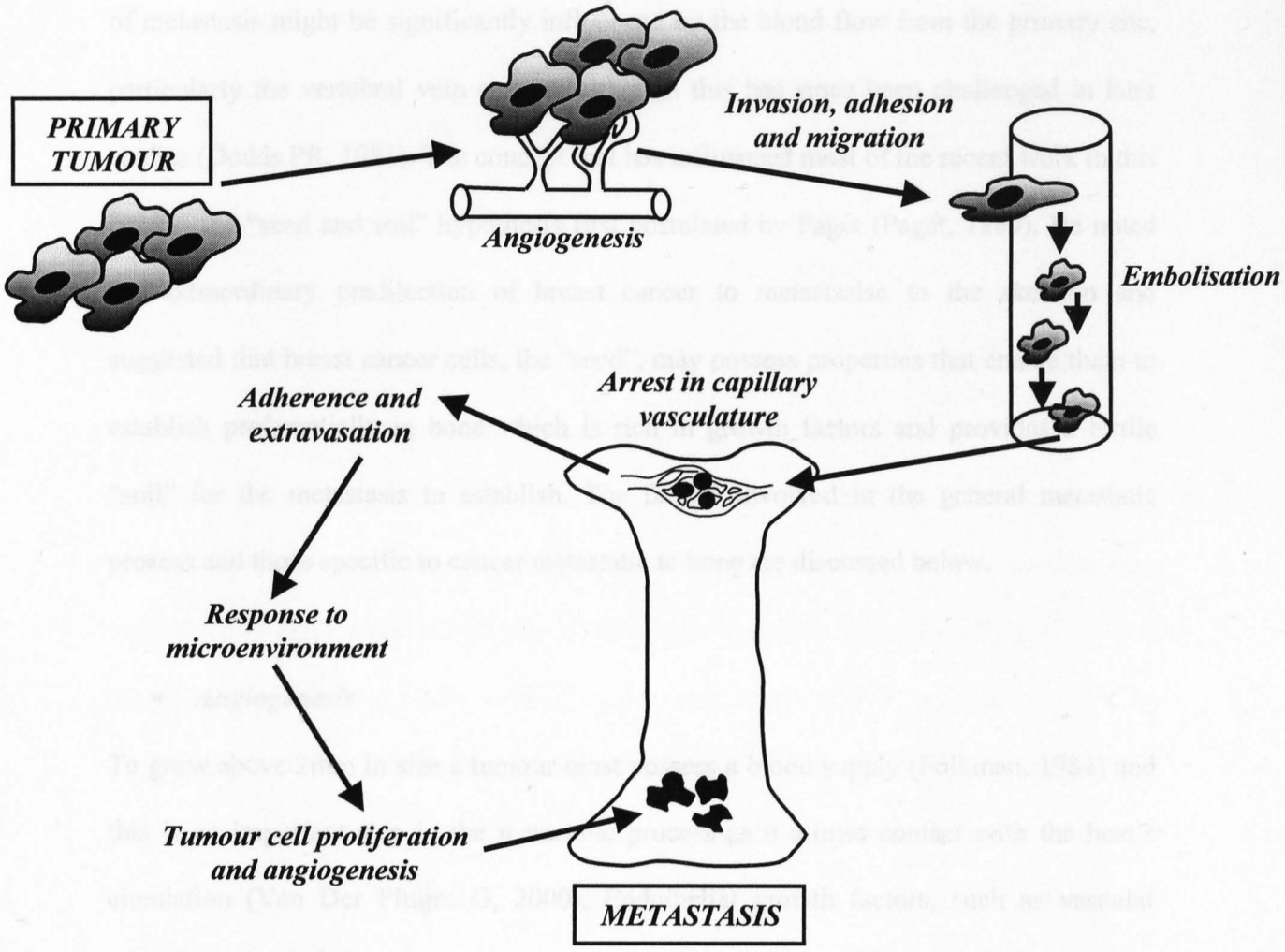


Figure 1.2 The pathogenesis of cancer metastases. Tumour cells must detach from the primary tumour, invade the tumour vasculature and enter in the circulation. Those that survive immune surveillance arrest in the capillary bed of distant organs, including bone and must extravasate to invade the host organ parenchyma. Tumour cells then respond to paracrine stimuli in the microenvironment to induce proliferation and promote angiogenesis to allow the metastasis to establish.

The metastatic process to bone appears to be selective and there are several theories as to the mechanisms behind this. The anatomical theory (Batson, 1940) suggests that the sites of metastasis might be significantly influenced by the blood flow from the primary site, particularly the vertebral vein system, although this has since been challenged in later studies (Dodds PR, 1981). The concept that has influenced most of the recent work in this field is the "seed and soil" hypothesis first postulated by Paget (Paget, 1889). He noted the extraordinary predilection of breast cancer to metastasise to the skeleton and suggested that breast cancer cells, the "seed", may possess properties that enable them to establish preferentially in bone which is rich in growth factors and provides a fertile "soil" for the metastasis to establish. The factors involved in the general metastatic process and those specific to cancer metastatic to bone are discussed below.

- ***Angiogenesis***

To grow above 2mm in size a tumour must possess a blood supply (Folkman, 1984) and this is an important step in the metastatic process as it allows contact with the host's circulation (Van Der Pluijm G, 2000). Endothelial growth factors, such as vascular endothelial growth factors (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factors (FGF), and endogenous anti angiogenic factors, such as angiostatin, regulate tumour neovascularisation. Of particular importance is VEGF which has been shown to be over-expressed by many tumours types. It has been shown to promote endothelial cell growth and chemotaxis (Ferrara N, 1997; Neufeld G, 1999) as well as the induction of the expression of proteases in endothelial cells (Pepper MS, 1991; Unemori EN, 1992). It may enhance the migration of tumour cells by increasing vascular

permeability (Chaplin DJ, 1997; Dvorak HF, 1995) and has also been implicated in lymphatic spread (Mandriota S, 2001). Tumour angiogenesis and growth can be inhibited by blocking the activity of VEGF using neutralising antibodies in tumour-bearing mice (Kim KJ, 1993) indicating that this factor plays a central role in tumour progression.

- ***Adhesion molecules***

Once neovascularisation has occurred, the next step in the metastatic process is the detachment of tumour cells from the primary site and subsequent attachment to endothelial cells and extracellular matrices. Cellular adhesion molecules such as integrins, cadherins, especially E-cadherin, and selectins mediate tumour cell interactions with substrata and can be differentially expressed to allow escape from the primary site, tumour cell migration and attachment to basement membranes at the target organ.

The cadherin family of cell adhesion molecules, particularly E-cadherin, plays an important role in epithelial cell-cell adhesion. Loss of E-cadherin expression in epithelial tumours is associated with the development of a highly invasive phenotype (Behrens, 1993; Behrens J, 1989). Invasive breast cancer cell lines, such as MDA-MB-231, express low levels of E-cadherin whereas non-metastatic cell lines are associated with high levels of expression (Sommers CL, 1991). Indeed, the de novo expression of E-cadherin in MDA-MB-231 cells reduces the ability of these cells to form osteolytic metastases in the bone of nude mice (Mbalaviele G, 1996). As well as changes in cadherin expression, cell-cell attachment may also be disrupted by the deletion of the cytoplasmic tail of the cadherin molecule or by alterations in catenin proteins which mediate interactions with the cytoskeleton. Hence complex alterations may occur to alter cell-cell adhesion.

The integrins are a diverse family of receptors that mediate adhesion between the cell membrane and the extracellular matrix or other cell adhesion molecules. They are composed of distinct α and β subunits, each of which has further subtypes, and the specificity with which ligands are bound is determined by the combination of subunits. Moreover, certain integrins may be activated or inactivated in response to other signals and hence modulate cell adhesion (Meyer T, 1998). Indeed, the function of integrins is not restricted to cell-substrata adhesion alone. Recent studies have suggested that these receptors may play a role in signal transduction and may influence cell motility and invasion (Hynes, 1992).

Members of the immunoglobulin superfamily, such as ICAM-1, ICAM-2, V-CAM and PECAM, have been implicated in the extravasation process (as reviewed by Meyer and Hart,) (Meyer T, 1998). The expression of these adhesion molecules, which contain one or more Ig-like domains, is upregulated by cytokines such as TNFα and interleukin-1 during the inflammatory process. Leucocytes recruited to the sites may interact with these adhesion molecules to arrest prior to extravasation. They may thus mediate interaction between the endothelium and tumour cells to enhance their migration through the vessel walls at metastastic sites. Similarly, expression of members of the selectin family of adhesion molecules is triggered during inflammation. The resultant low affinity binding initiates leucocyte rolling and this is followed by arrest and extravasation as described above. Similar processes may be implicated in tumour cell arrest and extravasation.

- ***Proteolytic enzymes***

Breast cancer cells must have a means of penetrating the basement membrane and invading the surrounding tissue and a host of proteolytic enzymes are vital to this process. The key groups of proteolytic enzymes implicated in tumour invasion are the serine proteases, including urokinase-type plasminogen activator (uPA) and plasmin, the matrix metalloproteinases and the cysteine proteases, cathepsin B and L (MacDougall JR, 1995).

The uPA/ urokinase plasminogen activator receptor (uPAR) system is involved in many physiological processes, including wound healing and angiogenesis, and is also implicated in tumour metastasis. Members involved in this system have been shown by immunohistochemistry to be located at the invasive edge of cancers and surrounding stromal cells, (Pyke C, 1991) suggesting that multifaceted interactions are occurring between tumour cells and the tissue environment to degrade the stroma. uPA, uPAR and PAI (plasminogen activator inhibitor) have recently been recognised as important prognostic factors in breast cancer. High breast tumour levels of uPA have been associated with higher relapse rates (Janicke F, 1990) and a worse prognosis (Duffy MJ, 1990). There is also evidence to suggest that this system may influence the metastatic potential of cancer cells. In one study, the *in vitro* invasive potential of rat breast carcinoma cells was enhanced by upregulating the expression of uPA and, *in vivo*, these resulted in larger mammary tumour growth with increased metastases (King RH, 1996). Interestingly, in a mouse syngeneic tumour model, monoclonal antibodies against uPA significantly reduced the development of lung metastases (Hearing VJ, 1998). Another important group of proteolytic enzymes are the matrix metalloproteinases. These are a

family of zinc-dependant endopeptidases which are divided into the gelatinases, collagenases and stromelysins according to the substrate upon which they act. Increased plasma levels of MMPs have been associated with higher levels of invasion and metastases in breast tumours (MacDougall JR, 1995). In bone in particular, Yoneda et al have reported that human breast cancer cells express the gelatinases MMP-2 and MMP-9 in their latent forms but that expression of the activated forms can be induced when cultured on extracellular matrix derived from osteoblasts (Yoneda T, 1997). In addition, MMP-9 has recently been shown to stimulate osteoclast-mediated bone resorption (Okada Y, 1995; Witty JP, 1996) and influence the differentiation of osteoclast precursors (Blavier L, 1995). It is possible, therefore, that MMPs produced by breast cancer cells may influence osteoclast differentiation and stimulate bone resorption to enhance the progression of bone secondaries. Another factor is the influence of ubiquitously occurring inhibitors of metalloproteinases, the tissue inhibitors of matrix metalloproteinases (TIMPs). These closely regulate the activity of MMPs and their over-expression in breast cancer cells markedly inhibits the formation of bone metastases in experimental studies (Yoneda T, 1997).

- ***Bone-specific proteins expressed by breast cancer cells***

The properties of metastastic cancer cells discussed so far are common to all metastastic tumours. There may be additional factors unique to osteotrophic cancers which aid their spread to bone in particular. Several proteins originally identified as bone matrix proteins are also expressed by certain cancers which have a predilection for bone such as breast

cancer and prostate cancer. These proteins include osteopontin (OPN), osteonectin (ON) and bone sialoprotein (BSP) (Koeneman KS, 1999; Rosol, 2000) (Jacob K, 1999).

- *Osteopontin*

OPN is a protein secreted by osteoblasts and placental trophoblasts and contains a conserved RDG (Arg-Gly-Asp) amino acid sequence. This allows binding to integrins and the ubiquitous CD44 cell adhesion protein (Weber GF, 1996; Xuan JW, 1995). Osteopontin expressed on breast cancer cells may mediate cell-cell or cell-matrix interaction and aid chemotaxis, thereby enhancing metastasis to bone.

- *Bone Sialoprotein*

Another bone matrix protein which is expressed by breast cancer cells, as well as other osteotrophic tumours, is bone sialoprotein (BSP) (Mundy G, 1996; Quinn JE, 1998) (Rubens, 1999). BSP is a glycoprotein which is secreted by osteoblasts and contains an integrin-binding RDG sequence which acts as a ligand for osteoclasts to potentiate bone resorption. It may facilitate interaction between breast cancer cells and osteoclasts as well as promoting cancer cell migration and proliferation (Sung V, 1998). BSP can be measured in the serum and may serve as a marker for osteotrophic cancers (Fedarco NS, 1999). Recent clinical studies have suggested that serum levels of BSP may be a reliable predictor of the development of subsequent bone metastases in patients with breast cancer (Bellahcene A, 1998). In addition, the expression of BSP in primary breast and prostate cancer has been associated with disease progression and a poorer prognosis (Bellahcene A, 1998; Mundy G, 1996) Yoneda T, 1997). Waltregny *et al* recently demonstrated a

differential increase in expression of BSP in bone metastases compared with visceral metastases from patients with breast and prostate cancer (Waltregny D, 2000). Therefore this protein may play a key role in the homing and proliferation of breast cancer cells in bone and in the development of bone metastases.

- *Osteonectin*

Osteonectin (ON) is a multifunctional glycoprotein which is involved in mineralization, cell-cell interactions and angiogenesis (Rosol, 2000). In addition to its production by osteoblasts, it is secreted by prostate and breast carcinoma cells. It has been shown to enhance MMP activity in both breast and prostate cancer thereby aiding invasion and metastasis (Jacob K, 1999).

- ***Breast cancer-bone interaction***

Bone is unique amongst metastastic sites as it is continually being renewed and remodelled. It is a storehouse for growth factors and these are constantly being released into the microenvironment as bone turnover occurs. Growth factors and cytokines are also released from bone marrow stromal cells and immune cells. TGFβ and IGFs 1 and 2 make up the majority of bone derived growth factors. Once released into the bone marrow milieu, these growth factors may have effects on breast cancer cells to aid the development of metastases. The tumour cell-bone interaction is complex and not fully understood but forms the focus of much research activity. Recent evidence suggests that metastastic breast cancer cells in bone may secrete soluble factors, for example PTHrP, which stimulate osteoclast activity and promote bone resorption (Figure 1.3) Recent

studies have suggested that RANKL may also play a role in this system (Thomas RJ, 1999). This, in turn, results in the release of growth factors into the bone marrow microenvironment which may act on breast cancer cells to promote further bone destruction and development of the metastasis. Thus, a spiral of events is triggered in which tumour derived mediators accelerate osteoclastic bone breakdown and the subsequently released growth factors act on breast cancer cells to stimulate the further production of soluble factors and promote further tumour cell chemotaxis. Evidence for the specific roles of cytokines and growth factors, such as PTHrP, in this process are discussed in the section below.

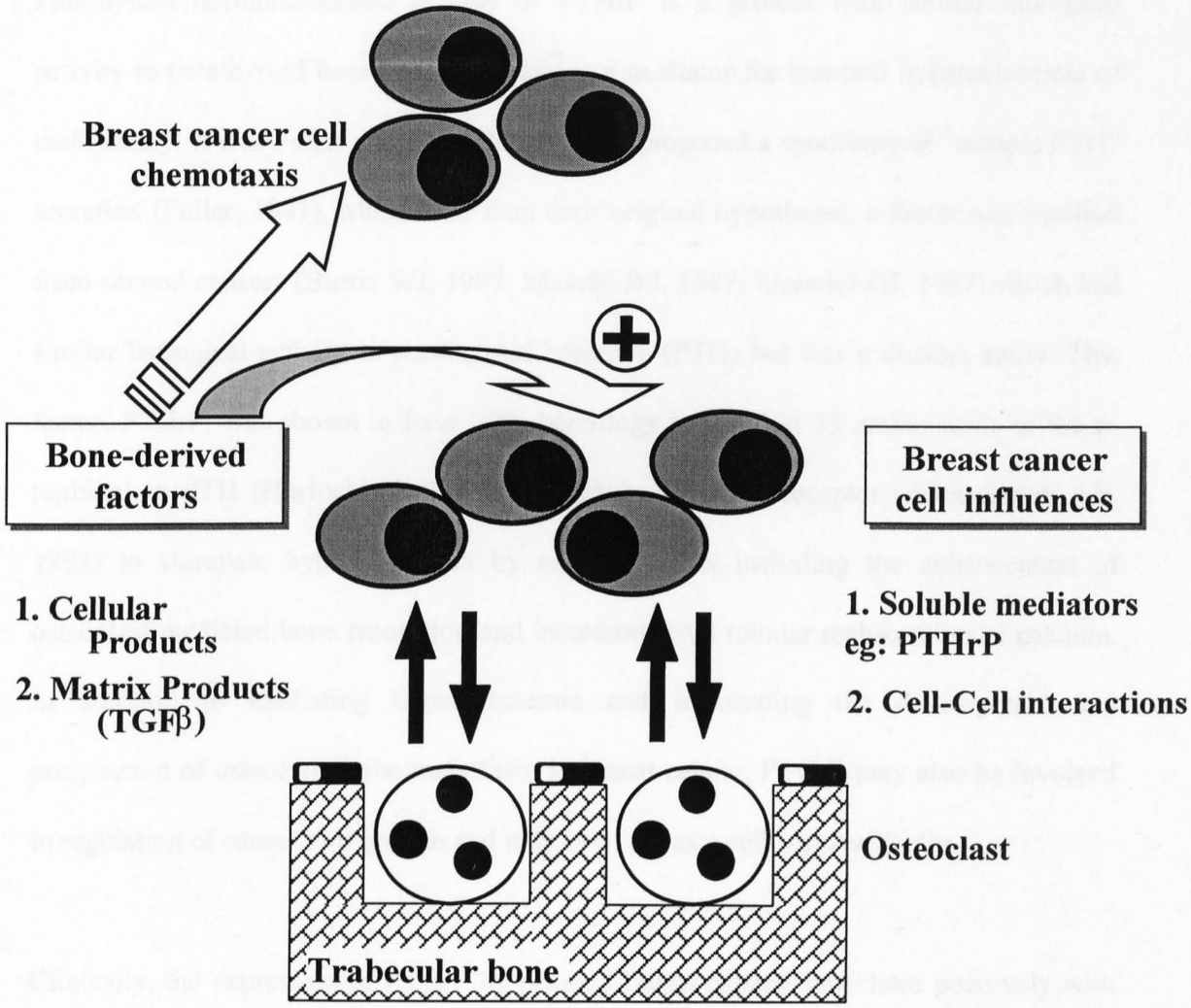


Figure 1.3 Interaction between breast cancer cells and the bone microenvironment. Tumour cells produce soluble mediators, such as PTHrP, which stimulate osteoclast activity and promote osteoclast-mediated bone resorption. This in turn results in the release of growth factors, such as TGF-β and IGFs, from the bone matrix which may promote further breast cancer cell chemotaxis and the release of cytokines from the tumour cells to further increase osteoclast activity. The net effect is to set up a cycle of bone destruction fueled by tumour-derived osteoclast activating cytokines and bone derived growth factors. In this environment, metastastic cells can establish and proliferate.

PTHrP

Parathyroid hormone-related peptide or PTHrP is a protein with similar biological activity to parathyroid hormone (PTH) and is a mediator for humoral hypercalcaemia of malignancy. It was Fuller and Albright who first proposed a syndrome of "ectopic PTH" secretion (Fuller, 1941). Much later than their original hypotheses, a factor was purified from several cancers (Burtis WJ, 1987; Mosely JM, 1987; Strewler GJ, 1987) which had similar biological activity to parathyroid hormone (PTH) but was a distinct entity. This factor, PTHrP, was shown to have 70% homology in the first 13 amino acids of the n-terminal to PTH (Horiuchi N, 1987). It binds to the PTH receptor (Abou-Samra AB, 1992) to stimulate hypercalcaemia by several means including the enhancement of osteoclast-mediated bone resorption and increased renal tubular reabsorption of calcium. In addition to mediating hypercalcaemia and influencing the development and progression of osteolytic bone metastases in breast cancer, PTHrP may also be involved in regulation of cancer cell growth and may even act as a cell survival factor.

Clinically, the expression of PTHrP by primary breast cancers correlates positively with the development of subsequent bone metastases and hypercalcaemia (Bundred NJ, 1991). PTHrP has been detected by immunohistochemistry in a higher proportion of patients with bone metastases from breast cancer compared to those with lung metastases or no metastastic disease (Kohno N, 1994). In animal studies, mice bearing MDA-MB-231 breast cancer cells that over-expressed PTHrP developed a significantly higher number of osteolytic bone metastases (Guise T, 1996). Treatment with monoclonal antibodies against PTHrP before injection of the tumour cells resulted in a dramatic reduction in the

number and size of bony metastases indicating that PTHrP has a central role in breast cancer bone metastasis.

- *IL-6.*

Interleukin 6 (IL-6) is a multifunctional regulator of immune response, haematopoesis and acute phase responses [Hirano, 1998 #193]. It has also been shown to influence the proliferation of both normal and tumour-derived cells. Several studies have demonstrated the secretion of IL-6 by mammary carcinoma cells (Chiu J, 1996; Pederson L, 1999) as well as other tumour types, particularly multiple myeloma (reviewed by Guise and Mundy (Guise T, 1998)and Klein *et al* (Klein B, 1995)and normal bone marrow stromal cells (Boyce B, 1999; Mohan S, 1991). In multiple myeloma, this cytokine acts as important growth regulator and survival factor in its ability to support myeloma cell proliferation (Kawano M, 1988) and indeed the stage of advancement of the disease has been shown to correlate with patient serum and bone marrow IL-6 concentrations (Bataille R *et al.*, 1992; Bataille R, 1989; Zhang GJ, 1999). In contrast, IL-6 has been shown to result in growth inhibition in mammary carcinoma cells (Novick D, 1992) and it has also been suggested that it may regulate the growth of normal and transformed human mammary epithelial cells differentially (Chiu J, 1996; Lai L, 1994). Chiu et al found that higher levels of IL-6 were secreted by ER negative breast cancer cells than ER positive cells and that this acted on ER positive cells to suppress growth in a paracrine fashion (Chiu J, 1996). Recent observations that IL-6 may disrupt breast cancer cell adhesion (Tamm I, 1994) and increase tumour cell migration (Badache A, 2001) together with the paracrine effects described above suggest that expression of this cytokine may signify the

development of a more aggressive and metastatic breast cancer cell phenotype. Indeed, elevated serum levels of IL-6 and sIL-6R have been observed in breast cancer patients compared with normal controls (Jablonska, 1998) and appear to correlate with more aggressive disease (Haverty A, 1997; Jiang X, 2000; Yokoe T, 1997). Interestingly, IL-6 has been implicated in the stimulation of osteoclast precursors, possibly through the activity of other factors such as PTHrP, which in turn would accelerate bone resorption (de la Mata J, 1995). The soluble form of the Il-6 receptor (sIL-6R) binds IL-6 and the complex interacts with the membrane-bound receptor, gp130, to stimulate signalling and enhance IL-6 effects.

- *TGF*β.

TGFβ is an important regulatory protein for the differentiation and proliferation of many human cells including bone cells (Centrella M, 1994). The TGFβ–1 isoform is highly expressed by mature osteoblasts and osteoclasts, stored in bone matrix and released into the microenvironment when bone resorption takes place (Pfeilschifter J, 1987). TGFβ signals via a series of receptors which interact with the Smad family of proteins to mediate signalling (Massague, 1998). The effects of TGFβ are cell specific, in some cases stimulating proliferation (mesenchymal cells) and in others inhibiting growth (epithelial cells) (Derynk, 1994). It also facilitates cell adhesion and the production of extracellular matrix protein.

Breast cancer cells, as well as cancer associated stromal cells, have been shown to express TGFβ (Van Roozendaal CEP, 1995). It may influence the production of other

important proteins, such as MMPs from breast cancer cells (Diudenvoorden W, 1999). In particular, TGFβ has been shown to stimulate the production of PTHrP from human breast cancer cells (Firek A, 1994). Using an *in vivo* model of osteolytic metastases, Yin *et al* elegantly demonstrated the relationship between TGFβ and PTHrP (Yin J, 1999). They inoculated mice with MDA-MB-231 breast cancer cells that had been transfected with genes encoding an inactive TGFβ receptor and found that these animals developed significantly fewer and smaller osteolytic lesions compared with controls or those bearing empty vectors. Taken together, these data suggest that TGFβ enhances the development of breast cancer bone metastases by stimulating the production of PTHrP and thereby increasing osteoclast differentiation and activity. The resulting bone resorption leads to the release of further growth factors from the bone matrix and the proliferation and chemotaxis of breast cancer cells.

- *Insulin-like Growth Factors*

The insulin-like growth factors are important proteins in bone formation. The system comprises two proteins which act as ligands, IGF-1 and IGF-II, two receptors and six binding proteins (Jones JI, 1995). IGFs I and II have a stimulatory effect on the functions of mature osteoblasts, as well as decreasing the rate of collagen degradation, indicating that they have a central role in the preservation of bone matrix integrity and in bone formation. There is evidence to suggest that they are involved in the development of the normal mammary gland, as well as being potentially mitogenic for breast cancer cells, as reviewed by Sachdev et al (Sachdev D, 2001).

1.2.5 Treatment of bone metastases

The current treatment strategies for metastastic bone disease in breast cancer focus mainly on symptom palliation although newer agents may help to prevent the occurrence or slow the progression of bone disease. Bone pain is managed with the use of analgesics, as well as local radiotherapy and bisphosphonates to slow down the progression of bone disease. Surgical intervention may be required if fracture is imminent or for spinal instability. Radiopharmaceuticals that target radiation to the sites of metastases are also in development. As discussed previously, many patients with bone only disease will have hormone receptor-positive tumours, therefore systemic endocrine therapy is widely used in these patients. Systemic chemotherapy is often reserved for those women with receptor-negative disease. Bisphosphonates, as discussed below in section 1.3, are the treatment of choice for hypercalcaemia of malignancy.

1.3 BISPHOSPHONATES

1.3.1 Introduction

Bisphosphonates are stable analogues of pyrophosphate and act as potent inhibitors of osteoclast-mediated bone resorption. They have been in clinical use for some time and have recently been established for the management of hypercalcaemia of malignancy and tumour induced bone disease. The structure of pyrophosphate and a generic bisphosphonate is shown below. Bisphosphonates are characterised by the replacement of the central oxygen atom by a carbon atom. This forms a central P-C-P bond (Figure 1.4).

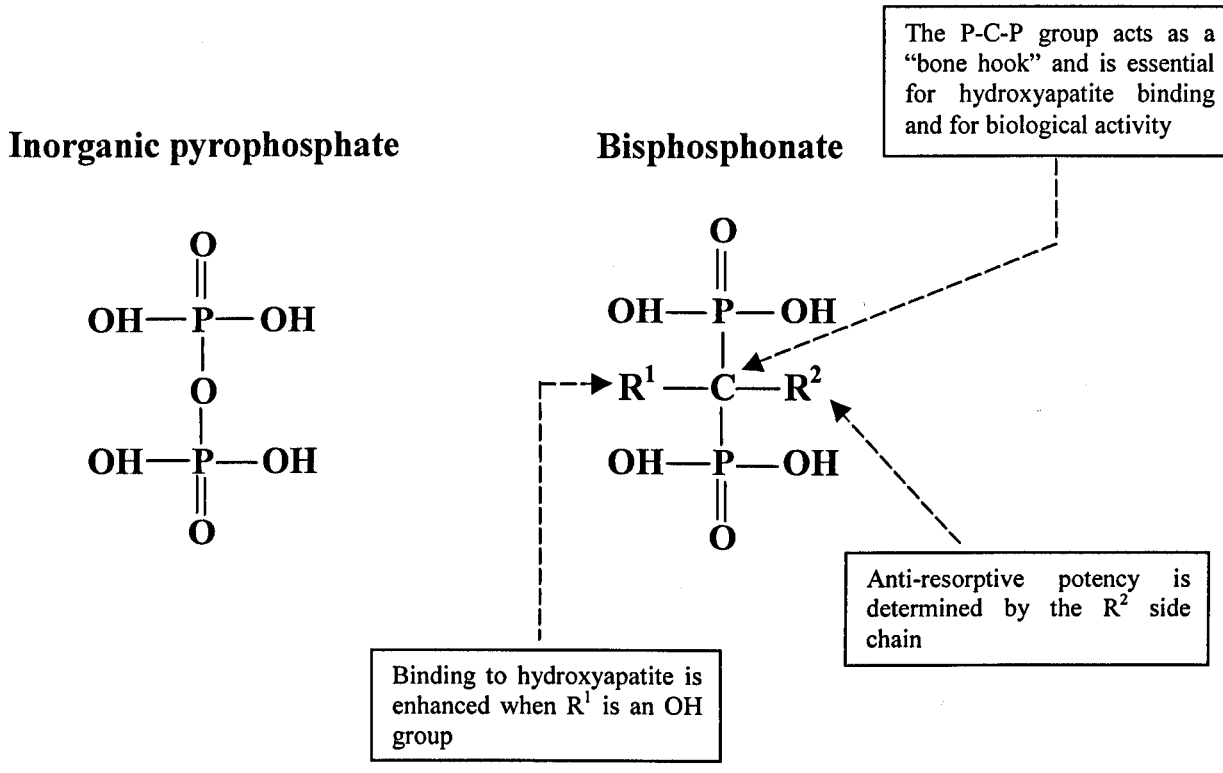


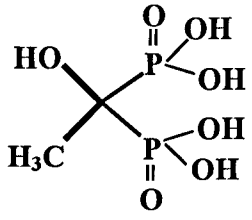
Figure 1.4 The structure of pyrophosphate and a generic bisphosphonate. Bisphosphonates are analogues of inorganic pyrophosphate where the central oxygen atom has been replaced with a carbon atom. The resulting P-C-P structure has a high affinity for hydroxyapatite. The affinity for bone and the anti-resorptive potency of bisphosphonates can be enhanced by altering the R^1 and R^2 side chains respectively.

1.3.2 Structure of bisphosphonates

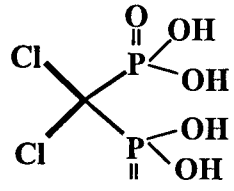
A characteristic that both bisphosphonates and pyrophosphate have in common is a high affinity for bone mineral along with the dissolution of hydroxyapatite *in vitro* (Jung A, 1972; Russell RGG, 1970). The phosphonate groups, through the chelation of calcium by the oxygen atoms, form the basis for this affinity. Placing a hydroxyl group (-OH) in the R^1 side chain increases the affinity of bisphosphonates for bone mineral even further

(Jung A, 1972). Pyrophosphate, in contrast to bisphosphonates, does not inhibit bone resorption and its relative instability when faced with enzymatic or chemical hydrolysis has been suggested as an explanation (Fleisch H, 1969; Russell RGG, 1970). Bisphosphonates, on the other hand, are more stable compounds. Interestingly, the affinity of bisphosphonates for bone mineral does not correlate with their anti-resorptive potency, either *in vitro* or *in vivo* (Shinoda H, 1983). The development of differing anti-resorptive potency depends on modification of the R^2 side chain (Geddes AD, 1994; Sietsema WK, 1989) (Figure1.5).

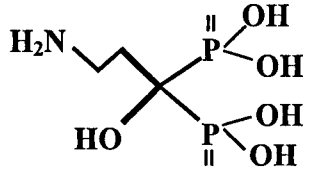
The anti-resorptive potency of bisphosphonates can be increased by the addition of a nitrogen group to the R^2 side chain. Compounds with this modification, such as in pamidronate and alendronate, show an increased potency *in vivo* by 1-3 orders of magnitude compared to the low potency bisphosphonate etidronate (Sietsema WK, 1989). Newer compounds have been developed to include the nitrogen group within a heterocyclic ring to further enhance the anti-resorptive potency. Both risedronate and zoledronic acid, which include this structure, have been found to be more potent *in vivo* than etidronate and pamidronate (Green JR, 1994; Sietsema WK, 1989) (figure 1.5).



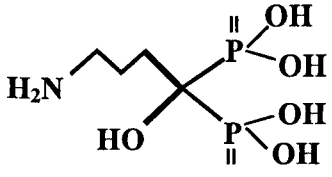
Etidronate (1)



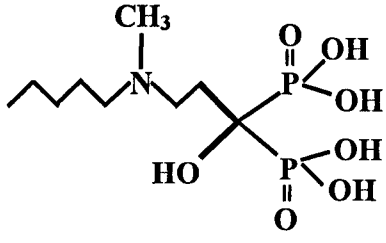
Clodronate (10)



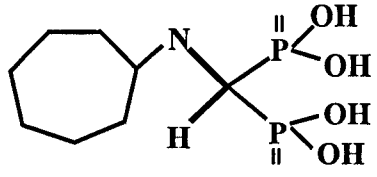
Pamidronate (100)



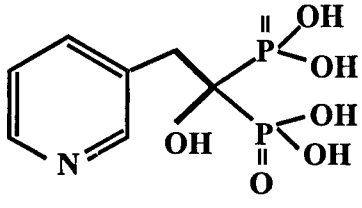
Alendronate (1,000)



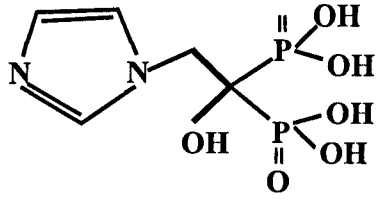
Ibandronate (10,000)



Incadronate (1,000)



Risedronate (5,000)



Zoledronic acid (94,000)

Figure1.5 The relationship between bisphosphonate structure and anti-resorptive potency. The relative *in vivo* anti-resorptive potencies are shown in brackets (Fleisch, 1991; Green JR, 1994). The introduction of an amino group in the R^2 side chain, as in pamidronate and alendronate, increases the anti-resorptive potency. This is further enhanced by methylation of the amino group, as in ibandronate, or by inclusion of a heterocyclic ring in the R^2 side chain. The most potent bisphosphonates, including risedronate and zoledronic acid, contain nitrogen atoms within the heterocyclic ring.

1.3.3 Cellular effects of bisphosphonates

Bisphosphonates have a high affinity for mineralised bone and adsorb to the bone surface. During the resorption process the bisphosphonate is released from the bone surface, exposing the osteoclast to high concentrations of the drug (Sato M *et al.*, 1991) with subsequent anti-resorptive effects (Figure 1.6). As well as effects on osteoclasts, however, bisphosphonates have also been shown to influence osteoclast precursors and osteoblasts, effects that may serve to augment their anti-resorptive action.

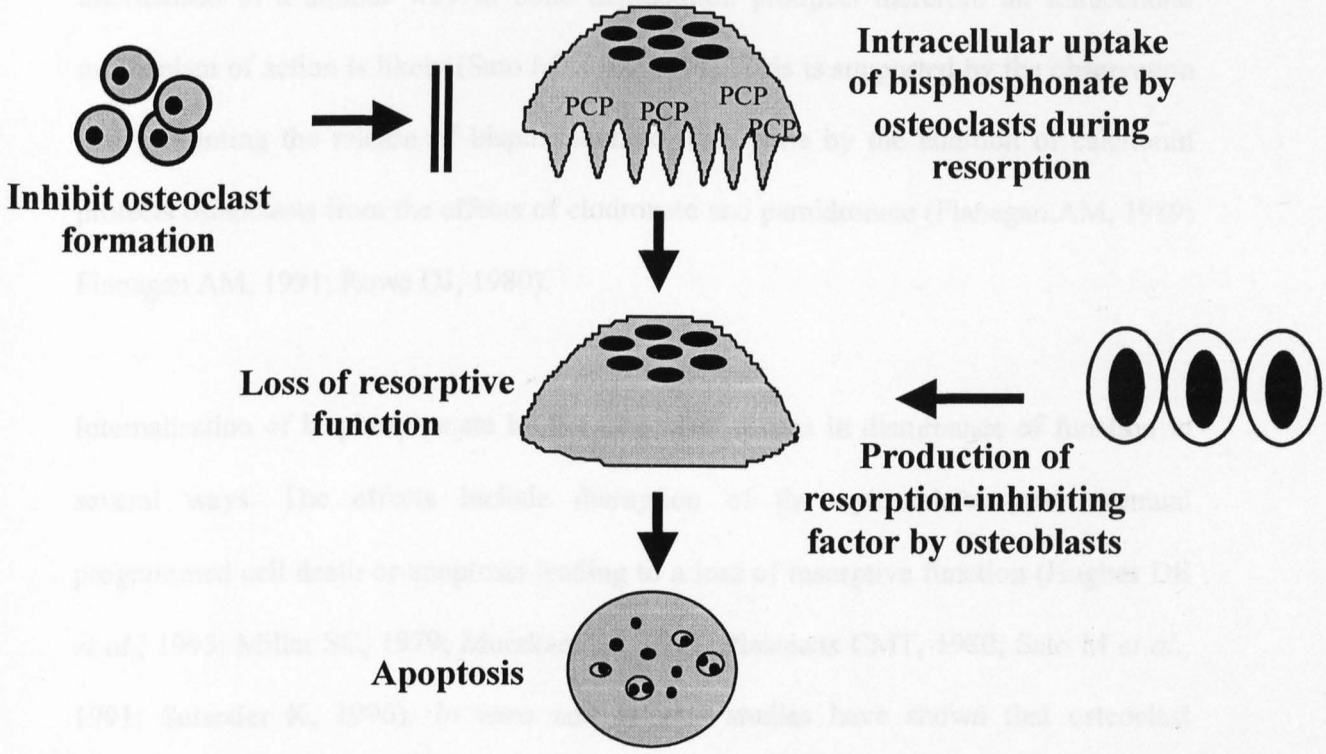


Figure 1.6 The effects of bisphosphonates on osteoclasts. Bisphosphonates inhibit osteoclast activity in several ways, including inhibiting the maturation of osteoclast precursors to mature osteoclasts, disrupting the osteoclast ruffled border and causing loss of resorptive function, and inducing osteoclast apoptosis. They also stimulate the production of a resorption-inhibiting factor by osteoclasts.

- ***Mature osteoclasts***

During the resorption process, the osteoclast appears to internalise bone degradation products by endocytosis at the ruffled border, transport them in vesicles into the cell cytoplasm and clear them from the anti-resorptive surface by exocytosis (Salo J, 1997). As acidification of the environment below the osteoclast occurs, bisphosphonates are released from the bone surface and relatively high concentrations accumulate below the osteoclast (Sato M *et al.*, 1991). *In vivo* studies suggest that radiolabeled alendronate is internalised in a similar way to bone degradation products therefore an intracellular mechanism of action is likely (Sato M *et al.*, 1991). This is supported by the observation that preventing the release of bisphosphonate from bone by the addition of calcitonin protects osteoclasts from the effects of clodronate and pamidronate (Flanagan AM, 1989; Flanagan AM, 1991; Rowe DJ, 1980).

Internalisation of bisphosphonate by the osteoclast results in disturbance of function in several ways. The effects include disruption of the cytoskeleton and eventual programmed cell death or apoptosis leading to a loss of resorptive function (Hughes DE *et al.*, 1995; Miller SC, 1979; Murakami H, 1995; Plasmans CMT, 1980; Sato M *et al.*, 1991; Selander K, 1996). *In vitro* and *in vivo* studies have shown that osteoclast cytoskeletal disruption and loss of the ruffled border at the osteoclast-bone interface occurs after bisphosphonate treatment (Plasmans CMT, 1980; Sato M *et al.*, 1991). In support of this, Murakami et al reported that tiludronate was selectively incorporated into

osteoclasts that were actively resorbing bone, i.e. those with a ruffled border, and disrupted the actin ring essential for osteoclast function (Murakami H, 1995).

The apoptotic effects of bisphosphonates on osteoclasts have recognised for some time. Early studies observed that degenerative changes in osteoclast nuclear morphology occurred following bisphosphonate treatment (Rowe DJ, 1976; Rowe DJ, 1980; Rowe DJ, 1983; Schenk R, 1973). Studies with clodronate and pamidronate confirmed that bisphosphonate treatment was associated with a reduction in osteoclast number on the bone surface and injury-associated changes in nuclear morphology (Flanagan AM, 1989; Flanagan AM, 1991; Marshall MJ, 1990) that were later to be confirmed as characteristic features of programmed cell death or apoptosis. (Wyllie R, 1980) The induction of osteoclast apoptosis by bisphosphonates, such as clodronate, pamidronate and risedronate, has recently been verified both *in vitro* and *in vivo* (Hughes DE *et al.*, 1995; Selander K, 1996).

- ***Osteoclast precursors***

Mature osteoclasts are necessary for bone resorption therefore interference with the maturation of osteoclasts from their precursors inevitably inhibits this process. There is evidence to suggest that, as well as altering mature osteoclast function, bisphosphonates may have effects on osteoclast precursors. *In vitro* studies of the effects of bisphosphonates on a human bone marrow culture system have demonstrated a concentration-dependent inhibition of formation of osteoclast-like cells, with the order of potency of bisphosphonates correlating closely with their *in vivo* anti-resorptive potency

(Hughes DE, 1989). Two mechanisms of action have been postulated. Low concentrations of nitrogen-containing bisphosphonates, such as pamidronate, have been reported to inhibit bone resorption by interfering with attachment and transformation of osteoclast precursors (Boonekamp P, 1986; Lowik C, 1988). A postulated mechanism for this is that bisphosphonates may interfere with the recognition of a bone matrix factor required for stimulation of osteoclast maturation. Higher concentrations of both amino-bisphosphonates and non-nitrogen-containing bisphosphonates have been found to affect mature osteoclast function and hence inhibit bone resorption. Certainly, the potent bisphosphonate alendronate has been found to inhibit osteoclast formation in vitro (Van Beek ER, 1997; (Fisher JE, 1999) although no effect has been demonstrated on the osteoclastgenesis potential of murine bone marrow *in vivo* (Van Beek ER, 1997).

- ***Osteoblasts***

Complex interactions occur between osteoclasts and osteoblasts to influence bone resorption. Bisphosphonates may also interfere with bone resorption by having effects on osteoblasts. *In vitro* studies have suggested that bisphosphonate treatment of osteoblasts may stimulate the production of a factor that inhibits osteoclast maturation or function. Pre-treatment of osteoblast-like cells CRP10/30 with either ibandronate or clodronate for short periods has been shown to reduce bone resorption when these cells were subsequently cultured with osteoclasts *in vitro* (Sahni M, 1993). Conditioned media from osteoblast-like cells treated with bisphosphonates also inhibited bone resorption by osteoclasts suggesting that this was a soluble factor releases by osteoblasts. Vitte *et al* suggested that this factor acted on osteoclast precursors to prevent maturation (Vitte C,

1996). Confirmatory studies with UMR106 osteoblast-like cells (Yu X, 1996) and mouse calvarial osteoclasts (Nikishawa M, 1996) have suggested that bisphosphonate treatment stimulates the production of a soluble osteoclast-inhibitory factor from these cells also.

1.3.4 Mechanisms of action

The mechanisms by which bisphosphonates elicit their anti-resorptive activity are only now becoming clear. There appear to be differences in mechanisms of action on osteoclast activity depending on the structure of the bisphosphonate. These fall into two distinct groups; those that are similar in structure to pyrophosphate, such as clodronate and etidronate, and those that contain nitrogen in their R2 side chain.

- ***Metabolism of bisphosphonates***

Studies looking at the effects of bisphosphonates on Dictyostelium discoideum slime mould demonstrated that growth was inhibited with a similar order of potency to that of the anti-resorptive potency of the bisphosphonate (Rogers MJ, 1994b). Bisphosphonates that were very similar in their structure to pyrophosphate, such as clodronate, were metabolised intracellularly into non-hydrolysable analogues of ATP containing methylene (Rogers MJ, 1994a; Rogers MJ, 1992) compared to those which contained nitrogen in the R2 side chain that were not metabolised. Aminoacyl tRNA synthetase enzymes were involved in incorporating the bisphosphonates into ATP analogues and it was postulated that inhibition of these enzymes by the bisphosphonate metabolites might be responsible for the cellular effects (Rogers MJ, 1994a). Similar observations have been made using human aminoacyl tRNA synthetase enzymes (Rogers MJ, 1996a). Frith

et al compared the actions of clodronate and alendronate on macrophages *in vitro* and found that clodronate, but not alendronate, is metabolised to non-hydrolysable toxic analogues of ATP (Frith JC *et al.*, 1997). Follow-up studies have confirmed that clodronate is also metabolised in the same way in osteoclasts *in vitro* and results in osteoclast apoptosis (Frith JC, 1998).

- ***Inhibition of the mevalonate pathway***

The mevalonate pathway is a biosynthetic pathway which is important for the biosynthesis of cholesterol and isoprenoid groups (Figure 1.7). Recently, evidence has suggested that bisphosphonates can inhibit the mevalonate pathway (Amin D, 1992; Amin D, 1996) causing loss of prenylated proteins such as Ras. Luckman et al (Luckman SP *et al.*, 1998) suggested that this mechanism may be responsible for apoptosis of macrophages after bisphosphonate treatment. This evidence raised the possibility that this pathway may also be targeted by bisphosphonates in osteoclasts.

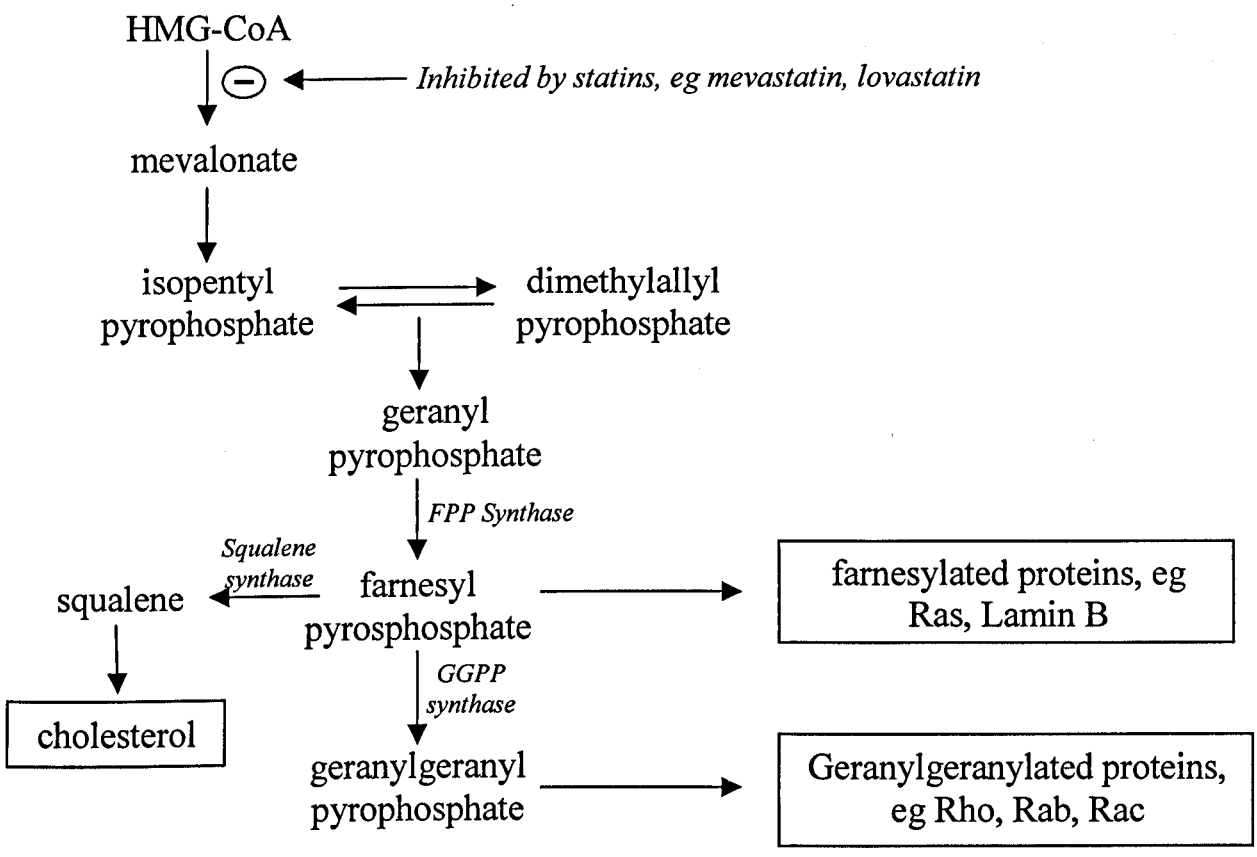


Figure 1.7 Outline of the mevalonate pathway involved in the synthesis of cholesterol, farnesyl diphosphate (FDP) and geranylgeranyl diphosphate (GGDP). FDP and GGDP act as substrates for the post-translation modification, or prenylation, of GTP-binding proteins such as Ras, Rho and Rac that are important in regulating cellular processes. Adapted from Russell and Rogers, 1999.

The initial step in the pathway is the production of mevalonate, which is regulated by the rate limiting enzyme HMG-CoA reductase (Zhang FL, 1996). Mevalonate is converted via intermediate steps to isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate, which are subsequently converted through geranyl pyrophosphate to farnesyl pyrophosphate (FPP). FPP can be incorporated into a number of end products, including cholesterol (via squalene), ubiquinone and dolichol. FPP can also be metabolised to geranylgeranyl pyrophosphate (GGPP) by geranylgeranyl synthase. FPP and GGPP are

isoprenyl- pyrophosphate intermediates which are used for protein farnesylation and geranylgeranylation respectively.

During protein isoprenylation, the covalent transfer of either 15-carbon farnesyl or 20-carbon geranylgeranyl groups (from FPP and GGPP respectively) to conserved cysteine residues found at or near the carboxy terminus. This is a post-translational form of modification of the protein which is facilitated by farnesyl transferase (FTase) and geranylgeranyl transferase (GGTase) respectively.

The precise role of protein prenylation is still being investigated. However it is clear that this process may be important for the membrane localisation of certain proteins as the lipid prenyl group acts to anchor the protein in the cell membrane (Zhang FL, 1996). It has also been found to be important for protein-protein interactions (Marshall, 1993). Proteins which undergo prenylation include Ras, Rho and Rac (Gibbs JF, 1997; Zhang FL, 1996). These are small GTP binding proteins which regulate a variety of processes including cell proliferation, apoptosis, alterations in cytoskeletal organisation and membrane ruffling (Zhang FL, 1996). Interestingly, the inhibition of the activity of Rho results in disrupted actin ring formation and inhibited bone resorption by osteoclasts in vitro. Hence the characteristic features of bisphosphonate-treated osteoclasts described earlier may occur as a result of inhibition of protein prenylation.

The first evidence that bisphosphonates may inhibit the mevalonate pathway was provided by Amin et al in 1992, who found that bisphosphonates inhibited both squalene

synthase and cholesterol biosynthesis (Amin D, 1992). The potent bisphosphonates incadronate and ibandronate were also found to inhibit cholesterol biosynthesis in J774 macrophages (Amin D, 1992). However, alendronate and pamidronate were less potent inhibitors of squalene synthase, but could still inhibit sterol biosynthesis in a cell-free system (Amin D, 1992) and therefore must inhibit enzymes of the mevalonate pathway other than squalene synthase. No inhibition of squalene synthase or cholesterol biosynthesis was found following treatment with etidronate or clodronate. These observations raised the possibility that the more potent nitrogen-containing bisphosphonates can inhibit not only squalene synthase but other enzymes of the mevalonate pathway also. As well as this, these data suggested that clodronate and etidronate had a different mechanism of action. Recent studies have confirmed that nitrogen-containing bisphosphonates inhibit enzymes of the mevalonate pathway with potencies corresponding to their anti-resorptive potencies, both *in vitro* and *in vivo* (Van Beek E, 1999b). In addition, van Beek et al have also demonstrated that the molecular target for nitrogen-containing bisphosphonates is the enzyme farnesyl pyrophosphate synthase (Van Beek E, 1999a).

- ***Effects on protein tyrosine phosphatases***

Protein tyrosine phosphatases form part of the signalling cascades that are essential for regulation of osteoclast differentiation and signalling. Inhibition of these pathways may therefore interfere with osteoclast function and hamper osteoclastic bone resorption (Endo N, 1996). The enzymatic activity of osteoclast protein tyrosine phosphatases (PTPε) has been shown to be inhibited by alendronate. Both alendronate and known PTP

inhibitors inhibited osteoclast differentiation and bone resorption in vitro, suggesting that the inhibition of PTPε is one of the mechanisms whereby alendronate may inhibit bone resorption (Schmidt A, 1996; Skory K, 1997) although this may not be the predominant mechanism.

- ***Inhibition of Osteoclast H^+ATPase***

The osteoclast proton ATPase pump, located in the ruffled border, is important in the acidification of the resorption cavity during bone resorption (Baron, 1989). There is evidence to suggest that bisphosphonates may have an inhibitory affect on the activity of this pump. David *et al* showed that tiludronate directly inhibited the activity of the proton H^+ATPase in vesicles derived from osteoclast plasma membranes (David P, 1996). Earlier studies by Carano *et al* suggested that the bisphosphonates etidronate, clodronate and pamidronate inhibited intracellular acidification by this pump by indirectly acting as metabolic inhibitors (Carano A, 1990). Zimolo *et al* similarly showed that etidronate and alendronate inhibit intracellular acidification, although this could be attributed to the prevention of insertion of H^+ATPase into the osteoclast membrane by the bisphosphonates (Zimolo Z, 1995).

1.3.5 Clinical uses

- ***Hypercalcaemia of malignancy***

Hypercalcaemia is a common complication of malignancy and often occurs in the late stages of disease. Approximately 30-40% if patients with breast cancer and 20% of patients with lung cancer experience hypercalcaemia. The median survival of patients

with solid tumours is 1 or 2 months and 70-80% have died within 1 year if left untreated (Kanis J, 1994). Several factors may contribute to tumour-induced hypercalcaemia, including the release of humoral factors from tumours which stimulate excessive bone resorption, local tumour-induced bone resorption and renal factors such as increased tubular reabsorption of calcium and impaired function of the glomeruli. Bisphosphonates, along with intravenous fluid replacement, have become the treatment of choice for hypercalcaemia of malignancy. Other treatments include calcitonin, phosphate and mithramycin (Ralston, 1992).

Bisphosphonates have proved to be effective in the treatment of hypercalcaemia of malignancy and the efficacy appears to be related to the relative anti-resorptive potencies. The least potent bisphosphonate in anti-resorptive terms, etidronate, is useful in inducing normocalcaemia in hypercalcaemic patients but the time to maximal response is 7 days and the effects are short-lived (Ralston S, 1989). Purohit *et al* showed that intravenous treatment with clodronate 1500mg resulted in more rapid achievement of normocalcaemia (within 3 days) with a longer duration of action (14 days) than had previously been seen with etidronate (Purohit O, 1995). A comparison between clodronate and pamidronate in this study, however, demonstrated the superiority of pamidronate over clodronate in the time to, and the duration of, normocalcaemia. All three of the agents discussed are licensed for the treatment of hypercalcaemia of malignancy. Other, bisphosphonates have also been investigated for this indication. Clinical studies with ibandronate (Ralston S, 1997) showed similar efficacy in hypercalcaemia of malignancy to that of pamidronate in earlier trials. Interestingly, two

large randomised trials comparing the potent third generation bisphosphonate zoledronic acid with pamidronate in patients with tumour-induced hypercalcaemia have shown that a single dose of 4mg or 8mg of zoledronic acid was significantly more effective in normalising serum calcium than 90mg of pamidronate (Major P, 2001). The newer, more potent agents may well become more clinically important as their potency allows lower doses to be given and also for their administration as short intravenous infusions over 15 minutes rather than 90 minutes or longer.

- ***Multiple Myeloma***

There has been much clinical and laboratory research interest in the role of bisphosphonates in multiple myeloma. It is a plasma cell malignancy which is characterised clinically by the development of osteolytic bone lesions, renal insufficiency, anaemia and a compromised immune system. Increased osteoclast formation and activity is a key feature. The osteolytic bone disease remains a major cause of morbidity in these patients and bisphosphonates have played a large part in the management multiple myeloma. Of the oral bisphosphonates studied in this context, etidronate was found to be the least beneficial (Belch AR, 1991). In contrast, Lahtinen *et al* reported that patients with newly diagnosed, untreated myeloma bone disease receiving oral clodronate 2.4g/day for 24 months showed a reduction in the rates of radiological osteolytic progression and vertebral fracture compared with placebo (Lahtinen R, 1992). A more recent study conducted by the MRC compared 1.6g/day of clodronate alongside standard chemotherapy to placebo (McCloskey *et al.*, 1998). This trial found a significant reduction in proportion of patients suffering new vertebral and non-vertebral fractures, as

well as a reduction in hypercalcaemia, bone pain and height loss. Interestingly, when the data was analysed retrospectively, a significant increase in survival was found in those patients who entered the trial without vertebral fractures and received clodronate. The question of the effects of bisphosphonates on survival in patients with multiple myeloma was addressed prospectively by Berenson *et al* (Berenson J *et al.*, 1996; Berenson JR *et al.*, 1998). This study randomised patients with Durie-Salmon stage III myeloma and at least one lytic lesion to receive either first-line chemotherapy or subsequent chemotherapy along with pamidronate 90mg or placebo. The proportions of patients experiencing SREs were significantly less in the bisphosphonate group than the placebo group. There was a significant improvement in pain and quality of life scores and the time to first skeletal event was significantly increased in the pamidronate group. Interestingly, median survival duration in the pamidronate group was 28 months vs 23 months in the placebo group (not statistically significant). Both oral clodronate and intravenous pamidronate are now used as part of the standard care of patients with multiple myeloma and bone disease.

- ***Bone metastases secondary to breast cancer***

In recent years bisphosphonates have become important in the management of cancer-induced bone disease and are now well established in the management of patients with bone metastases secondary to breast cancer (Paterson AHG, 1993; Hortobagyi GN, 1996; Berenson J, 1996; McCloskey, 1998; Diel IJ, 1998; Theriault RL, 1999, Powles T, 2002).

Clinical studies have confirmed that bisphosphonate treatment can result in re-calcification of lytic bone lesions secondary to breast cancer in a significant number of patients (Body JJ, 1995; Coleman RE, 1988b; Morton AR, 1988; Thiebaud D, 1991) and both oral and intravenous bisphosphonates have been investigated for their effects in the metastastic setting.

Oral bisphosphonates have been in use for some time and the majority of data on their clinical benefits in breast cancer come from trials using either pamidronate or clodronate, although newer compounds are also emerging. In a large, unblinded study, Van Holten *et al* suggested that the daily use of oral pamidronate 600mg resulted in an approximately 50% reduction in total skeletal complications initially (Van Holten-Verzantvoort A, 1987) and 38% reduction on update of the data (van Holten-Verzantvoort AT *et al.*, 1993). There was also a significant reduction in the requirements for a change in systemic therapy or radiotherapy although no significant changes were seen in time to further skeletal events, survival or radiological appearance of the lesions. 300mg pamidronate dose was also investigated in this study but was not found to be of benefit, in contrast to 600mg pamidronate. The drawback, however, was that 600mg of pamidronate was poorly tolerated due to increased gastrointestinal side effects therefore this dose could not be maintained. More, recently, Coleman *et al* observed that both 150mg and 300mg of oral pamidronate resulted in symptomatic improvement in 32% and 25% respectively of patients treated for metastatic breast cancer (Coleman RE, 1998). This was associated with a reduction in bone resorption and radiological stabilization or sclerosis of metastastic lesions.

A large, randomized, placebo controlled, double-blind trial has also been carried out with clodronate (Paterson AHG *et al.*, 1993). Patients randomized to receive 1600mg clodronate daily experienced a significant reduction in skeletal complications, including incidence of hypercalcaemia, rate of vertebral fracture and vertebral deformity. There was a trend towards a reduction in radiotherapy requirements for patients in the clodronate arm and the rate of skeletal events was significantly lower in the clodronate- treated group (219 skeletal events per 100 patient years) compared with placebo (305 skeletal events per 100 patient years) ($p<0.001$). Interestingly, there was no significant difference in survival between the two groups in this study. The issues of poor absorption and bioavailability of orally administered bisphosphonates, as well as patient compliance and gastrointestinal side effects, remain problematic. Recently, ibandronate, a potent nitrogen-containing bisphosphonate, has been shown to be well tolerated in oral formulation at doses that inhibit bone resorption (Coleman RE, 1999) and may prove to be a useful addition to the range of oral bisphosphonates available.

Several studies have been conducted with intravenous bisphosphonates in the context of breast cancer metastatic to bone. Two studies addressed the role of intravenous pamidronate along with chemotherapy and compared this to chemotherapy alone (Conte PF, 1996; Hultborn R, 1996). These studies used doses of 45mg 3 weekly and 60mg 3-4 weekly, respectively. Both found a significant increase in the time to progression of bone metastases in the bisphosphonate arm. Two further randomized placebo-controlled studies were carried out in which pamidronate 90mg iv alongside systemic chemotherapy

or endocrine therapy was compared to systemic therapy plus placebo (Hortobagyi GN *et al.*, 1996; Theriault RL *et al.*, 1999). Hortobagyi *et al* found a delay of 13.1 months in first skeletal related event (SRE) in the pamidronate group compared to 7.0 months in the placebo group (Hortobagyi GN *et al.*, 1996). They also found a reduction in the proportion of patients experiencing SREs in the bisphosphonate group (46%) compared with placebo (65%). There was no difference in overall survival. Theriault *et al* reported a similar reduction in the proportion of patients suffering SREs in the pamidronate-treated group (56%) compared with placebo (67%). The timing of first SRE in patients receiving pamidronate was delayed (10.4 months) compared with the placebo group (6.9 months) (Theriault RL *et al.*, 1999). Patients receiving pamidronate experienced additional benefits in quality of life and improved analgesic control compared with the placebo groups. A recent study comparing the potent third generation bisphosphonate zoledronic acid (4mg or 8mg iv) with 90mg iv pamidronate in patients with lytic bone lesions due to breast cancer or multiple myeloma reported that zoledronic acid was equivalent to pamidronate in terms of skeletal related events and tolerability. Intravenous bisphosphonates now have an established role in the management of skeletal complications of breast cancer and multiple myeloma.

- ***Adjuvant use of bisphosphonates in breast cancer***

Furthermore, in the adjuvant situation, studies with oral clodronate suggest that the use of bisphosphonates in combination with chemotherapy or endocrine therapy may delay first recurrence in bone (Diel IJ, 1998). In addition, Diel *et al* have shown that treatment with oral clodronate may be associated with reduced rates of visceral metastases and improved

survival compared with those receiving standard anti-neoplastic therapy alone (Diel IJ, 1998). Powles *et al* (Powles T, 2002) conducted a larger study of 1069 women with early breast cancer who were randomized to receive either clodronate 1600mg daily or placebo for 2 years. The incidence of bone metastases during the treatment period was significantly lower in the patients on clodronate treatment compared to those on placebo, although on longer follow-up this difference became non-significant statistically. Interestingly, there was a significant reduction in mortality in the clodronate group over the follow-up period. However, the role of bisphosphonates in the adjuvant setting remains to be clarified as Saarto *et al* reported an increase in the frequency of bone and visceral metastases with poorer survival in clodronate treated patients when compared with control (Saarto T, 2001). Large scale studies investigating the adjuvant use of both clodronate and the potent third generation bisphosphonate, zoledronic acid, are currently underway.

1.4 ANTI-TUMOUR EFFECTS OF BISPHOSPHONATES

Several studies have been done to investigate the anti-tumour effects of bisphosphonates in breast cancer. The clinical evidence for possible survival benefits in breast cancer and multiple myeloma has stimulated investigation into the direct effects of bisphosphonates on tumour cells, in addition to osteoclast effects.

- ***In vitro***

Shipman *et al* have demonstrated that the nitrogen-containing bisphosphonates, Incadronate and pamidronate, could induce apoptosis of human multiple myeloma cells

in vitro, (Shipman CM, 1997) an effect that was mediated by inhibition of the mevalonate pathway (Shipman CM, 1998). This has recently been confirmed by Aparicio *et al* (Aparicio A *et al.*, 1998). Pamidronate has very recently been shown to induce apoptosis in human melanoma cells (Riebeling C, 2002) and inhibit growth in human osteosarcoma cells (Sonnemann J, 2001). Studies have also suggested that bisphosphonates can inhibit the adhesion and invasion of breast cancer cells to bone matrices (Van der Pluijm G, 1996; Magnetto S, 1999; Boissier, 1997) and can enhance the ability of anti-neoplastic agents to inhibit breast cancer cell invasion (Magnetto S, 1999).

- ***In vivo***

Although bisphosphonates have effects on osteoclasts, macrophages and tumour cells *in vitro*, the direct anti-tumour activity of these compounds *in vivo* remains uncertain. Interestingly, there have been several *in vivo* studies that suggest that anti-tumour effects may occur with bisphosphonate treatment. Wingen *et al* observed a reduction in tumour growth in bone in a rat model of mammary carcinoma after treatment with pamidronate (Wingen F, 1988). Furthermore, when pamidronate was combined with melphalan, a chemotherapeutic agent, there was a greater reduction in tumour burden than with either agent alone. Studies with risedronate in a murine model of osteolytic bone disease show a selective reduction in the burden of MDA-MB-231 breast carcinoma cells in bone after bisphosphonate treatment (Sasaki A, 1995). Interestingly, the animals treated with risedronate had a significant improvement in survival compared with control animals. However, these effects were attributed to indirect effects on tumour cells due to inhibition of bone resorption as no inhibition of tumour cell proliferation was observed with

risedronate treatment *in vitro*. Subsequent studies with a potent third generation bisphosphonate YH529 in the same *in vivo* model showed that treatment resulted in fewer new bone and non-osseous metastases and inhibition of progression of established bone lesions (Sasaki A, 1998). Similar effects, inhibition of new bone metastases and slowed progression of established lesions, have been observed with ibandronate *in vivo* and the effects were enhanced when ibandronate was combined with tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) (Yoneda T, 1997).

1.5 AIMS OF THIS WORK

It has long been recognized that bisphosphonates inhibit osteoclast activity and osteoclast-mediated bone resorption. However, the demonstration that these compounds can induce apoptosis in cells other than osteoclasts, and that the treatment of breast cancer patients with bisphosphonates may improve survival, raises the intriguing possibility that bisphosphonates may have direct anti-tumour effects in breast cancer cells. The aims of the work presented in this thesis are described below:

- The first aim of this work was to determine whether the third generation bisphosphonate zoledronic acid had a direct anti-tumour effect on breast cancer cells *in vitro*.
- Since bisphosphonates are often used in conjunction with anti-neoplastic agents in the clinical setting, and may influence patient survival, the second aim was to investigate zoledronic acid had additive or synergistic effects with anti-neoplastic agents, such as paclitaxel, at clinically relevant concentrations.

- The third aim was to relate these data to the *in vivo* situation and investigate whether intravenous bisphosphonate treatment in patients with breast cancer and bone metastases resulted in anti-tumour effects *in vivo*.
- It is possible that by inhibiting bone resorption, bisphosphonates prevent the release of local factors from osteoclasts and bone which could affect the behavior of tumour cells. Therefore, our final aim was to determine the *in vivo* effects of intravenous bisphosphonate treatment, in the same group of patients, on cytokines and growth factors present in the local bone marrow environment.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

2.1.1 Zoledronic Acid

Zoledronic acid 1- Hydroxy-2-[(1H-imidazol-1-yl) ethylidene] bisphosphonic acid monohydrate was supplied as the hydrated disodium salt by Novartis Pharma AG (Basel, Switzerland) (Green JR, 1994). A stock solution was prepared in phosphate buffered saline (PBS) (pH7.4) and filter-sterilised using a 0.22-μM filter (Gelman Sciences, Northampton, UK).

2.1.2 Mevalonate Pathway Reagents

Farnesol (FOH) (mixed isomers) and geranylgeraniol (GGOH) were purchased from Sigma Chemical Co. (Poole, UK) and American Radiolabeled Chemicals Inc. (St Louis, MO), respectively. Stock solutions were prepared in 100% ethanol.

All other chemicals, unless otherwise stated, were obtained from Sigma Chemical Co.

2.2 CELL CULTURE

2.2.1 Phosphate-buffered saline and tissue culture media

Cells were cultured in single strength RPMI 1640 media purchased from Life Technologies (Paisley, UK). 10x concentrate of phosphate-buffered saline (PBS) from Life Technologies (Paisley, UK) was diluted with dd.H_2O to prepare single strength solutions under sterile conditions.

2.2.2 Foetal Calf Serum

Foetal calf serum (FCS) was purchased from Life Technologies (Paisley, UK) and was heat inactivated by placing in a water bath at 56^0C for at least 30 minutes. Every new batch of FCS was routinely tested to ensure that cell growth was adequately supported and that there were no alterations in cell behaviour.

2.2.3 Human breast carcinoma cell lines

The human breast carcinoma cell line MCF-7 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Professor R. Nicholson (University of Cardiff, Wales, UK) kindly provided the MDA-MB-231 human breast carcinoma cell line. The MCF-7 cell line was derived from the pleural effusion of a patient with metastatic breast carcinoma (Soule HD *et al.*, 1973) as was the MDA-MB-231 cell line (Cailleau R *et al.*, 1974).

Cells were cultured in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 10% FCS, 1mM glutamine, 1mM sodium pyruvate and 1x MEM nonessential amino acids (Life Technologies) (tissue culture media). They were maintained at 37^0C in a humidified atmosphere of 95% air and 5% CO_2. Cells were harvested using trypsin/ ETDA 0.5%/ 0.2% (Life Technologies) and passaged twice a week.

2.3 DETERMINATION OF CELL NUMBER

2.3.1 Haemocytometer.

The haemocytometer (Improved Neubauer) was used to determine cell number and density by counting the number of cells in a chamber of known depth. The coverslip was adhered to the haemocytometer, signified by the appearance of "Newtons Rings". Breast cancer cells were harvested and resuspended in a known volume of fresh tissue culture media. 7.5µl was taken from this cell suspension and introduced into the haemocytometer chamber. The number of cells in four 1mm areas was determined and the mean of this value was taken. This signified the cell number x 10^4/ml and was used to calculate initial cell densities for each experiment.

2.4 ANALYSIS OF NUCLEAR MORPHOLOGY

2.4.1 Introduction

Programmed cell death or apoptosis is a phenomenon that can be readily distinguished from other forms of cell death due the characteristic nuclear morphology displayed. In the initial stages condensation of the nuclear chromatin occurs to form distinct granular masses against the nuclear membrane (Kerr JFR, 1994; Wyllie R, 1980). During this stage, condensation of the cytoplasm can also be seen along with convolution of the cell and nuclear membrane. In solid tissues, separation of the apoptotic cells from the adjacent healthy cells now occurs. Once the nuclear and cell membranes have become convoluted, the nucleus breaks up into discrete fragments consisting either entirely of condensed chromatin or of chromatin forming crescent shapes around the nuclear periphery.

Membrane-bound apoptotic bodies are also formed by budding of the cell. Apoptosis primarily affects single cells whereas necrosis characteristically affects groups of cells, resulting in inflammation of the adjacent viable tissue. Necrosis is associated with irregular clumping of chromatin, swelling of the nucleus and organelles such as mitochondria and swelling of the cytoplasm, which results in rupture of both the plasma and organelle membranes (Wyllie R, 1980). Apoptotic cells are therefore clearly identifiable on the basis of changes in nuclear morphology, and can easily be distinguished from both normal and necrotic cells.

2.4.2 Method of analysis of nuclear morphology

Breast cancer cells were cultured in the presence or absence of zoledronic acid and also in the presence or absence of paclitaxel for up to 96 hours. After harvesting, the cells were fixed in 4% (v/v) formaldehyde in PBS for 10 minutes at room temperature and cytospun onto glass slides using a Shandon cytospin 3 at 500rpm for 5 minutes. These were then stained with 2μg/ml 4'-6-diamidino-2-phenyl-indole (DAPI) 4'-6-diamidino-2-phenyl-indole (DAPI) in PBS and mounted in Citifluor (Agar Scientific, Stanstead, UK). To prevent evaporation the coverslip edges were sealed with nail varnish and the cell were examined with either a 40x objective or a 100x objective (oil immersion) using a DMRB fluorescence microscope (Leica) with a Photometrics CCD camera and SmartCapturerR image analysis system (Vysis (UK) Ltd, Richmond, UK).

Apoptotic cells were identified on the basis of characteristic changes in nuclear morphology (Wyllie R, 1980). 5 random fields were counted per slide (four replicates per

treatment unless otherwise stated) and the percentage of apoptotic cells was calculated. Each experiment, unless otherwise stated, was performed on a minimum of two separate occasions.

2.5 DETECTION OF DNA FRAGMENTATION.

2.5.1 *In situ* nick translation assay.

Apoptotic cells were also identified by using an *in situ* fluorescence nick translation assay, which detects DNA strand breaks in apoptotic nuclei. Internucleosomal DNA fragmentation is thought to arise due to activation of endogenous endonuclease and is characteristic of apoptosis (Arends MJ *et al.*, 1990). The *in situ* nick translation assay detects endonuclease-mediated DNA strand breaks by the addition of dATP, dCTP, dGTP and biotinylated dUTP to free 3' hydroxyl ends of the DNA strands (Gorczyca W, 1993). This reaction is catalysed by DNA polymerase. The biotinylated dUTP is then detected by R-phycoerythrin (R-PE), directly conjugated to streptavidin, which binds to biotin. The level of R-PE incorporation in the cells is measured by flow cytometry. This technique has been shown to detect cells that also demonstrate the characteristic nuclear morphology of apoptosis (Rogers, 1996; Shipman CM, 1997).

Flow cytometry is a technique where the cells are passed as a single cell suspension through a sensing point allowing measurements to be made on each individual cell rather than the population as a whole. The cells are excited by a light source, usually a laser, and the cells can be labeled with single or multiple fluorescent probes that are excited by a given wavelength of light. When excited, different fluorescent probes will emit light

(fluoresce) at different wavelengths, enabling several parameters to be measured. The scattered and fluorescent light emitted by cells following excitation is collected by photodetectors and converted into electronic signals that enable graphical and statistical analysis. The *in situ* nick translation assay involves the incorporation of R-PE-labeled dUTP into DNA strand breaks in apoptotic cells. R-PE is excited at 495nm-564nm of light and emits light at approximately 576nm. The levels of R-PE fluorescence act as a measure of apoptosis, with apoptotic cells having higher levels of R-PE fluorescence than non-apoptotic cells (figure 2.1).

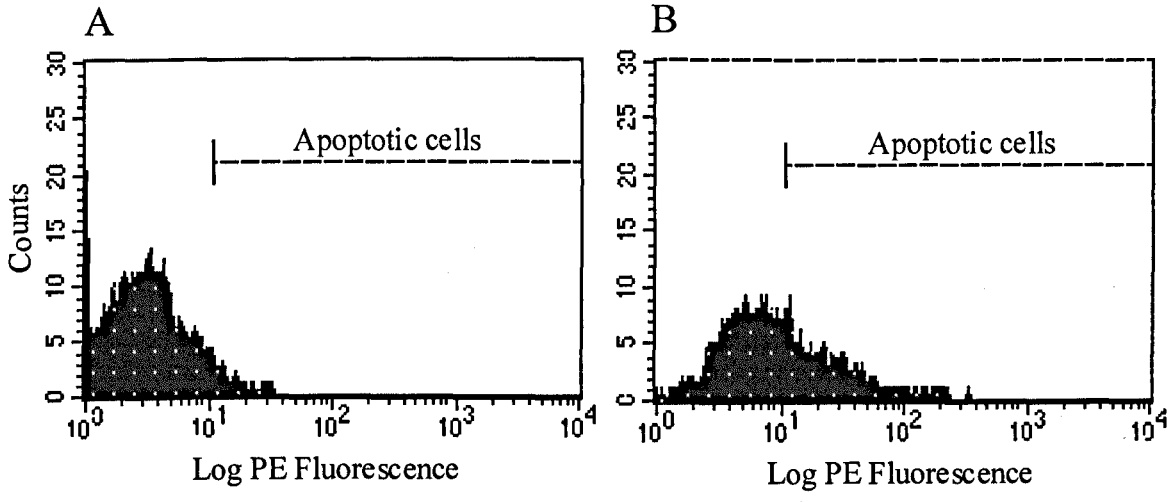


Figure 2.1 The *in situ* nick translation assay allows the detection of apoptotic breast cancer cells. MCF-7 cells were treated with vehicle control (A) and 100μM zoledronic acid (B). Healthy cells appear in the control as a peak below the first log decade (A). A small additional peak of apoptotic cells appears between the first and second log decades after treatment with zoledronic acid (B).

Cells were harvested following bisphosphonate treatment, washed once with sterile PBS, and then fixed in 4% formaldehyde in PBS for 15 minutes on ice. Cells were then washed again in PBS, resuspended in 0.5ml ice-cold ethanol and stored at -20^0C until analysis. To label DNA strand breaks in apoptotic cells, the cells were again washed in PBS and incubated at room temperature with a buffer containing 50mM tris, 2.5 mM magnesium chloride, 10μg/ml bovine serum albumen, 10 mM β-mercaptoethanol, 0.2nM of unlabeled dATP, dGTP and dCTP and 0.2nM biotin-16-dUTP in the presence of 1 unit DNA polymerase for 4 hours with intermittent gentle agitation (Gorczyca W, 1993) (Rogers MJ, 1996b). The cells were then washed in sterile PBS and incubated with a second buffer containing 4x saline-sodium citrate buffer (SSC), 0.1% Triton-X 100, 5% (w/v) fat free powdered milk and 1% (v/v) R-phycoerythrin (R-PE) - conjugated streptavidin (DAKO) for 30 minutes at room temperature in the dark. After washing with PBS, cells were resuspended in 300μl of PBS and analysed on a Becton Dickinson FACSort using the FL2 detector with a 585nm bandpass filter (42nm bandwidth). 3,000 events were counted for each sample run and the proportion of cells positively labelled with R-PE was determined. This proportion represented the proportion of cells with DNA fragmentation. All experiments were repeated on a minimum of two occasions.

2.6 PRIMARY CELLS

2.6.1 Isolation of primary cells from the bone marrow by erythrocyte lysis.

The erythrocyte lysis method makes use of a buffer containing 10mM potassium bicarbonate, 0.155M ammonium chloride and 0.1mM ethylenediamine tetraacetic acid (EDTA) in distilled water to lyse red cells whilst preserving the mononuclear cells present in a peripheral blood or bone marrow sample. 1.25ml blood or bone marrow in EDTA was centrifuged at 300g (1200rpm, 5min) and the plasma removed carefully with a Pasteur pipette (leaving buffy layer). The pellet of cells was resuspended in 4.5ml lysis buffer in a 15ml polypropylene tube (Corning, High Wycombe, UK) and incubated at room temperature for 10 minutes (spinning tubes using a blood rotator whilst incubating). The sample was then mixed with 20ml RPMI tissue culture media, overlaid onto 10ml 5% (w/v) albumin solution in RPMI, and centrifuged at 300g for 10minutes without braking. The pellet was washed twice in media and resuspended in 1ml sterile PBS to allow determination of cell number using the haemocytometer as previously described.

2.6.2 Fixation and permeabilisation

Following erythrocyte lysis as described above, the cells were fixed in 1% (v/v) formaldehyde for 15 minutes on ice and stored in 70% (v/v) ethanol at -20^{0}C until later flow cytometry analysis.

2.7 STATISICAL ANALYSIS

Results were expressed as the mean ± SEM. The relationship between the concentration of zoledronic acid, time of incubation with zoledronic acid, and effects on cell number and apoptosis were analysed using the non-parametric Kruskal-Wallis Analysis by Rank test. Experiments using the *in situ* nick translation assay were performed with a single sample per treatment. These data were therefore not analysed for statistical significance. All other data were analysed using non-parametric Mann Whitney *U* test unless otherwise specified. All experiments were repeated on at least two separate occasions.

CHAPTER 3

THE ANTI-TUMOUR EFFECTS OF ZOLEDRONIC ACID ON BREAST CANCER CELLS *IN VITRO*

3.1 SUMMARY

Bisphosphonates are well established in the management of breast-cancer induced bone disease. Recent studies have suggested that these compounds are effective in preventing the development of bone metastases. However, it is unclear whether this reflects an indirect effect via an inhibition of bone resorption or a direct anti-tumour effect. The aim of the work presented in this chapter was to investigate whether bisphosphonates have direct effects on breast cancer cells *in vitro* by investigating the effects of the potent bisphosphonate zoledronic acid on cell number and apoptosis in human breast carcinoma cell lines.

Zoledronic acid caused a dose- and time-dependent decrease in cell number and a concomitant increase in tumour cell apoptosis in MCF-7 and MDA-MB-231 human breast carcinoma cells. Short-term exposure to zoledronic acid was sufficient to cause a significant reduction in cell number and an increase in apoptosis in MCF-7 cells. These effects could be prevented by incubation with geranylgeraniol, suggesting that zoledronic acid-induced apoptosis is mediated by inhibiting enzymes of the mevalonate pathway.

The data presented here demonstrate that, in addition to inhibiting bone resorption, zoledronic acid can induce breast cancer cell apoptosis *in vitro*, indicating that further investigation into the direct effects of bisphosphonates on tumour cells *in vivo* is warranted.

3.2 INTRODUCTION

Bisphosphonates are now well established in the management of patients with multiple myeloma and bone metastases secondary to breast cancer and have been shown to reduce skeletal morbidity and improve quality of life (Berenson J *et al.*, 1996; Diel IJ *et al.*, 1998; Hortobagyi GN *et al.*, 1996) McCloskey, 1998, Paterson AHG *et al.*, 1993; Theriault RL *et al.*, 1999). In addition, there is evidence that treatment of primary breast cancer patients with oral clodronate alongside chemotherapy and hormonal therapy may delay first skeletal recurrence (Diel IJ *et al.*, 1998, Powles *et al*, 2002). Furthermore, Diel et al suggested that adjuvant clodronate treatment might also confer a survival benefit when compared with standard anti-neoplastic therapy alone. Interestingly, another similar study with oral clodronate did not reveal any benefit on skeletal or extra-skeletal disease, (Saarto T *et al.*, 2001) indicating that the role of bisphosphonates in the adjuvant setting remains to be clarified.

The mechanisms by which bisphosphonates elicit their anti-resorptive activity are only now becoming clear. Recent data suggest that bisphosphonates may have different mechanisms of action depending on their structure. Bisphosphonates that are similar to pyrophosphate in structure, such as clodronate and etidronate, are metabolised to cytotoxic analogues of ATP which, in turn, are responsible for inhibiting osteoclast activity (Frith JC *et al.*, 1997). However nitrogen-containing bisphosphonates induce apoptosis in osteoclasts and other cells, such as macrophages and myeloma cells, by interfering with the mevalonate pathway (Hughes DE *et al.*, 1995; Luckman SP *et al.*,

1998; Shipman CM *et al.*, 1998). Bisphosphonates have also been shown to have other, more subtle, effects on tumour cells in particular. In some studies, bisphosphonate treatment has resulted in the inhibition of breast cancer cell adhesion to substrates (Van der Pluijm G, 1996; Boissier, 1997; Magnetto S, 1999) and subsequent invasion (Boissier S, 2000). Thus, the demonstration that these compounds can induce apoptosis in cells other than osteoclasts, and that the treatment of patients with bisphosphonates may improve survival, raises the intriguing possibility that bisphosphonates may also have direct anti-tumour effects in breast cancer cells. The aim of the work presented in this chapter was to determine whether the third generation bisphosphonate zoledronic acid has direct effects on human breast carcinoma cells *in vitro*.

3.3 METHODS

3.3.1 Chemicals

Chemicals were obtained and prepared as described in Chapter 2, Material and Methods.

3.3.2 Measurement of cell number.

MCF-7 and MDA-MB-231 breast cancer cells were plated at a density of $5x10^4$ cells/ml in 24-well plates in replicates of four wells per treatment and allowed to adhere and proliferate for 72 hours. Cells were then incubated with fresh media containing increasing concentrations of zoledronic acid (0.1-100μM) for 72 hours. In some studies, MCF-7 cells were seeded as described above and incubated with 100μM zoledronic acid for 24, 48, 72 and 96 hours. MCF-7 cells were also incubated with 100μM EDTA for 72 hours to determine the effects of calcium chelation on cell number. In some experiments, MCF-7 cells were treated with 100μM zoledronic acid in the presence or absence of 100μM mevalonate, 50μM farnesol or 50μM geranylgeraniol, for 72 hours, in order to determine the effects of intermediates of the mevalonate pathway on zoledronic acid-induced apoptosis. To assess the effects of acute exposure to zoledronic acid, MCF-7 cells were prepared as described above and incubated with or without zoledronic acid for 2, 6, or 24 hours. The media was then replaced with fresh media without zoledronic acid and the cells incubated for a total of 72 hours. In each experiment, following incubation, cells were harvested, suspended in PBS and cell number determined using a haemocytometer (Improved Neubauer) (Materials and Methods).

3.3.3 Identification of apoptotic cells by examination of nuclear morphology.

MCF-7 and MDA-MB-231 breast cancer cells were cultured as described above for assessment of nuclear morphology with four replicates per treatment. Cells were incubated with zoledronic acid or vehicle as above. Both cells that were still adherent and those in the supernatant were then harvested, fixed with 4% formaldehyde (v/v) in PBS and cytospun onto glass slides. Following bisphosphonate treatment, cells were stained with DAPI as described in Materials and Methods.

3.3.4 Identification of apoptotic cells by the *in situ* nick translation assay.

MCF-7 and MDA-MB-231 breast cancer cells were seeded at 1x 10^5 cells/ml in 6-well plates, allowed to adhere and proliferate for 72 hours and then treated as described in the previous section. Cells were harvested, washed in sterile PBS, fixed in 4% formaldehyde for 15 minutes on ice and permeablised by incubation with 100% ethanol overnight. This was followed by analysis of DNA fragmentation by fluorescence *in situ* nick translation assay as described in the Materials and Methods, section 2.5.1.

3.3.5 Statistical analysis

Stastistical analysis was carried out as described in the Materials and Methods section.

3.4 RESULTS

3.4.1 Zoledronic acid causes a reduction in breast cancer cell number.

Treatment of MCF-7 breast cancer cells with increasing concentrations of zoledronic acid for 72 hours caused a dose-dependant decrease in cell number (Figure 3.1A) ($p<0.001$). 0.1-1μM zoledronic acid had little effect on the number of MCF-7 cells. In contrast, zoledronic acid at 10 and 100μM caused a significant reduction in the proportions of MCF-7 cells (49.54 $\pm$ 5.43% and 23.55 $\pm$ 5.43% of control, respectively) ($p<0.05$). Zoledronic acid had little effect on MDA-MB-231 cells at concentrations of 0.1-10μM, whereas the 100μM concentration resulted in a significant reduction in cell number (25.33 $\pm$ 6.0% of control) ($p<0.05$). Treatment of breast cancer cells with 100μM zoledronic acid for increasing periods of time resulted in a 63.5% reduction in MCF-7 cell number at 72 hours ($6.8\pm 5.3 \times 10^4$ vs$18.6\pm 3.2 \times 10^4$) and an 87.1% reduction at 96 hours ($3.81\pm 1.9 \times 10^4$ Vs $29.6 \pm 4.5 \times 10^4$) ($p<0.05$ in each case) (Figure 3.1B).

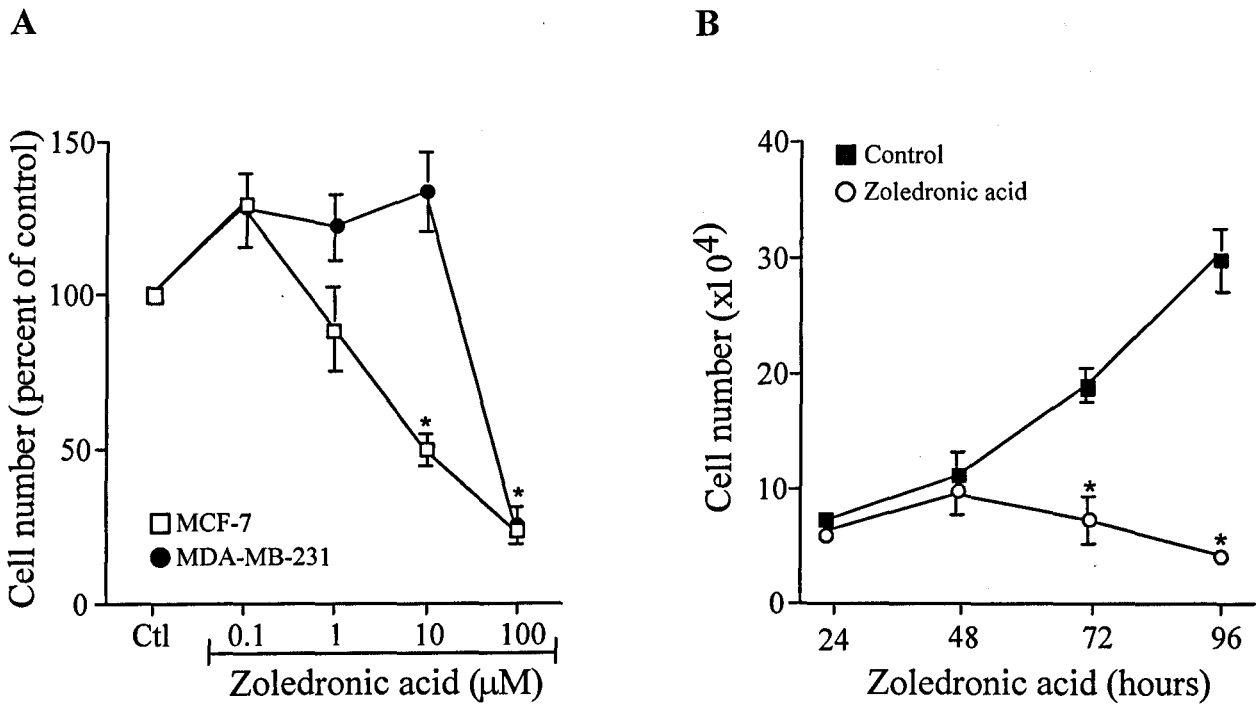


Figure 3.1. Effect of (A) increasing concentrations of zoledronic acid (0.1-100μM) on MCF-7 and MDA-MB-231 cell number after incubation for 72 hours and (B) increasing time of incubation of MCF-7 cells (24-96 hours) with 100μM zoledronic acid. Results are expressed as percent of control or absolute cell numbers. Data are the mean and SEM (n=4). * $p<0.05$ compared with control.

Incubation of MCF-7 cells with 100μM EDTA resulted in a small but significant reduction in cell number when compared with control ($16.08 \times 10^4 \pm 4.2$ vs. $24.52 \times 10^4 \pm 4.3$). However, treatment with zoledronic acid caused a greater reduction in cell number ($7.00 \times 10^4 \pm 1.5$ vs. $24.52 \times 10^4 \pm 4.3$) and this was significantly lower than that caused by EDTA ($p<0.05$ when zoledronic acid was compared to EDTA) (Table 3.1).

Table 3.1. Comparison of the effect of zoledronic acid and EDTA on MCF-7 cell number and apoptosis

Treatment[a]	Cell Number (x 10^4)[b]	Apoptosis (%)
Control	24.52 ± 4.3	1.09 ± 0.5
EDTA (100μM)	16.08 ± 4.2*	1.30 ± 0.5
Zoledronic acid (100μM)	7.00 ± 1.5*†	8.60 ± 1.2*

[a] Cells were incubated with control, zoledronic acid or EDTA for a total of 72 hours.
[b] Results are expressed as mean ± SEM (n=4).
* $p<0.05$ compared with control. † $p<0.05$ compared with EDTA.

3.4.2 Zoledronic acid induces apoptosis of breast cancer cells.

Treatment of MCF-7 and MDA-MB-231 cells with zoledronic acid caused an increase in the proportion of cells with nuclear morphology characteristic of apoptosis. These characteristics included nuclear fragmentation, chromatin condensation and the formation of dense, rounded apoptotic bodies (Figure 3.2). Treatment of cells with increasing concentrations of zoledronic acid (0.1-100μM) resulted in a dose-dependant increase in the proportion of cells undergoing apoptosis ($p<0.005$). Concentrations of 0.1 and 1μM zoledronic acid had little effect on MCF-7 cell apoptosis. In contrast, treatment with 10μM zoledronic acid resulted in a greater than four-fold increase in MCF-7 cell apoptosis (431.26 $\pm$ 32.21% of control) whereas the 100μM concentration induced a six-fold increase in the proportion of apoptotic cells (652.54 $\pm$ 71.49% of control) (Figure 3.3A). Treatment of MDA-MB-231 cells with concentrations of 0.1μM, 1μM and 10μM bisphosphonate had little effect on apoptosis. However, MDA-MB-231 cells showed a fifteen-fold increase in apoptosis (1515.24 $\pm$ 139.33% of control) after treatment with 100μM zoledronic acid ($p<0.05$). Treatment of MCF-7 cells with 100μM zoledronic acid for 24-96 hours caused a significant time-dependant increase in apoptosis ($p<0.005$) (figure 3.3B). In contrast, treatment of cells with 100μM EDTA had no effect on tumour cell apoptosis when compared to control (1.3 $\pm$ 0.5% vs. 1.09 $\pm$ 0.5% respectively) (Table3.1).

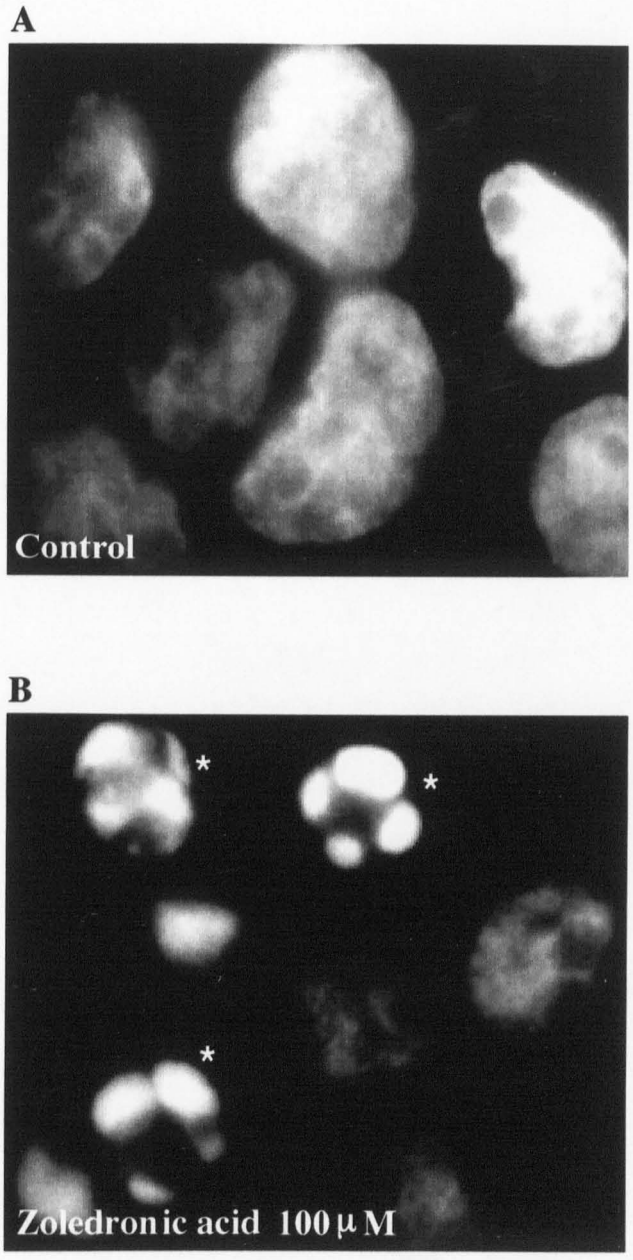


Figure 3.2. Effect of zoledronic acid on apoptosis of MCF-7 and MDA-MB-231 breast cancer cells. Fluorescence micrographs of MCF-7 cells incubated with (A) vehicle control and (B) 100μM zoledronic acid for 72 hours demonstrating characteristic changes in nuclear morphological. Cells were cytospun, stained with 4'-6-diamidino-2-phenyl-indole (DAPI) and examined with a x100 oil immersion objective. Apoptotic nuclei are identified with an asterisk.

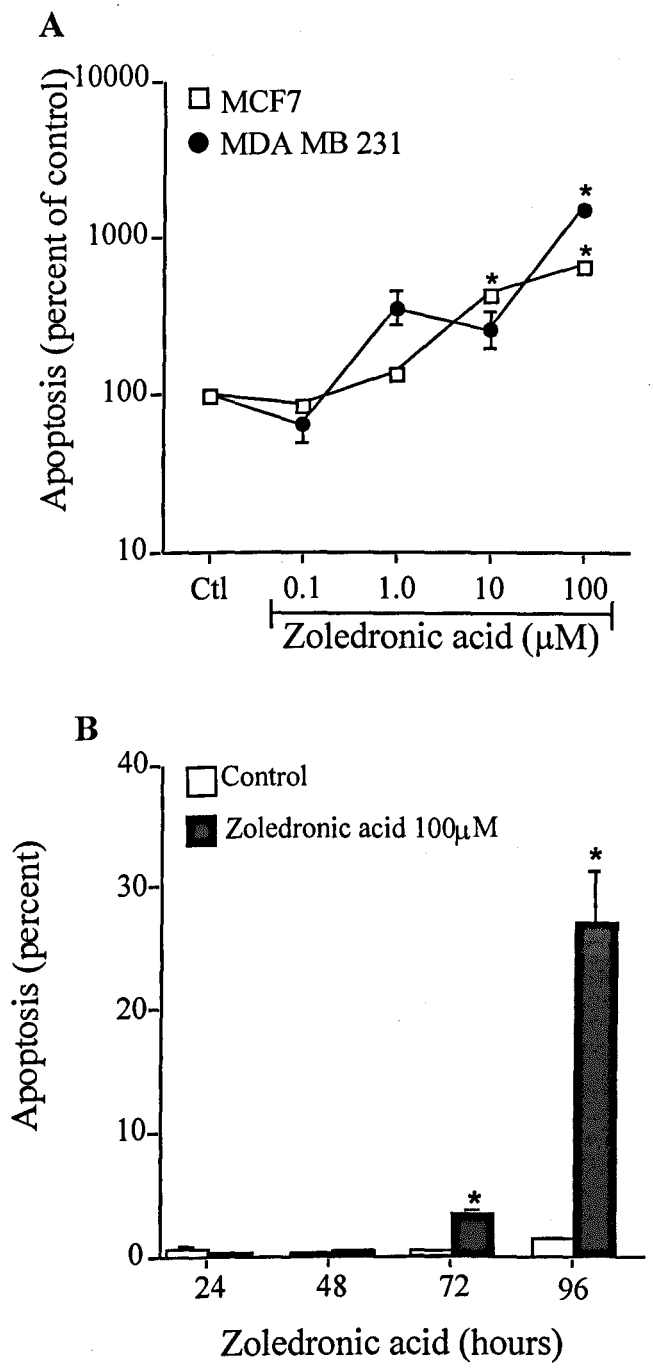


Figure 3.3. The effect of increasing concentrations of zoledronic acid (0.1-100μM) on MCF-7 and MDA-MB-231 cell apoptosis after 72 hours treatment with 100μM zoledronic acid (A). The effect of increasing time of incubation (24-96 hours) with 100μM zoledronic acid on MCF-7 apoptosis (B). Results are expressed as percent of the appropriate control or as absolute percentages. Data are the mean ± SE (n=4). * $p<0.05$ compared with control.

The induction of apoptosis in MCF-7 and MDA-MB-231 cells by zoledronic acid was confirmed using a fluorescence *in situ* nick translation assay. Treatment of MCF-7 cells with a range of zoledronic acid concentrations had little effect on apoptosis at 0.1 and 1.0μM, however, an increase in the proportion of apoptotic cells was observed with 10μM and 100μM zoledronic acid compared with control (28.7% and 70.7% vs. 22.57%, respectively). Treatment of MDA-MB-231 cells with 0.1-1μM zoledronic acid did not cause an increase in apoptosis although treatment with the 10 and 100μM zoledronic acid resulted in an increase in the proportions of apoptotic cells (126.6% and 126.6% of control) (Table 3.2). A time-dependant increase in MCF7 cell apoptosis was confirmed when cells were incubated with 100μM zoledronic acid for 24-96 hours (Table 3.3).

Table 3.2. The effects of increasing concentrations of zoledronic acid on MCF-7 and MDA-MB-231 cell apoptosis (*in situ* nick translation assay)

Zoledronic acid (μM)[a]	Apoptosis [b] (% of control)	
	MDA-MB-231	MCF-7
Control	100	100
0.1	98.98	96.89
1.0	82.53	115.06
10.0	126.63	127.02
100.0	126.63	314.26

[a] Cells were incubated with control and zoledronic acid for a total of 72 hours.
[b] Apoptosis was assessed by *in situ* nick translation assay and results expressed as percent of control.

Table 3.3. The effects of 100μM zoledronic acid on MCF-7 cell apoptosis after 24, 48, 72 and 96 hours of incubation (*in situ* nick translation assay)

Time (hours)	Zoledronic acid (% of control)[a]
24	120.9
48	126.8
72	215.8
96	226.9

[a] Apoptosis was assessed by the *in situ* nick translation assay and results were expressed as percent of control.

3.4.3 Induction of apoptosis of breast cancer cells by zoledronic acid occurs via inhibition of enzymes of the mevalonate pathway.

The effects of the addition of intermediates of the mevalonate pathway on zoledronic acid-induced MCF-7 cell number and apoptosis were determined. MCF-7 cell number was reduced compared with control on treatment with 100μM zoledronic acid (28.09 ± 2.1% of control) (figure 3.4). This was not affected greatly after addition of 100μM mevalonate (24.24% of control) or 50μM farnesol (19.21 ± 1.98% of control). However, the addition of 50μM geranylgerniol significantly protected against the effect of zoledronic acid on cell number (51.94 ± 5.57% of control) ($p<0.05$).

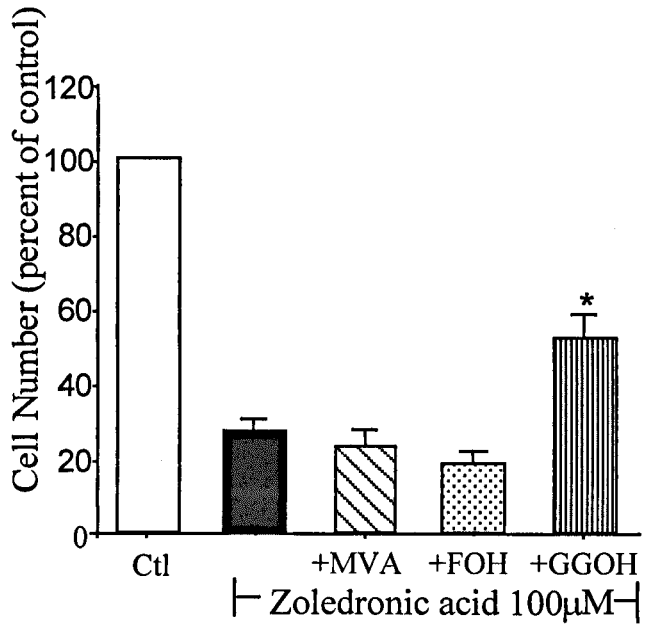


Figure 3.4. Effect of intermediates of the mevalonate pathway on zoledronic acid-induced reduction in breast cancer cell number. MCF7 cells were treated with 100μM zoledronic acid in the presence or absence of 100μM mevalonate (MVA), 50μM farnesol (FOH) and 50μM geranyl geraniol (GGOH) for 72 hours. Cells were then harvested, resuspended in PBS, and the cell number determined using a haemocytometer. Results are expressed as percent of control. Data are mean ± SEM (n=4).* $p<0.05$ when compared with zoledronic acid.

Analysis of nuclear morphology demonstrated that treatment of MCF-7 cells with 100μM zoledronic acid resulted in a significant increase in apoptosis (18.68 ± 2.5%) compared with control (0.26 ± 0.1%). The addition of 50μM farnesol resulted in a partial inhibition of apoptosis (14.31 ± 0.3%), whereas the addition of 50μM geranylgeraniol inhibited apoptosis to levels close to control (2.27 ± 0.2%) ($p<0.05$ compared to zoledronic acid 100μM) (figure 3.5A). These effects were confirmed using the *in situ* nick translation

assay (figure 3.5B). Treatment of MCF-7 cells with 100μM zoledronic acid resulted in a significant increase in apoptosis compared to control (60.57% and 16.6% apoptosis, respectively). The addition of 100μM mevalonate or 50μM farnesol to zoledronic acid had little effect on zoledronic acid-induced apoptosis (54.83% and 49.17% respectively). In contrast, the addition of 50μM geranylgeraniol inhibited zoledronic acid-induced apoptosis to levels below that of control (12.11%).

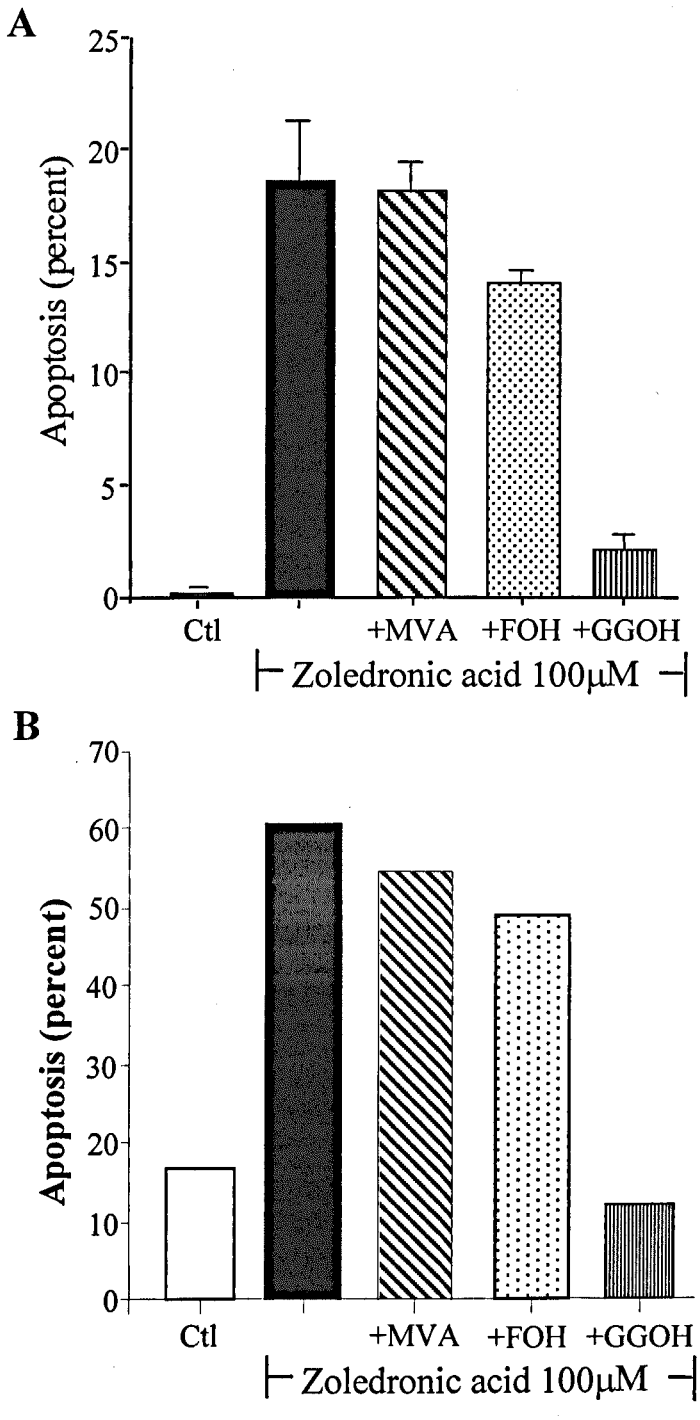


Figure 3.5. Effect of intermediates of the mevalonate pathway on zoledronic acid-induced breast cancer cell apoptosis. MCF7 cells were treated with 100μM zoledronic acid in the presence or absence of 100μM mevalonate (MVA), 50μM farnesol (FOH) and 50μM geranyl geraniol (GGOH) for 72 hours. Apoptosis was determined by analysis of nuclear morphology after staining cells with DAPI (A) and by the *in situ* nick translation assay (B). Results are expressed as absolute percentages. Data for nuclear morphology experiments are mean ± SEM (n=4).* $p<0.05$ when compared with zoledronic acid.

3.4.4 Acute exposure to zoledronic acid causes a reduction in breast cancer cell number and an increase in apoptosis.

Zoledronic acid inhibits breast cancer cell proliferation and induces apoptosis *in vitro* in a dose- and time-dependent fashion. However *in vivo*, breast cancer cells are probably exposed to micromolar concentrations of bisphosphonates for only a few hours. Therefore, in order to look at conditions that may more accurately reflect the situation *in vivo*, we examined the effects of acute exposure to 100μM zoledronic acid on MCF-7 cell number and apoptosis (figure 3.4). Two hours of exposure to zoledronic acid resulted in reduction in cell number to 77.1 $\pm$ 19% of control ($p<0.05$). This was associated with an increase in the proportions of apoptotic cells from 1.23 $\pm$ 0.5% in the control samples to 8.05 $\pm$ 0.6% in zoledronic acid-treated cells ($p<0.05$). Six and twenty-four hours of exposure resulted in a reduction in cell number of 44.75 $\pm$ 7.9% and 30.04 $\pm$ 9.8% of control, respectively ($p<0.05$). The proportions of apoptotic cells at the six and twenty-four hour time points were similar to that at two hours (8.24 $\pm$ 0.7% and 8.24 $\pm$ 1.1% respectively) ($p<0.05$) indicating that acute exposure to zoledronic acid results in significant induction of apoptosis. The induction of apoptosis by zoledronic acid at 2, 6, and 24 hours was confirmed using the *in situ* nick translation assay. Two hours of exposure to 100μM zoledronic acid showed an increase in apoptosis of 236.03% of control. This proportion increased to 289.15% after 6 hours of exposure and 643.62% after 24 hours (values expressed as percent of appropriate controls).

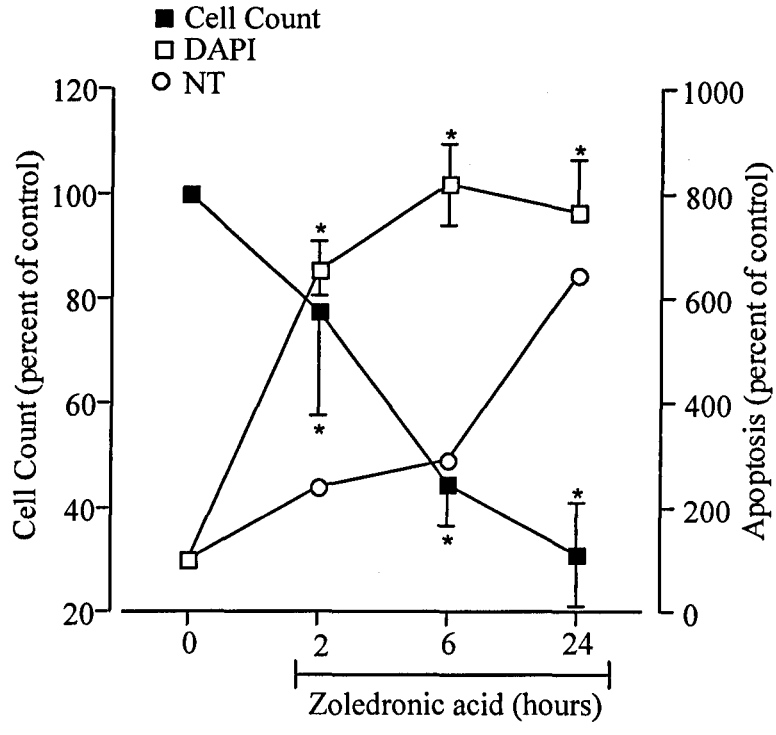


Figure 3.4. Effect of short-term exposure of MCF-7 cells to 100μM zoledronic acid. MCF-7 cells were incubated with zoledronic acid for 2, 6, 24, and 72 hours. The media was then replaced and the cells were maintained for a total of 72 hours. The effects on cell number (n=4) and apoptosis as assessed by *in situ* nick translation assay (NT) and changes in nuclear morphology (DAPI, n=4) are shown. Results are expressed as percent of control and are the mean ± SE. * $p < 0.05$ compared with control.

3.5 DISCUSSION

In the present study we have shown that zoledronic acid has a dose- and time-dependant effect on breast cancer cell number *in vitro*. These observations are consistent with previous studies that have demonstrated that bisphosphonates cause a significant reduction in osteoclast number (Hughes DE, 1995; Fisher JE, 1999). Importantly, studies in human myeloma cells have shown that nitrogen-containing bisphosphonates, including zoledronic acid, also inhibit cell proliferation (Shipman CM, 1997; Shipman CM, 1998; Aparicio A, 1998; Derenne S, 1999). The effects of zoledronic acid on MCF-7 and MDA-MB-231 cell number were associated with concomitant dose- and time-dependant changes in nuclear morphology that were characteristic of apoptosis (Wyllie, 1980). These effects on tumour cell apoptosis are consistent with previous reports that have demonstrated the induction of apoptosis, in macrophages, osteoclasts and myeloma cells, by bisphosphonates such as pamidronate, alendronate, incadronate, and zoledronic acid (Hughes DE, 1995; Rogers, 1996; Shipman CM, 1997; Shipman CM, 1998; Aparicio A, 1998; Fisher JE, 1999). Similarly, Senaratne *et al* have recently reported that a panel of bisphosphonates, including zoledronic acid, induces caspase activity and apoptosis in human breast cancer cells *in vitro* (Senaratne SG, 2000). The fluorescence *in situ* nick translation technique has been shown to detect cells that also demonstrate the characteristic nuclear morphology of apoptosis (Rogers, 1996; Shipman CM, 1997). Although there were differences between the levels of apoptosis detected by the two different techniques, the relative changes induced by bisphosphonate treatment were similar. The reasons for the discrepancy between methods are unclear but may reflect the conservative assessment of nuclear morphology as only cells displaying definitive

morphological characteristics were counted as apoptotic. Alternatively, the fluorescence *in situ* nick translation assay may be identifying cells in an earlier stage of apoptosis.

In the present study concentrations of 10-100μM zoledronic acid for 72 hours were required to induce apoptosis of breast cancer cells. However, bisphosphonates have a high affinity for mineralised bone and localise to bone rapidly, thus in a clinical setting it is likely that primary breast cancer cells or those in visceral metastases may be exposed to these compounds for shorter periods. Indeed, peak serum concentrations are likely to be in the range of 1-3μM and maintained for only a few hours (Berenson JR, 2000). In bone metastases, where osteoclasts are resorbing bone loaded with bisphosphonate, and thereby releasing it, prolonged exposure to high local concentrations of the compound would be predicted (Sato M, 1991). However, exposure times of 2, 6 and 12 hours may more accurately reflect those seen by cells other than osteoclasts *in vivo*. Incubation of MCF-7 cells with 100μM zoledronic acid for these periods resulted in a similar reduction in cell number and increase in apoptosis, suggesting that acute exposure is sufficient to have an anti-tumour effect in these cells. This is consistent with observations from a study in J774 macrophages which demonstrated a time-dependant increase in caspase-3-like enzyme activity, associated with apoptosis, when acutely exposed to nitrogen-containing bisphosphonates (Coxon FP, 1998).

Although zoledronic acid clearly has cytotoxic effects on breast cancer cells, these may be mediated by their ability to chelate calcium. However, studies with EDTA, which will also chelate calcium, demonstrated that this has only a small effect on the reduction in

cell number and remained significantly different from the effect observed following zoledronic acid treatment. Unlike the effect on cell number, EDTA treatment did not result in a significant increase in apoptosis compared to control suggesting that the apoptosis induced by zoledronic acid is not mediated by chelation of calcium. The reasons for the differences in these effects on cell number and apoptosis are unclear; however, calcium chelation with EDTA may have cytostatic effect on breast cancer cells rather than a cytotoxic effect. Interestingly, MCF-7 and MDA-MB-231 cells showed different levels of sensitivity to zoledronic acid, the reasons for which remain unclear. However, this may reflect the differing expression of *p53* as MCF-7 cells express wild type *p53* whilst MDA-MB-231 cells express a mutant *p53* sequence, which may confer increased resistance to apoptotic stimuli (O'Connor PM, 1997). Alternatively, MDA-MB-231 cells may produce a number of cytokines or local growth factors that could act as survival factors and inhibit zoledronic acid-induced apoptosis (Fromigue, 2000). There may also be differences, between the cell lines, in the way in which zoledronic acid is taken up.

We have also demonstrated that zoledronic acid-induced apoptosis of MCF-7 breast cancer cells can be inhibited by addition of intermediates of the mevalonate pathway, which is consistent with observations in osteoclasts, macrophages and myeloma cells (Fisher JE, 1999; Luckman SP, 1998; Shipman CM, 1998). This pathway has been shown to be important in the post-translational modification of small GTP-binding proteins including Ras, by the process of farneslyation, and Rho and Rac by geranylgeranylation. These proteins are involved in a range of important cellular processes including

proliferation (Ras), membrane trafficking and cytoskeletal organisation (Rho and Rac). In the present study, the addition of geranylgeraniol, rescued MCF-7 cells from zoledronic acid-induced apoptosis, whereas the addition of farnesol only partially inhibited tumour cell death. Recent observations by Fisher *et al* suggest that this may also be the case in osteoclasts where disruption of the cytoskeleton and loss of the osteoclast ruffled border, as well as apoptosis, are seen with bisphosphonate treatment (Fisher JE, 1999). However, this contrasts with previous studies in J774 macrophages and the JJN3 myeloma cells in which bisphosphonate-induced apoptosis could be prevented by the addition of both geranylgeraniol and farnesol. Our findings also contrast with the results of a recent study by Senaratne *et al* (Senaratne SG 2002) who demonstrated that the addition of farnesol to zoledronic acid-treated MDA-MB-231 cells protected the against loss of viability and loss of membrane localization of Ras, as well as protecting MCF-7 cells from the apoptotoc effects of zoledronic acid. The reasons for the differences between these studies are unclear; however, farnesylated proteins such as Ras may be less important in breast cancer cell and osteoclast function than in macrophages and myeloma cells. The effects of intermediates of the mevalonate pathway on zoledronic acid-induced reduction in cell number are less clear. The addition of geranylgeraniol to the system partially negates this effect but there is still a marked reduction compared with control. The reasons for this are unclear but it is possible that the addition of geranylgeraniol is insufficient to prevent zoledronic acid-induced inhibition of adhesion to the substratum and subsequent cell growth. In addition, the calcium chelating action of zoledronic acid, which would not be altered by intermediates of the mevalonate pathway, may affect cell number as suggested earlier.

In summary, the data presented in this chapter strongly suggest that zoledronic acid can have an important anti-tumour effect in breast cancer cells *in vitro* by promoting tumour cell apoptosis. It is therefore possible that the beneficial effects seen in patients receiving bisphosphonate therapy may be due to anti-tumour effects as well as inhibition of osteoclast-mediated bone resorption. These observations warrant further investigation *in vitro* and *in vivo* in patients receiving bisphosphonates.

CHAPTER 4

ZOLEDRONIC ACID AND PACLITAXEL HAVE SYNERGISTIC EFFECTS ON HUMAN BREAST CANCER CELLS.

4.1 SUMMARY

Bisphosphonates are commonly used alongside chemotherapy and endocrine agents in the clinical arena. Recent clinical data suggests that bisphosphonate treatment may influence non-skeletal as well as skeletal metastases and may also confer a survival advantage. This raises the possibility that bisphosphonates may act on breast cancer cells in synergy with anti-neoplastic agents. In the previous chapter we have demonstrated that the potent bisphosphonate zoledronic acid can have direct anti-tumour effects on breast cancer cells *in vitro*. The aim of the work presented in this chapter was to investigate the effects of combining zoledronic acid and paclitaxel, a commonly used chemotherapy agent on breast cancer cell number and apoptosis.

Treatment with zoledronic acid and clinically achievable concentrations of paclitaxel resulted in a four to five-fold increase in MCF-7 cell apoptosis. Isobologram analysis revealed synergistic effects on MCF-7 cell number and apoptosis when zoledronic acid and paclitaxel were combined. The synergistic effects on apoptosis were confirmed using the MDA-MB-231 cell line.

These data suggest that zoledronic acid can act in synergy with paclitaxel on breast cancer cells *in vitro* and this may provide one explanation for the beneficial effects of bisphosphonates experienced by breast cancer patients. These effects require further *in vivo* study.

4.2 INTRODUCTION

Bisphosphonates are effective in the management of bone metastases from breast cancer and multiple myeloma and are often used in conjunction with standard anti-neoplastic therapies in the clinic. Several large clinical studies investigating the effects of bisphosphonates on skeletal related events in both breast cancer and multiple myeloma have demonstrated benefits in quality of life and reduction of skeletal morbidity (Berenson J *et al.*, 1996; Diel IJ *et al.*, 1998; Hortobagyi GN *et al.*, 1996; McCloskey *et al.*, 1998; Paterson AHG *et al.*, 1993; Theriault RL *et al.*, 1999, Powles T *et al*, 2002). Importantly, recent data from randomised controlled trials have indicated that the use of bisphosphonates in patients with breast cancer, in addition to anti-cancer treaments, may confer a survival advantage as well as skeletal benefits (Diel IJ *et al.*, 1998). This raises the intriguing possibility that bisphosphonates may somehow act synergistically with cancer therapies to affect tumour burden.

The investigation of synergistic effects between two compounds requires detailed and rigorous analysis. The isobologram method makes use of combined drug dose-response data to demonstrate the presence of synergy regardless of the mechanism of action of the drugs under investigation (Gessner, 1988). The simple comparison of the sum of the individual drug effects with the observed combined effect does not give an accurate indication of the interaction between the two drugs. The reason for this is that the dose-response curves for most drugs are non-linear (Berenbaum, 1989). The isobologram method concisely evaluates drug interactions independent of the shape of the dose-response curves and hence alleviates this problem. This method has been utilised for both

in vitro (Leonessa F, 1994; Voigt W, 2000) and *in vivo* (Church MW, 1988) studies. The quantification of the interactions identified by the isobolgram method requires further rigorous analysis of the dose-response data (Greco WR, 1995) and this can be done using a mathematical model (Appendix 1). The degree of synergy or antagonism between two compounds can thus be determined and can be used to compare the effects of different drug combinations. The aim of the work presented in this chapter, therefore, was to determine whether the combination of zoledronic acid with the commonly used chemotherapeutic agent paclitaxel synergistically enhanced the anti-tumour effects observed in Chapter 3.

4.3 METHODS

4.3.1 Chemicals

Zoledronic acid was prepared as described previously in Materials and Methods (section 2.1.3). Paclitaxel was purchased from Sigma Chemical Co (Poole, UK) and a stock solution of 0.1mM (w/v) was made by dissolving paclitaxel in dimethyl sulphoxide (DMSO) and then filter-sterilised using a 0.22μm filter. All other chemicals were purchased from Sigma Chemical Co unless otherwise stated.

4.3.2 Identification of apoptotic cells

In preliminary studies to identify possible synergistic effects between zoledronic acid and paclitaxel MCF7 cells were seeded at a density of $5x10^4$ cells/ml in 24-well plates (Costar) in replicates of four wells per treatment and allowed to adhere and proliferate for 72 hours. Cells were incubated with fresh media in the presence or absence of 10μM zoledronic acid and/or 2nM paclitaxel acid for a total of 72 hours.

Some preliminary studies to assess the proportion of apoptotic cells were also carried out using the *in situ* nick translation assay. MCF-7 cells were plated at a density of $1x10^5$/ml in 6 well plates (Costar) with one replicate per treatment and allowed to proliferate for 72 hours. The cells were then treated with 10μM zoledronic acid and 2nM paclitaxel as above. The proportion of apoptotic cells was determined by flow cytometric analysis following the incorporation of fluorescently-labelled dUTP into DNA strand breaks, as described previously (Materials and Methods).

For more detailed characterisation of synergistic effects and to enable the construction of dose-response curves and isobolograms, MCF-7 and MDA-MB-231 cells were seeded as above (n=8). They were subsequently incubated with increasing concentrations of zoledronic acid (0.1-100μM) in the presence or absence of

increasing concentrations of paclitaxel (0.01-10nM) for 72 hours. Following treatment, the cells were harvested, fixed in 4% formaldehyde (v/v) in PBS and cytospun onto glass slides. Once this was done, cells were stained with the nuclear stain DAPI as described earlier (Materials and Methods, section 2.4.2).

4.3.3 Analysis of cell number

MCF-7 and MDA-MB-231 breast cancer cells were plated at a density of $5x10^4$ cells/ml in 24-well plates (Costar) (n=8) and allowed to adhere and proliferate for 72 hours. Cells were incubated as above with fresh media contained with increasing concentrations of zoledronic acid (0.1-100μM) in the presence or absence of increasing concentrations of paclitaxel (0.01-10nM) for 72 hours. After harvesting, cells were resuspended in PBS and cell number was determined using a haemocytometer. Dose response curves and isobolograms were constructed using this data.

4.3.4 Isobologram analysis

The isobologram method of analysis was used to assess whether synergistic effects were seen when paclitaxel and zoledronic acid were combined. This method allows the identification of interactions between two drugs, regardless of mechanism of action of the individual drug (Berenbaum, 1989; Greco WR, 1995). Dose response curves were plotted for the effects of paclitaxel and the effects of zoledronic acid on MCF7 cell number. From these, the combined drug IC_{75} (inhibitory concentration) values (75% of maximum effect, 25% remaining) were determined for each curve. The ratio of the combined drug IC_{75} to the IC_{75} value of each drug alone was calculated and plotted as an isobologram (D/D75). The analysis was repeated for the effect of

paclitaxel and zoledronic acid on MDA-231 cell number and again the IC_{75} values were used to plot the isobologram. The data obtained from experiments determining the effect of paclitaxel and zoledronic acid on apoptosis were also analysed in the same way. In this case, IC_{10} values for MCF-7 and MDA-MB-231 cells and the corresponding ratios were calculated from the dose response curves. The analysis was kindly carried out by Dr Amin Rostami, Dept of Pharmacology, University of Sheffield Medical School.

4.3.5 Further Isobologram analysis

As discussed above, the isobolgram analysis makes use of the ratio of the IC values of paclitaxel and zoledronic acid to generate a curve which indicates the presence of synergy, additivity or antagonism between the two drugs. The presence, or otherwise, of a synergistic effect determined using this method should not depend on which particular IC value was chosen as it is the ratio that is important. We tested this on a sample of our data looking at the effect of both drugs in combination on MDA-MB-231 cell apoptosis. We identified the points on the dose-response curves that corresponded to IC_5 and IC_{10} and plotted two separate isobolograms with each of these IC values respectively.

4.3.6 GraFit Analysis

The model shown in Appendix 1 (Equation 1) was used for analysis of synergy using the GraFit Version 3.00 software (Erithacus Software Ltd, Surrey, UK). This software allows 3-dimensional analysis so that the data for the zoledronic acid and paclitaxel effects can be analysed simultaneously. This general model for non-additivity assumed an interaction term α. If $\alpha=0$ then the effect of the combining the two drugs could be considered additive only. In contrast, negative values indicated antagonism and positive values demonstrated synergy. The sigmoidicity function γ indicates the shape of the curve. This analysis was also kindly carried out by Dr Amin Rostami.

4.4 RESULTS

4.4.1 Combined treatment with zoledronic acid and paclitaxel, at pharmacologically achievable concentrations, results in apparent synergistic effects on breast cancer cell apoptosis.

In preliminary studies, we investigated the effect of zoledronic acid in combination with paclitaxel on apoptosis of MCF-7 cells. Analysis of nuclear morphology demonstrated that combining zoledronic acid and paclitaxel caused a greater than two-fold increase in the proportion of apoptotic MCF-7 cells when compared with either drug alone ($p<0.02$) (data not shown). Treatment with 10μM zoledronic acid and 2nM paclitaxel resulted in a fivefold increase in apoptosis (774.8% of control) compared to zoledronic acid alone (155.71%) and a fourfold increase compared to paclitaxel alone (189.68%) (figure 4.1) on analysis with the in situ nick translation assay.

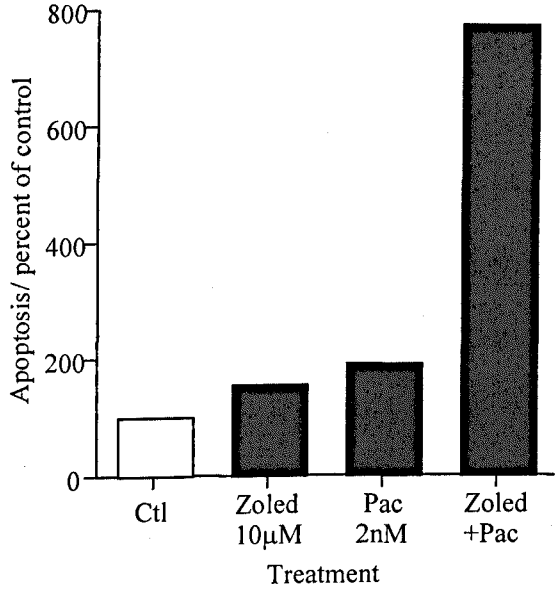


Figure 4.1 Effect of combined treatment of MCF-7 cells with 10μM zoledronic acid and 2nM paclitaxel. Cells were incubated with 10μM zoledronic acid in the presence or absence of 2nM paclitaxel for a total of 72 hours. Apoptosis was assessed using the *in situ* nick translation assay. Results are expressed as percent of control.

4.4.2 Isobologram analysis of combined effects of zoledronic acid and paclitaxel on breast cancer cell number demonstrated synergy.

Since the above data suggested that these compounds might have synergistic effects, a more detailed isobologram analysis was undertaken. The combination of paclitaxel and zoledronic acid was shown to result in synergistic effects on MCF-7 cell number (figure 4.2). Dose response curves were plotted for the effect of paclitaxel (4.2A) and zoledronic acid (4.2B) on cell number and the IC_{75} values calculated. The resulting data points were plotted on an isobologram (4.2C). The D/D75 ratios for paclitaxel alone (1) and zoledronic acid alone (1) are shown and connected by a diagonal line. Points lying on this line indicate a purely additive effect. Data points forming a curve lying above this line indicate antagonism between the two drugs whereas a curve lying below this line suggest a synergistic interaction. The combined D/D75 ratios derived from the dose-response data clearly formed a curve which lay below the additive effect line, indicating synergistic effects between paclitaxel and zoledronate on tumour cell number.

These experiments were repeated with the MDA-MB-231 cell line. As shown in figure 4.3A and 4.3B, and dose response curves were plotted for the effects of increasing concentrations of paclitaxel and increasing concentrations of zoledronic acid. The IC_{75} values for effect on cell number were calculated and were plotted as an isobologram, figure 4.3C. Isobologram analysis demonstrated that there was also a synergistic effect on MDA-MB-231 cell number when paclitaxel and zoledronic acid are combined.

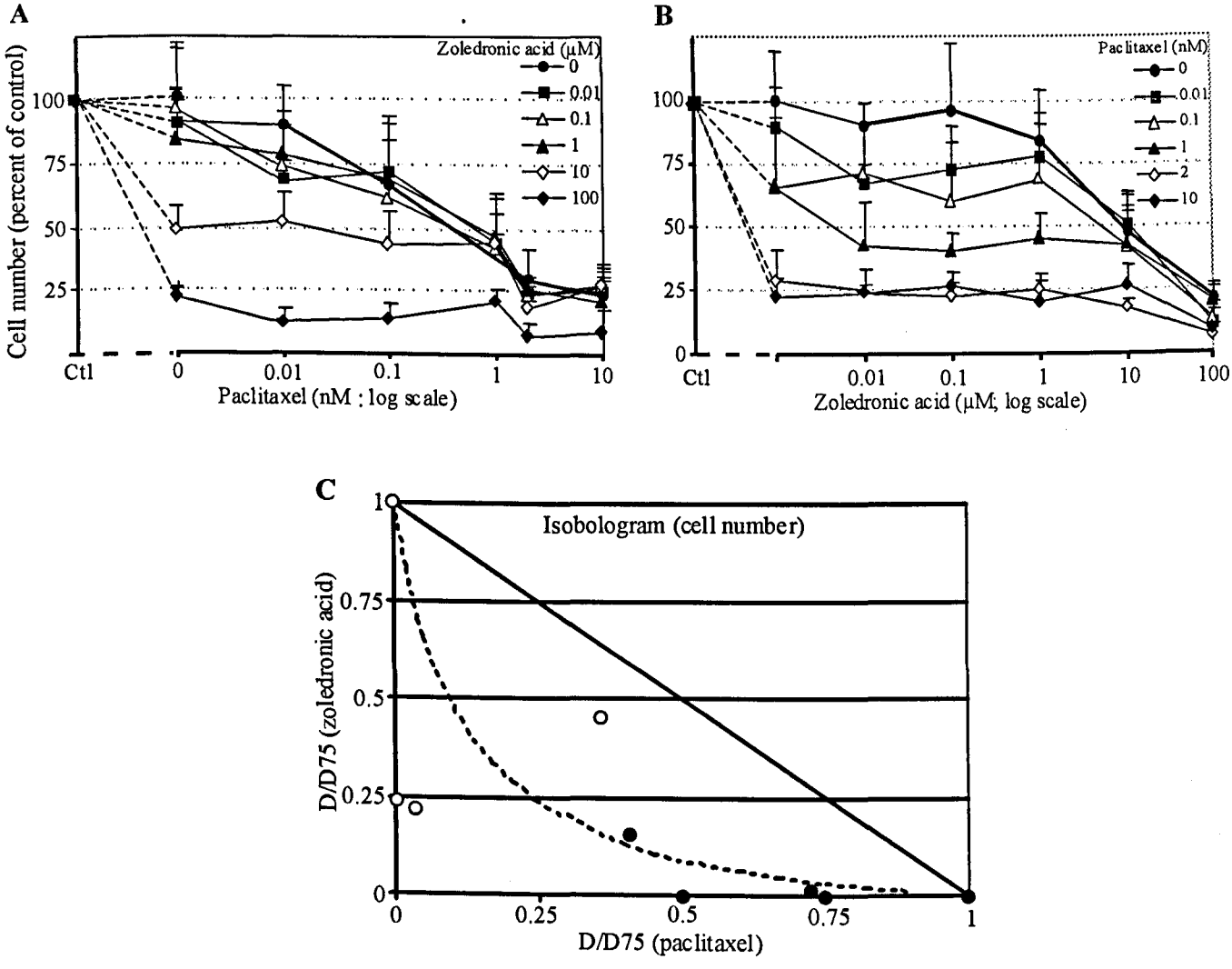


Figure 4.2 Dose response curves and isobologram plot for the interaction between paclitaxel and zoledronic acid on MCF7 cell number. Cells were incubated with increasing concentrations of zoledronic acid (0.01-100μM) in combination with increasing concentrations of paclitaxel (0.01-10nM) for a total of 72 hours. Cells were harvested to determine cell number (n=8) and dose response curves were plotted for effect of paclitaxel (A) and zoledronic acid (B) on cell number. Combined drug IC_{75} values were determined from these data and used to construct an isobologram (I.C) for the interaction between paclitaxel (●) and zoledronic acid (O). Dose response results expressed as percent of control and are means ± SD.

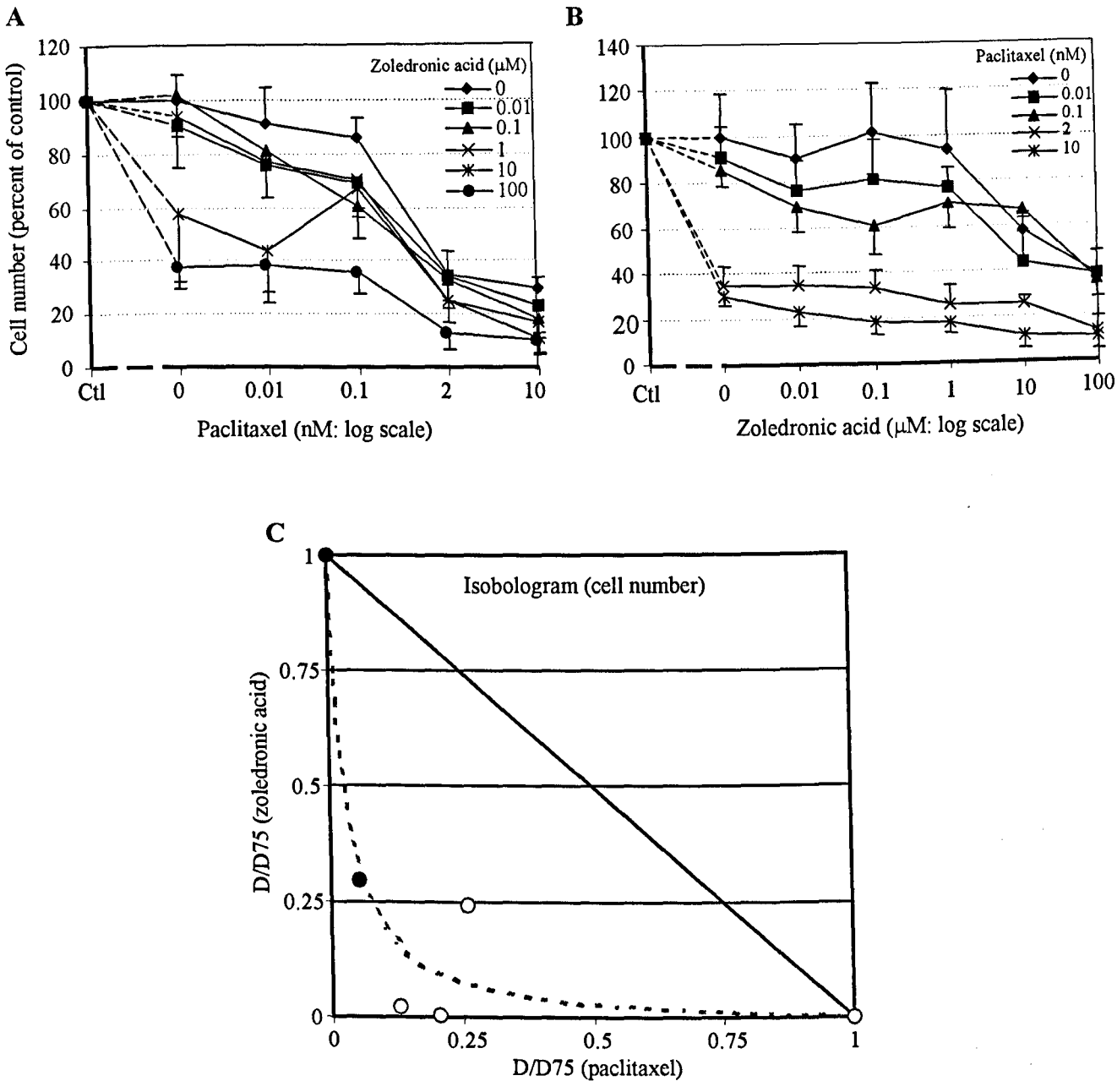


Figure 4.3 Dose response curves and isobologram plot for the interaction between paclitaxel and zoledronic acid on MDA-MB-231 cell number. Cells were incubated with increasing concentrations of zoledronic acid (0.01-100μM) in combination with increasing concentrations of paclitaxel (0.01-10nM) for a total of 72 hours. Cells were harvested to determine cell number (n=8) and dose response curves were plotted for effect of paclitaxel (A) and zoledronic acid (B) on cell number. Combined drug IC_{75} values were determined from these data and used to construct an isobologram (C) for the interaction between paclitaxel (●) and zoledronic acid (○). Dose response results expressed as percent of control and are means ± SD.

4.4.3 Isobologram analysis of the combined effects of zoledronic acid and paclitaxel on breast cancer cell apoptosis demonstrated synergy.

Combined treatment of MCF-7 cells with paclitaxel and zoledronic acid resulted in a synergistic increase in tumour cell apoptosis (figure 4.4). Combined drug IC_{10} values were determined from dose response curves plotted for paclitaxel (4.4A) and zoledronate (4.4B). These were then used to construct an isobologram (4.4C) as described above. Again, the combined D/D10 ratios, when plotted on the isobologram, formed a curve that lay below the additive effect line indicating a synergistic effect on tumour cell apoptosis. The analysis of the combined effect of paclitaxel and zoledronic acid on MDA-MB-231 cell apoptosis also demonstrated the presence of synergy (figure 4.5). In this case, IC_5 and IC_{10} values were calculated from the dose response curves 4.5A and 4.5B. These were then used to construct two isobolgrams as before (4.5C and 4.5D). Again the isobologram curve confirmed that paclitaxel and zoledronic acid had synergistic effects on MDA-MB-231 cell apoptosis. In addition, the synergistic effects were present in both IC_5 and IC_{10} isobolograms, indicating that qualitative assessment of synergy using this method is not dependent on the IC value used.

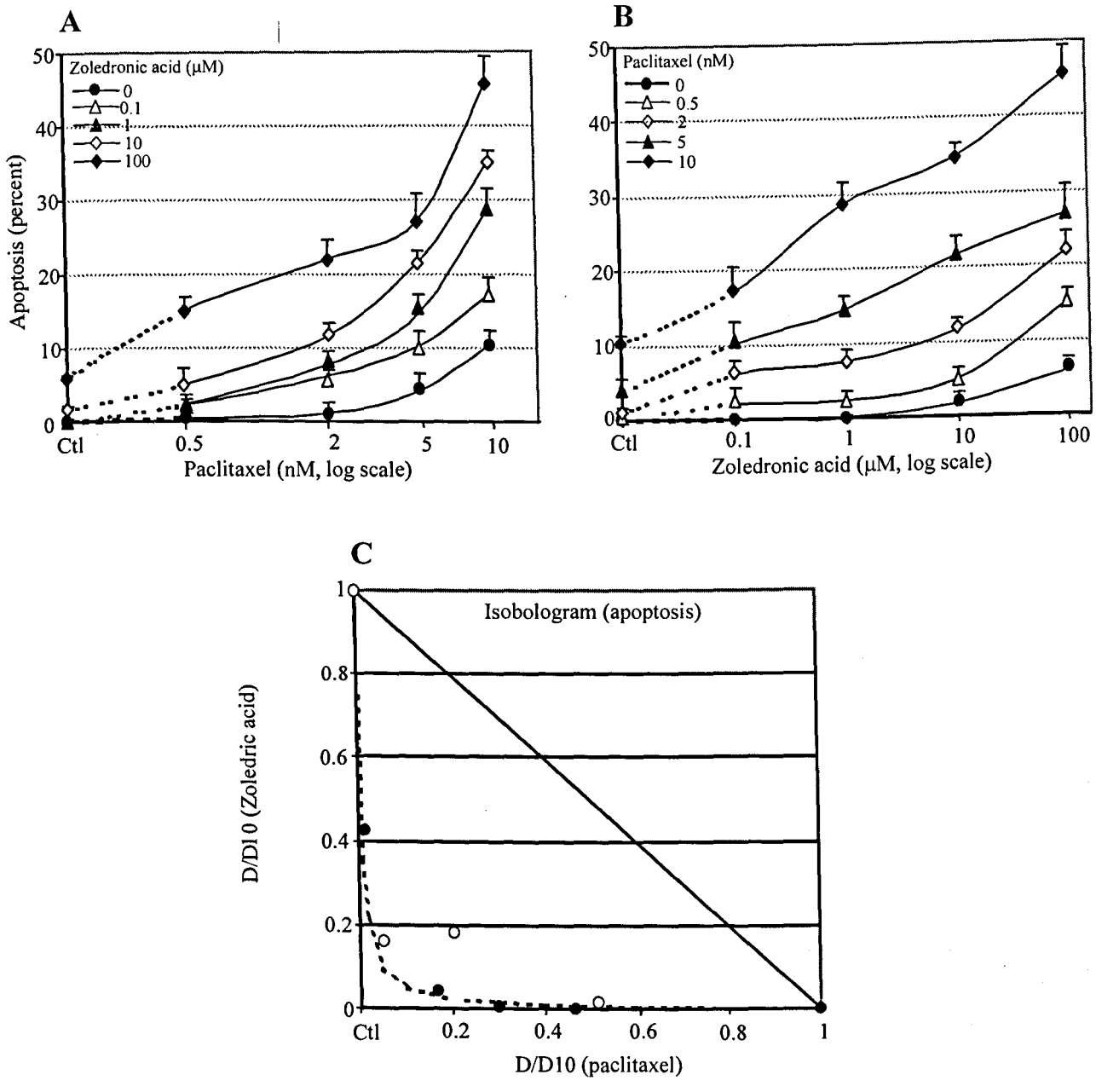


Figure 4.4 Dose response curves and isobologram plot for the interaction between paclitaxel and zoledronic acid on MCF-7 cell apoptosis. Cells were incubated with increasing concentrations of zoledronic acid (0.01-100μM) in combination with increasing concentrations of paclitaxel (0.01-10nM) for a total of 72 hours. Cells were harvested to determine cell number (n=8) and dose response curves were plotted for effect of paclitaxel (A) and zoledronic acid (B) on apoptosis. Combined drug IC_{10} values were determined from these data and used to construct an isobologram (I.C) for the interaction between paclitaxel (●) and zoledronic acid (O). Dose response results expressed as percent of control and are means ± SEM.

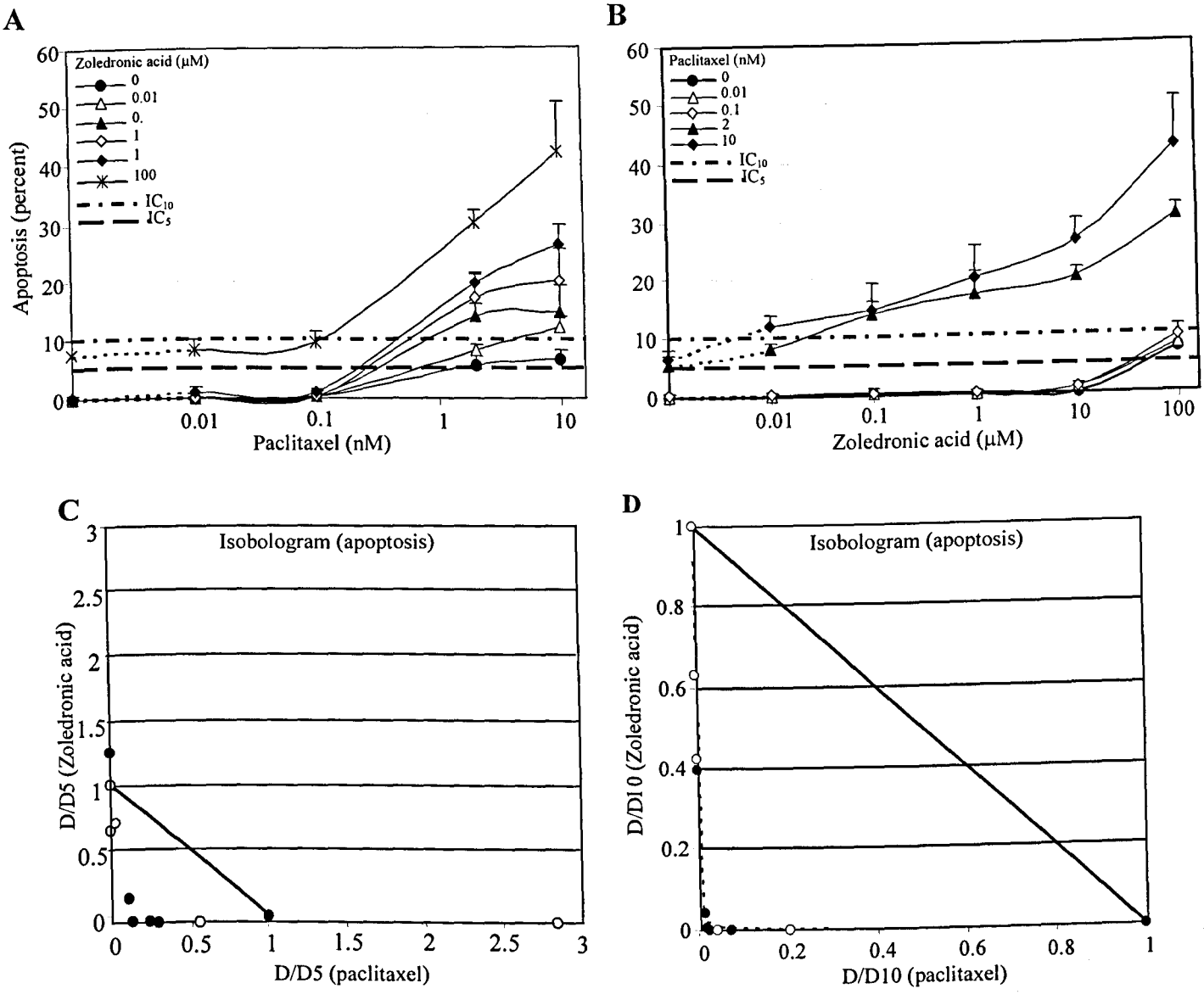


Figure 4.5 Dose response curves and isobologram plot for the interaction between paclitaxel and zoledronic acid on MDA-MB-231 cell apoptosis. Cells were incubated with increasing concentrations of zoledronic acid (0.01-100μM) in combination with increasing concentrations of paclitaxel (0.01-10nM) for a total of 72 hours. Cells were harvested to determine cell number (n=8) and dose response curves were plotted for effect of paclitaxel (A) and zoledronic acid (B) on apoptosis. Combined drug IC_5 and IC_{10} values were determined from these data and used to construct an isobolograms (C) and (D) for the interaction between paclitaxel (●) and zoledronic acid (O). Dose response results expressed as percent of control and are means ± SEM. Isobolograms at both IC values indicated the presence of synergy.

4.4.4 Analysis of the combined effects of zoledronic acid and paclitaxel on breast cancer cell number and apoptosis using the GraFit software.

The use of the model, Equation 1, suggested a synergistic effect on MCF-7 cell number when paclitaxel and zoledronic acid were combined (Table 4.1). The calculated value of α indicated a threefold synergistic effect of the combined drugs on MCF-7 cell number. This combination of drugs also appeared to have a synergistic effect on MDA-MB-231 cell number. However, neither of these values were statistically significant ($\alpha = 3.31 \pm 2.33$ and 5.42 ± 5.08 for MCF-7 and MDA-MB-231 cells respectively). There was also no significant difference between the two cell lines in terms of the effect on cell number.

Table 4.1 Synergy analysis for the effect of paclitaxel and zoledronic acid on cell number

Cell line	α value ($\pm$ SE)
MCF-7	3.31 ± 2.3
MDA-MB-231	5.42 ± 5.08

When the dose response data for breast cancer cell apoptosis was analysed using the model, the combination of paclitaxel and zoledronic acid clearly had a synergistic effect on cell death (Table 4.2). The calculated α values were 67.29 ± 25.9 for MCF-7 cell apoptosis and 295.68 ± 90.6 for MDA-MB-231 cells.

Table 4.2 Synergy analysis for the effect of paclitaxel and zoledronic acid on apoptosis

Cell line	α value ($\pm$ SE)
MCF-7	67.29 ± 25.9
MDA-MB-231	295.68 ± 90.6

4.5 DISCUSSION

In the clinical arena, bisphosphonates are often used in combination with chemotherapy or endocrine therapy. Randomised controlled studies in multiple myeloma and breast cancer patients have suggested that bisphosphonates may confer a survival benefit as well as influencing skeletal morbidity when combined with chemotherapeutic agents (Berenson JR *et al.*, 1998; Diel IJ *et al.*, 1998; McCloskey EV & MacLennan ICM, 1996). This raises the intriguing possibility that bisphosphonates may have synergistic effects on tumour cells when combined with anti-neoplastic agents.

The present study has shown that treatment of MCF-7 cells with zoledronic acid and 2nM paclitaxel, a concentration readily achievable in the serum of patients (Wilson WH, 1994) results in an increase in apoptosis above the expected additive effect. To investigate this more fully and to demonstrate synergy, the isobologram method was used in our studies. This method allows for the analysis of the interaction between two drugs independent of their mechanism of action (Berenbaum, 1989; Greco WR,

1995). It provides a qualitative measure of synergy and has been used both *in vitro* and *in vivo* (Gessner, 1988; Leonessa F, 1994). The mathematical model in Equation 1 (Appendix 1) describes the relationship between the effect of each drug alone and that of the two combined. An interactivity term α can be determined by entering the IC values obtained from the dose response curves for each drug. If α is positive, there is a synergistic interaction between the two drug. Conversely, a negative value for α indicates antagonism. This model can also be used to compare the degrees of synergy between different agents, for example, the synergistic interaction between paclitaxel and zoledronic acid can be compared to the interaction between zoledronic acid and tamoxifen although this is beyond the scope of this thesis.

The analysis of the combined effects of paclitaxel and zoledronic acid on MCF-7 cell number suggested the presence of synergy by the isobologram method. However, although there was at least an additive effect on cell number, the mathematical modelling data did not confirm synergy. However, paclitaxel has been shown to inhibit breast cancer cell proliferation by blocking mitosis (Blagosklonny MV, 1999; McCloskey DE, 1996; Yeung TK, 1999). This, in combination with the effects of bisphosphonates on tumour cell number demonstrated by ourselves, (Chapter 3,) (Jagdev SP, 2001) and others, (Shipman CM, 1997; Shipman CM, 1998; Aparicio A, 1998; Derenne S, 1999) may result in a possible additive effect on cell number. A similar synergistic effect on MDA-MB-231 cell number was suggested on isobologram analysis when the cells were treated with this combination. The mathematical modelling data also hinted at a synergistic effect although, again, the results for the MDA-MB-231 cells were not statistically significant.

In our earlier studies we showed that the bisphosphonate zoledronic acid clearly induced apoptosis in breast cancer cells *in vitro* (Chapter 3). The present studies demonstrate that the combination of paclitaxel and zoledronic acid results in synergistic increases in MCF-7 and MDA-MB-231 cell apoptosis. Dose response studies showed increasing proportions of apoptotic cells as the combined concentrations of paclitaxel and bisphosphonate went up. Isobologram analysis for both cell lines indicated that the effects on breast cancer cell apoptosis were indeed synergistic. The mathematical modelling data confirmed this. Bisphosphonates have been shown to act in synergy with certain agents including taxanes. Tassone et al demonstrated that the combination of zoledronic acid and dexamethasone inhibited human myeloma cell growth and resulted in synergistic induction of apoptosis (Tassone P, 2000). In addition, Magnetto et al investigated the effects of a combination of ibandronate and taxanes on breast cancer cells and suggested that the combination synergistically inhibited breast cancer cell adhesion and invasion (Magnetto S *et al.*, 1999). In contrast to our studies, they did not demonstrate any synergistic activity on breast cancer cell apoptosis. However, investigations in animal models also appear to confirm the possibility of synergistic interactions between bisphosphonates and anti-neoplastic agents. Stearns and Wang found that the combination of alendronate and taxol inhibited the formation of bone metastases induced by prostate cancer cells in SCID mice (Stearns ME, 1996). Similarly, the use of both incadronate and zoledronic acid with oral UFT (a combination of uracil and a fluorouracil prodrug) inhibited the formation of bone metastases and visceral metastases in nude mice inoculated with MDA-MB-231 cells (Yoneda T, 1999; Yoneda T, 2000). The mechanism by which paclitaxel and zoledronic acid synergistically induced apoptosis in our studies was not determined but inhibition of

the mevalonate pathway due to zoledronic acid treatment and the prevention of chromosome segregation and cell division by paclitaxel may result in a cumulative apoptotic insult. As the data discussed above suggest, it is possible that they may also act together to affect other aspects of tumour cell function such as invasion and adhesion. These mechanisms require further study both *in vitro* and *in vivo*. It would also be important to investigate possible synergy with other anti-neoplastic agents, as well as to explore temporal relationships, in future studies.

CHAPTER 5

INVESTIGATION OF THE *IN VIVO* ANTITUMOUR EFFECTS OF BISPHOSPHONATES USING THE DUAL FLUORESCENCE LABELLING TECHNIQUE

5.1 SUMMARY

The work presented in the chapters 3 and 4 demonstrates that zoledronic acid clearly has anti-tumour effects on breast cancer cells *in vitro*. The important question is whether these effects are observed in patients with breast cancer-induced bone metastases who are receiving bisphosphonate treatment. To address this issue, it is important to identify the levels of apoptosis in tumour cells that are part of or in contact with secondary bone lesions and this can be done using bone marrow aspirate samples. The aim of the work presented in this chapter, therefore, was to develop a technique to identify apoptotic breast cancer cells in the mixed cell population present in bone marrow. This technique was then used to investigate the changes in bone marrow tumour cell apoptosis before and after intravenous bisphosphonate treatment in samples from breast cancer patients.

A fluorescence dual labelling method was developed by combining an epithelial tumour marker (BerEP4) with a marker for apoptosis, the fluorescence *in situ* nick translation assay. Samples of peripheral blood were mixed with MCF-7 cells and used to test and optimise the assay. Having established a technique capable of detecting apoptotic breast cancer cells in a mixed cell population, 5 patient bone marrow samples were analysed before and 3 days after treatment with intravenous pamidronate or zoledronic acid. No tumour cells were detected in these patients therefore the assay was modified by replacing the BerEP4 antibody with a fluorescence-labelled anti-cytokeratin antibody, MNF116. Three of the initial 5 patients and a further 9 patients were analysed using the new antibody and 33% of patient samples were positive for tumour cells. However, there did not appear to be an increase in bone marrow tumour cell apoptosis after bisphosphonate treatment although the number of patients with paired samples that could be examined successfully was very small.

The data presented in this chapter indicated that the dual labelling assay was able to successfully identify and quantify the levels of apoptosis in bone marrow breast cancer cells. In these few patients, treatment with intravenous bisphosphonates does not appear to promote apoptosis of bone marrow tumour cells although a larger study is warranted to establish definitively the effect of bisphosphonates on breast cancer cell apoptosis *in vivo*.

5.2 INTRODUCTION

The use of bisphosphonates in breast cancer and multiple myeloma patients has been shown to have a beneficial effect on skeletal morbidity (Berenson J, 1996; Diel IJ, 1998; Hortobagyi GN, 1996; Kanis JA, 1996; McCloskey, 1998; Theriault RL, 1999; Paterson AHG, 1993, Powles T, 2002). In addition, recent data in patients with multiple myeloma suggest that treatment with bisphosphonates confers a survival benefit (Berenson J, 1996). Similar survival effects may also be apparent in breast cancer as Diel et al have recently reported that adjuvant bisphosphonate treatment influences extra-skeletal as well as skeletal metastases and may increase survival (Diel IJ, 1998). Taken together, these data suggest that bisphosphonates may have anti-tumour effects in addition to potently inhibiting osteoclast-mediated bone resorption. The work presented in chapters 3 and 4 supports the hypothesis that bisphosphonates can have direct effects on breast cancer cells at least *in vitro*. The important question is whether these effects are observed in patients with breast cancer-induced bone metastases who are receiving bisphosphonate treatment. To address this issue, it is important to identify the levels of apoptosis in tumour cells that are part of, or in contact with, secondary bone lesions and this can be done using bone marrow aspirate samples.

The aim of the work presented in this chapter was to develop a technique that would allow the identification of apoptotic breast cancer cells in the mixed cell population present in the bone marrow. The assay was developed using MCF-7 breast cancer cells mixed with peripheral blood and, once established, was used to analyse paired bone marrow samples from patients with breast cancer before and after intravenous bisphosphonate treatment. Breast cancer cells are of epithelial origin and can therefore be recognised by epithelial cell markers. One such marker is the human epithelial antigen which is expressed on the surface and in the cytoplasm of epithelial

cells and we initially used an antibody raised against this antigen, the BerEP4 antibody (Latza U, 1990) to identify tumour cells. Although this antibody performed well during the development of the technique, it did not identify any positive cells from the first set of bone marrow samples tested. It was therefore replaced with the MNF116 antibody raised against a panel of cytokeratins which are expressed by cells of epithelial origin, both in normal and in malignant tissue (Goddard MJ, 1991). This was then combined with the fluorescence *in situ* nick translation assay for apoptosis to enable the detection of apoptotic breast cancer cells in the bone marrow of patients receiving bisphosphonate treatment.

5.3 MATERIALS AND METHODS

5.3.1 Chemicals

The FITC-conjugated antibodies, IgG1 isotype control, BerEP4 and MNF116, were purchased from DAKO Ltd (Cambridgeshire, UK) and stored at 4^0C until use. Both antibodies were supplied at a concentration of 100mg/L and were used at the recommended dilution of 1:10. Human AB serum was obtained from the Blood Transfusion Service, Northern General Hospital, Sheffield, UK. All other chemicals were purchased from Sigma Chemicals Co (Poole, Dorset, UK).

5.3.2 Cell Culture

The human breast carcinoma cell line MCF-7 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in RPMI 1640 (Life Technologies, Inc., Paisley, UK) supplemented with 10% FCS, 1mM glutamine, 1mM sodium pyruvate and 1x MEM nonessential amino acids (Life Technologies Inc). Cells were harvested using trypsin/ ETDA 0.5%/ 0.2% (Life Technologies).

5.3.3 Bisphosphonate treatment of breast cancer cells

In some studies, MCF-7 cells were seeded at a density of $1x10^6$ cells/ml in T25 flasks and allowed to grow to confluence. They were then incubated with fresh media containing PBS vehicle control or 500μM zoledronic acid for a further 72 hours before they were harvested.

5.3.4 Patient Samples

Pre- and post-treatment bone marrow aspirate samples were taken from 14 patients with breast cancer and bone metastases who were due to receive intravenous bisphosphonate treatment with

either pamidronate (90mg) or zoledronic acid (4mg or 8mg) for the first time at the Cancer Research Centre, Weston Park Hospital, Sheffield, UK. No concomitant chemotherapy treatment or treatment with agents that altered bone metabolism was allowed, although some patients were on long-term hormonal therapy for their breast cancer. A bone marrow aspirate sample was taken from the posterior iliac crest before and three days after bisphosphonate treatment. The first four patients had samples taken consecutively from the same iliac crest. Subsequent patients had samples taken from opposite sides before and after treatment to reduce the possibility of trauma-related apoptosis in the samples. Ethics approval was obtained for the study from the South Sheffield Ethics Committee and full written informed consent was obtained from each patient before the samples were taken. Samples of peripheral blood for development of the assay were taken at the Outpatient Department, Royal Hallamshire Hospital, Sheffield, UK.

5.3.5 Development of the dual labelling technique

5.3.5.1 Breast cancer cell identification

The FITC-conjugated antibody BerEP4 was used to identify breast cancer cells. This antibody is raised in the mouse against the Human Epithelial Antigen, a surface antigen present predominantly on the surface but also in the cytoplasm of cells of epithelial origin such as breast cancer cells [Latza U, 1990 #44]. MCF-7 cells were plated at $1x10^6$/ml in T25 tissue culture flasks (Costar), cultured until confluent, and were then harvested. Half the sample was fixed in 4% formaldehyde (v/v) and half the sample was analysed whilst fresh. To confirm that this antibody binds to breast cancer cells, the MCF-7 cells were washed in ice-cold wash buffer consisting of PBS and 1% FCS and resuspended in 100µl of wash buffer containing either 10mg/L isotype control or 10mg/L BerEP4 for 30 minutes on ice in the dark. The cells were then washed again and resuspended in 300µl of PBS for FACSort analysis. The positively labelled tumour cells were detected using the FL1 detector (530nm bandpass filter, 30nm bandwidth). The

isotype control was used to mark the background level of fluorescence in the sample and the positively labelled cells were detected above this gate.

To confirm that MCF-7 cells stained positively with the BerEP4 antibody, cells were harvested, fixed with 4% (v/v) formaldehyde in PBS and cytospun onto glass slides. They were then stained with 4'-6-diamidino-2-phenyl-indole (DAPI) at 2μg/ml in PBS for 15 minutes, washed and stained with 10% (v/v) isotype control or 10% (v/v) BerEP4 in PBS containing 1% FCS (v/v) for 30 minutes. Cells were examined using a DMRB fluorescence microscope (Leica).

5.3.5.2 Isolation of breast cancer cells from a mixed cell population

MCF-7 cells were harvested, mixed with peripheral blood and subsequently processed according to the Erythrocyte Lysis Method described in Materials and Methods. The mononuclear cell population was then labelled with isotype control or BerEP4 as described in section 5.3.5.1 above and analysed for the presence of tumour cells by flow cytometry. The positively labelled tumour cells were detected using the FL1 detector (530nm bandpass filter, 30nm bandwidth). The isotype control was used to mark the background level of fluorescence in the sample and positively labelled cells were detected above this gate.

5.3.5.3 Identification of apoptotic tumour cells from a mixed cell population with the dual fluorescence labelling technique

MCF-7 cells were seeded as above and incubated in the presence or absence of 500μM zoledronic acid for 72 hours. The cells were then harvested and mixed with peripheral blood, after which the samples were processed according to the Erythrocyte Lysis method (Materials and Methods). Cells were then fixed in 4% formaldehyde (v/v) and frozen in 100% ethanol and stored at -20^0C until they were analysed. To allow simultaneous labelling for DNA strand breaks

associated with apoptosis and tumour cells, the samples were incubated at room temperature with a buffer containing 50mM tris, 2.5 mM magnesium chloride, 10μg/ml bovine serum albumen, 10 mM β-mercaptoethanol, 0.2nM of unlabeled dATP, dGTP and dCTP and 0.2nM biotin-16-dUTP in the presence of 1 unit DNA polymerase for 4 hours with intermittent gentle agitation [Gorczyca W, 1993 #11]. The cells were then washed in sterile PBS and incubated with a second buffer containing 4x saline-sodium citrate buffer (SSC), 0.1% Triton-X 100, 5% (w/v) fat free powdered milk,1% (v/v) phycoerythrin- conjugated streptavidin (DAKO) and either 10% (v/v) FITC-conjugated isotype control (DAKO) or 10% (v/v) FITC-conjugated BerEP4 antibody for 30 minutes at room temperature in the dark. After washing with PBS, cells were analysed using flow cytometry. The R-PE-positive cells (apoptotic cells) were detected using the FL2 detector (585nm bandpass filter, 42nm bandwidth) and the FITC-positive cells (tumour cells) were detected using the FL1 detector (530nm bandpass filter, 30nm bandwidth). Results were expressed as log PE fluorescence against log FITC fluorescence. The FITC-conjugated isotype control was used as a negative control for each sample and signified the background level of fluorescence in each sample. Antibody positive cells (breast cancer cells) appeared above the marker for background FITC fluorescence.

5.3.5.4 Dual fluorescence labelling of patient bone marrow

Bone marrow aspirate samples were collected from breast cancer patients before and 72 hours after intravenous bisphosphonate treatment (pamidronate or zoledronic acid) into tubes containing EDTA. Samples were centrifuged at 1000rpm for 5minutes and the plasma removed and stored at -80^0C for future analysis. The remaining cells were resuspended in erythrocyte lysis buffer and processed according to the Erythrocyte Lysis method described in Materials and Methods. The cells were then fixed in 4% (v/v) formaldehyde in PBS and stored in 100% ethanol at -20^0C until analysed. To identify apoptotic tumour cells from the mixed mononuclear cell

population in the bone marrow, samples were processed according to the dual fluorescence labelling method described in section 5.3.5.3 above with the addition of 4% (v/v) human AB serum to the wash buffer. The cells were resuspended in 300µl PBS and analysed on the FACSort (Becton Dickinson). As before, the R-PE-positive cells (apoptotic cells) were detected using the FL2 detector (585nm bandpass filter, 42nm bandwidth) and the FITC-positive cells (tumour cells) were detected using the FL1 detector (530nm bandpass filter, 30nm bandwidth). Results were expressed as log PE fluorescence against log FITC fluorescence. As before, the FITC-conjugated isotype control was used as a negative control for each sample and signified the background level of fluorescence in each sample.

5.3.5.5 Modifications to the fluorescence antibody labelling technique

The dual fluorescence technique was used to analyse 5 sets of paired bone marrow samples from patients with breast cancer-induced bone metastases. No tumour cells were detected in any of these samples. The reasons for this were unclear but it was possible that the BerEP4 antibody was not sensitive enough to detect the presence of low levels of tumour cells in the bone marrow. This antibody was therefore substituted with a FITC-conjugated antibody that had been raised against a panel of cytokeratins and could identify epithelial cells, the MNF116 antibody (Moll R, 1982; Goddard MJ, 1991). To confirm positive staining, MCF-7 cells were mixed with peripheral blood, the sample processed according to the Erythrocyte Lysis method (Materials and Methods), and the remaining cells prepared for antibody staining. The cells were washed in wash buffer consisting of 1% (v/v) FCS in PBS and incubated with 100µl of wash buffer containing either 10% isotype control (v/v) or 10% MNF116 (v/v) for 30 minutes on ice in the dark. The cells were then washed again and resuspended in 300µl of PBS for FACSort analysis. The positively labelled tumour cells were detected using the FL1 detector (530nm bandpass filter, 30nm

bandwidth). The isotype control was used to mark the background level of fluorescence in the sample as before.

5.3.5.6 Sensitivity of tumour cell detection with the modified assay

To determine the sensitivity of tumour cell labelling with the MNF116 antibody, MCF-7 cells were mixed with peripheral blood in increasing proportions (tumour cells to mononuclear cells) (0.5- 50%). The proportions were based on the assumption that peripheral blood contains $6x10^6$-$1x10^7$ mononuclear cells /ml. The samples were then prepared for flow cytometry analysis after undergoing erythrocyte lysis and fluorescence antibody staining as described in section 5.3.5.5 above. The proportions of antibody positive cells detected by the technique were identified using the FL1 detector on the FACSort machine.

5.3.5.7 Re-analysis of patient samples with MNF116 antibody

The modified tumour cell labelling method described in section 5.3.5.5 appeared to be a sensitive method for identifying breast cancer cells in a mixed cell population. We therefore re-analysed three of the previous patient samples using this technique (with the inclusion of 4% (v/v) human AB serum in the wash buffer). After labelling with the antibody, samples were analysed on the FACSort as in section 5.3.5.5.

5.3.5.8 Analysis of apoptotic tumour cells in patient bone marrow using the modified dual fluorescence labelling technique

The dual labelling technique described in section 5.3.5.4 was modified by substituting the BerEP4 antibody with the MNF116 antibody in the second step of the assay. A further 9 paired patient samples were analysed using this assay for the presence of apoptotic tumour cells.

5.3.6 Statistical analysis

The data was found to be non-parametric therefore the difference between pre- and post treatment levels of total cell apoptosis in the bone marrow samples was analysed using the Wilcoxon matched pairs test. Analysis of pre- and post-treatment apoptosis of breast cancer cells was not possible, as the number of patients with detectable tumour cells in both pre- and post-treatment samples was too small.

5.4 RESULTS

5.4.1 BerEP4 antibody positively identified breast cancer cells

MCF-7 cells were labelled with FITC-conjugated isotype control and FITC-conjugated BerEP4 (Figure 5.1). Visualisation using a Leica DMRB fluorescence microscope confirmed the presence of positive staining of MCF-7 cells with BerEP4 (Figure 5.1B) compared with isotype control (Figure 5.1B).

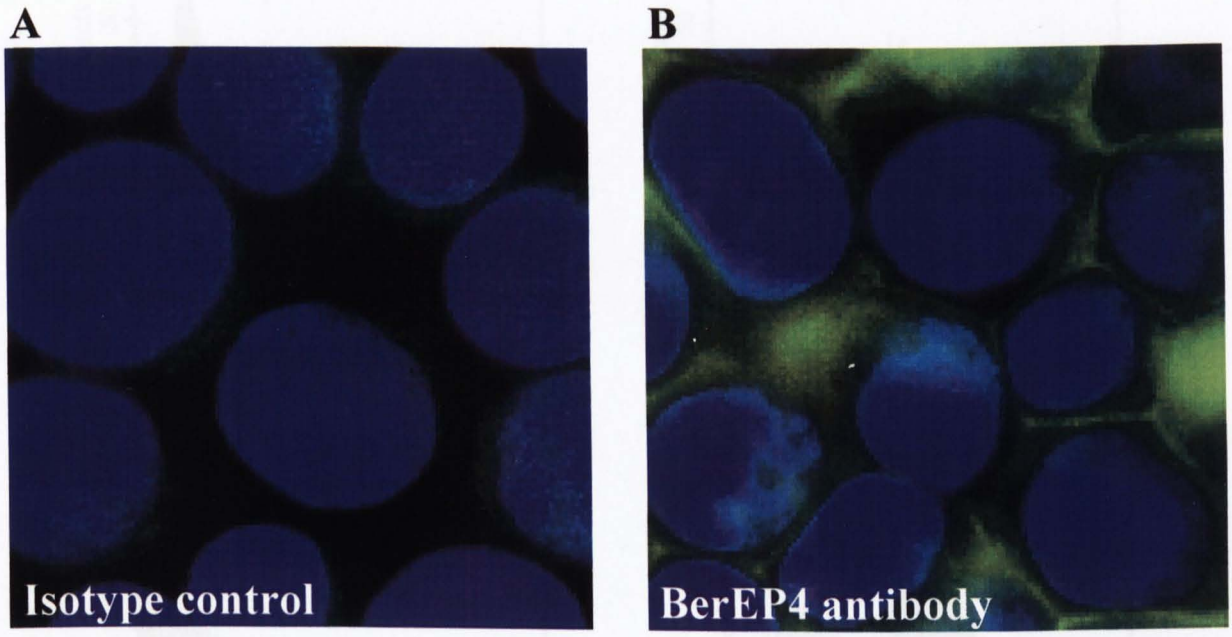


Figure 5.1. MCF-7 staining with (A) isotype control and (B) BerEP4; original magnification x100. Cells were cytospun onto glass slides, stained with 10% (v/v) FITC-conjugated isotype control or FITC-conjugated BerEP4 antibody in wash buffer and counterstained with 4'-6-diamidino-2-phenyl-indole (DAPI) at 2µg/ml in PBS. Cells were visualised using a Leica DMRB microscope.

Flow cytometry analysis of fresh MCF-7 cells revealed a shift in peak fluorescence in cells stained with BerEp4 to the right compared with those stained with isotype control (figure 5.2A and 5.2B). Similar levels of staining were achieved in MCF-7 cells that had been fixed in 4% (v/v) formaldehyde before the antibody labeling procedure (figure 5.2C and D).

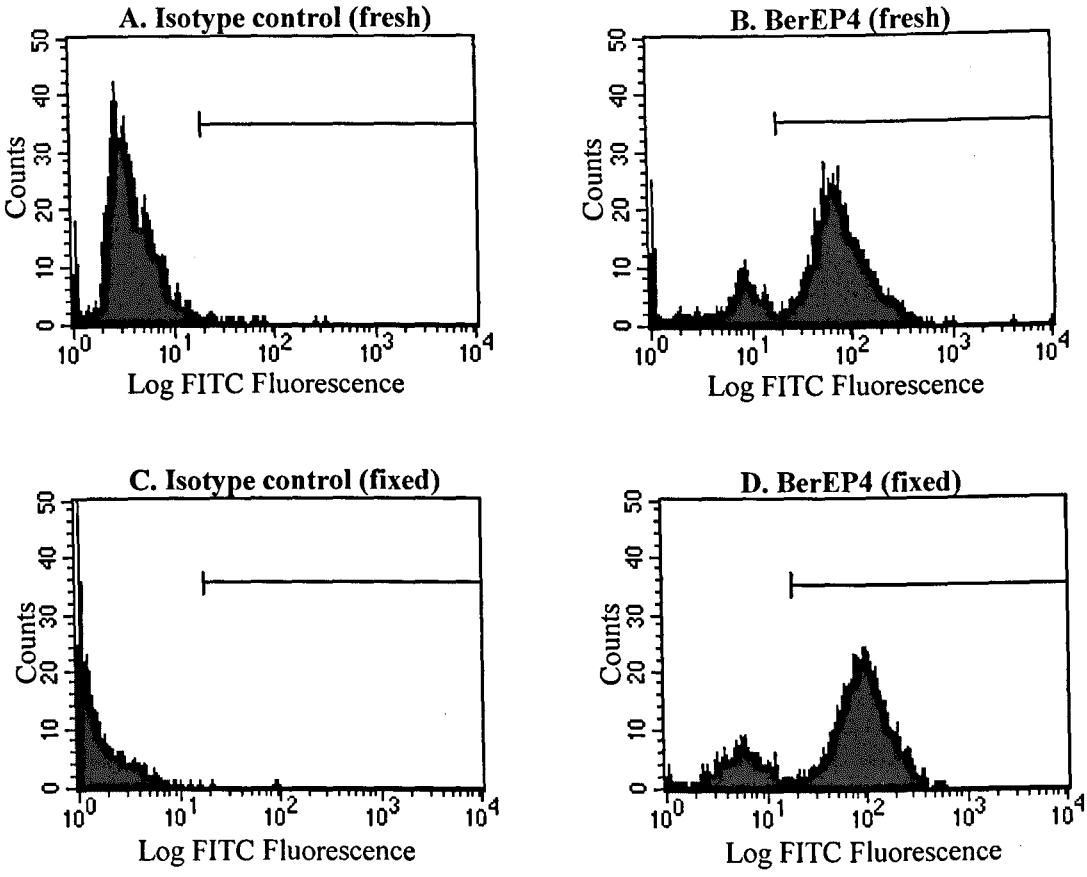


Figure 5.2. Flow cytometric analysis of MCF7 cells stained with isotype control and BerEP4 whilst fresh (A and B) and after fixation in 4% (v/v) formaldehyde (C and D). Results are expressed as Log FITC fluorescence against cell counts.

5.4.2 Fluorescence antibody labeling method identified tumour cells in a mixed cell population

MCF-7 cells were mixed with peripheral blood and labeled with either isotype control or BerEp4 after undergoing erythrocyte lysis. Analysis of the isotype-labeled sample by flow cytometry identified the background level of binding and was marked with a marker just below the second log decade for FITC fluorescence (figure 5.3A). The analysis of the BerEp4 labeled sample revealed a population of positively-labeled cells above the second log decade for FITC fluorescence (figure 5.3B) indicating that this antibody can identify breast cancer cells in a mixed cell population.

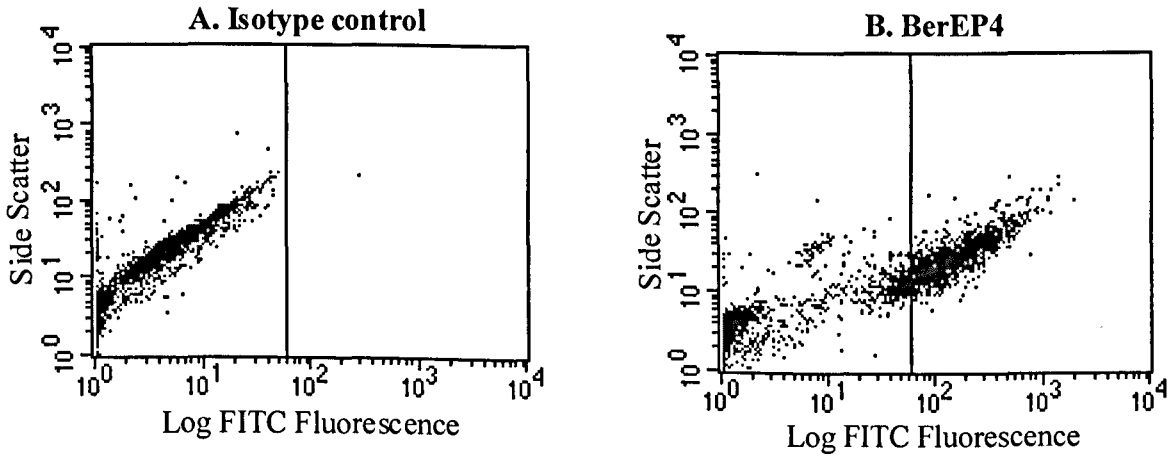
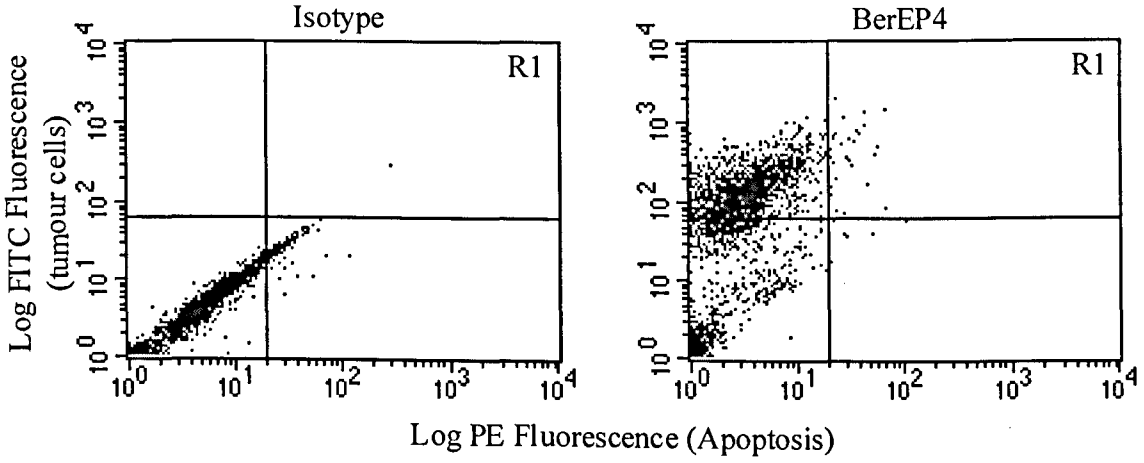


Figure 5.3. Flow cytometry analysis of MCF-7 cells mixed with peripheral blood and labeled with isotype control (A) or BerEP4 (B) after erythrocyte lysis. Results are expressed as Log FITC fluorescence against side scatter.

5.4.3 Fluorescence dual labeling assay detected apoptotic breast cancer cells in a mixed cell population

MCF-7 cells were treated with PBS control or 500μM zoledronic acid for 72 hours after which they were loaded into peripheral blood. Dual labeling of the control samples as described in section 5.3.5.3 revealed an antibody positive population of cells around the second log decade for FITC fluorescence in the BerEp4 labeled samples compared to the isotype control labeled samples (Figure 5.4A). Cells that were positive for both FITC and PE conjugated markers, i.e. the apoptotic tumour cells, should appear in the right upper quadrant of the plots, R1. 0.2% of antibody-positive cells were detected in this region in sample A (PBS control). In contrast, 30% of BerEP4-positive cells (17.1% of total sample population) appeared in region R1 after treatment with zoledronic acid (Figure 5.4B) indicating that apoptotic tumour cells can be identified by this technique.

A. Control



B. Zoledronic acid

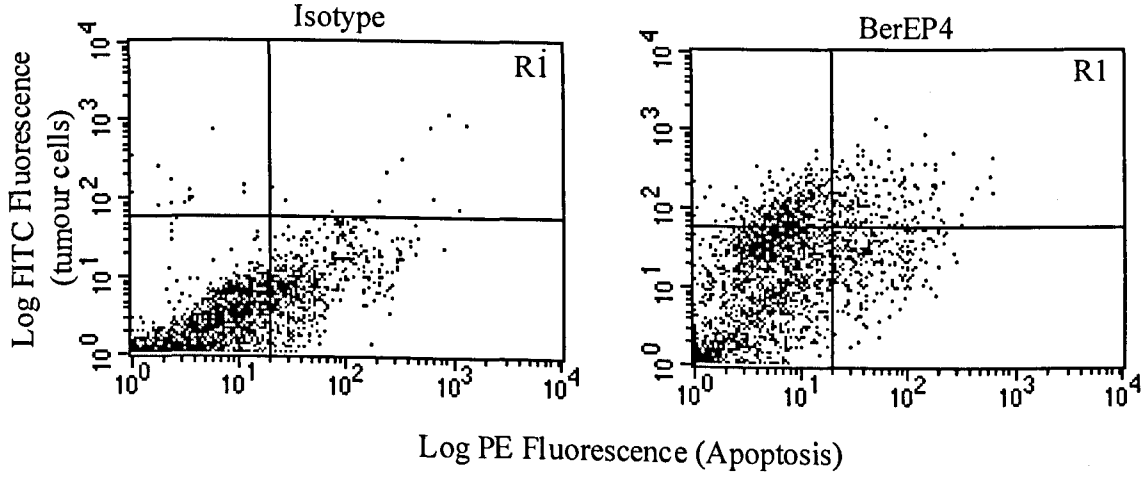


Figure 5.4. Dual fluorescence labelling technique. MCF-7 cells were treated with PBS (A) or 500μM zoledronic acid (B) and loaded into peripheral blood. After erythrocyte lysis, samples were labelled with a dual fluorescence labelling assay (consisting of the BerEP4 tumour cell label combined with *in situ* nick translation assay) and analysed by flow cytometry. Results are expressed as Log PE fluorescence (apoptosis) against log FITC fluorescence (breast cancer cells). Cells positive for both labels (apoptotic tumour cells) fall in region R1.

5.4.4 Paired patient bone marrow samples were analysed using the fluorescence dual labelling technique.

Five sets of paired patient bone marrow samples were analysed using the dual fluorescence labelling assay (Table 5.1). Of these, four patient samples showed an increase in apoptosis in all cell populations following intravenous bisphosphonate treatment (patients 1, 2, 3, and 5). Patient 4 showed greater levels of apoptosis in the pre-treatment sample compared with post-treatment. No tumour cells were detected in any of these samples.

Table 5.1. Analysis of patient bone marrow samples before and after bisphosphonate treatment using the dual fluorescence labelling assay.

Patient	Treatment	Overall Apoptosis (%)	Antibody-positive cells (%)
1	Pre	0.08	X
	Post	11.44	X
2	Pre	0.86	X
	Post	2.91	X
3	Pre	2.57	X
	Post	8.01	X
4	Pre	24.49	X
	Post	15.67	X
5	Pre	6.90	X
	Post	12.92	X

5.4.5 The modified fluorescence antibody labelling technique sensitively detected tumour cells in a mixed cell population

As the dual fluorescence labelling assay described above did not identify tumour cells in the five sets of samples analysed, the method was modified to incorporate a FITC-conjugated anti-cytokeratin antibody (MNF116) in place of the BerEP4 antibody. Tumour cell staining with this antibody was confirmed by labelling MCF-7 cells mixed with peripheral blood as described in section 5.3.5.5. The results are shown in figure 5.5 below. The background level of binding was determined with the isotype control (figure 5.5A). A marker was placed at the upper limit of the background population. On analysis of the MNF116-labelled sample, a clearly positive population of cells appeared above the second log decade for FITC fluorescence (figure 5.5B).

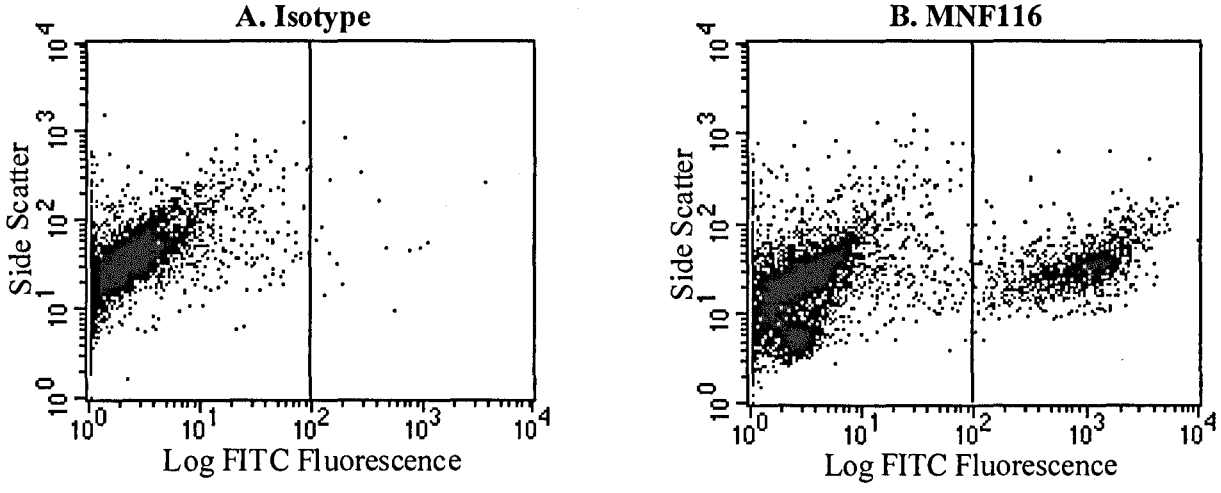


Figure 5.5. Fluorescence antibody labelling of MCF-7 cells mixed with peripheral blood with isotype control (A) and MNF116 (B). Results are expressed as log FITC fluorescence against side scatter.

The sensitivity of the MNF116 in detecting tumour cells was determined by mixing increasing proportions of MCF-7 cells into peripheral blood and then staining and analysing the samples after erythrocyte lysis. Flow cytometry analysis revealed that the antibody was able to detect breast cancer cells in a mixed cell population down to levels of around 1% (Table 5.2). There was no statistically significant difference between the percentage of cells loaded and those recovered.

Table 5.2 Sensitivity of MNF116 antibody in detecting breast cancer cells in a mixed cell population.

Breast cancer cells loaded (%)	Breast cancer cells recovered (%)
0	0.07
0.5	1.17
1	1.63
2.5	2.07
5	5.94
10	14.25
20	22.12
50	59.16

Three of the previous five patient bone marrow samples (patients 2, 3 and 5) were re-analysed using the new antibody to determine whether breast cancer cells could be detected. Analysis of the sample from patient 3 revealed the presence of 5.08% MNF116-positive cells (breast cancer cells) in the pre-treatment sample and 18.99% tumour cells in the post-treatment sample compared with no positive staining with the previous antibody (figure 5.6). In addition, 3.22% and 20.08% antibody-positive tumour cells were detected in the pre-treatment and post-treatment

samples respectively of patient 5. Re-analysis of patient 2 did not demonstrate the presence of breast cancer cells. Unfortunately, there were insufficient numbers of cells remaining in these samples to stain with the full dual fluorescence labelling assay.

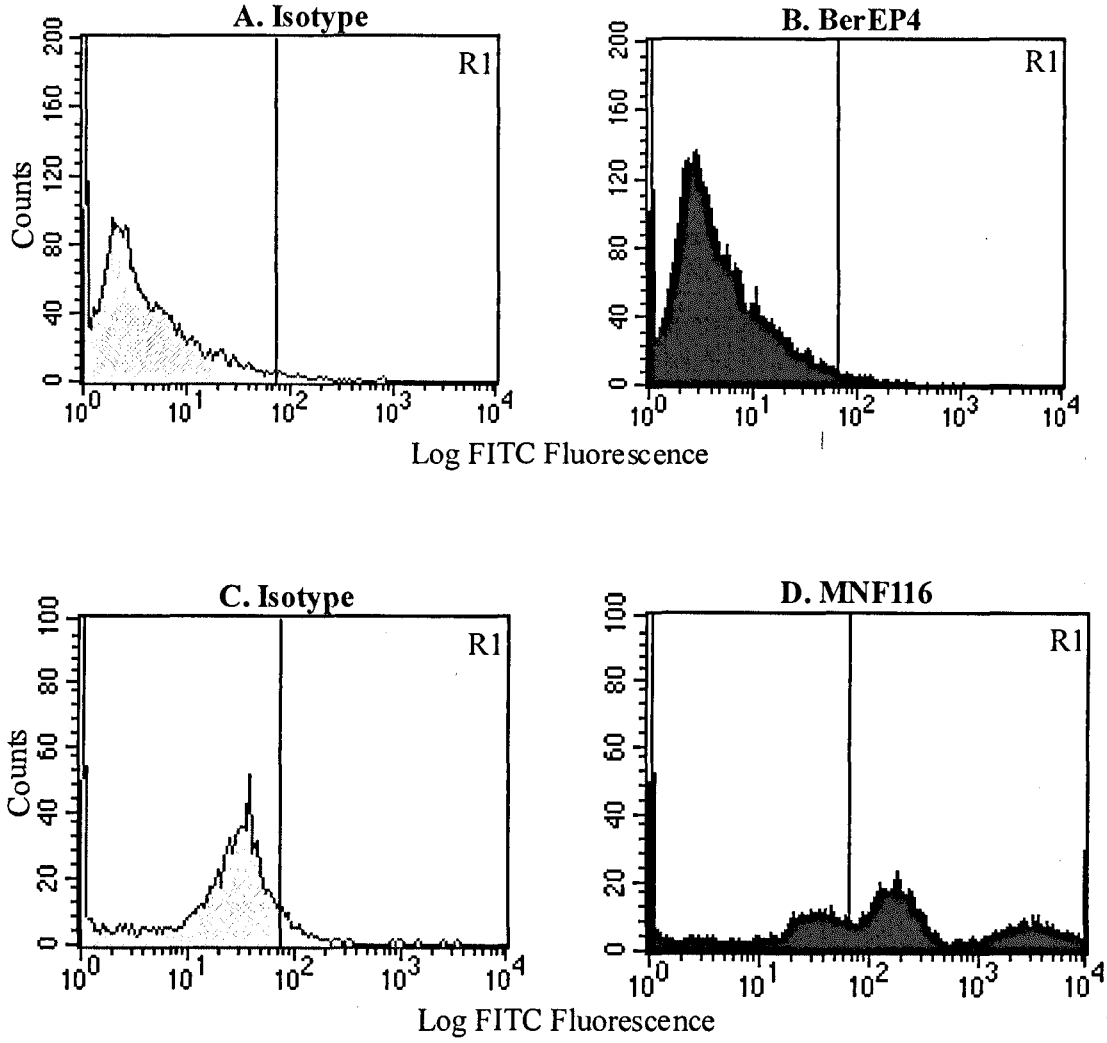


Figure 5.6. BerEP4 and MNF116 antibody staining of patient 3's bone marrow cells. Patient bone marrow sample was stained with isotype control (A) and BerEP4 (B) and analysed by flow cytometry. The same sample was subsequently stained with isotype control (C) and MNF116 (D). Results were expressed as Log FITC fluorescence against cell counts. The positively stained cells appeared above the marker in region 1 (R1).

5.4.6 The assessment of primary tumour cell apoptosis in the bone marrow of patients receiving bisphosphonate treatment

Nine further paired patient samples were analysed using the modified dual labeling technique. The flow cytometry results of patient 8 are illustrated in figure 5.7 below.

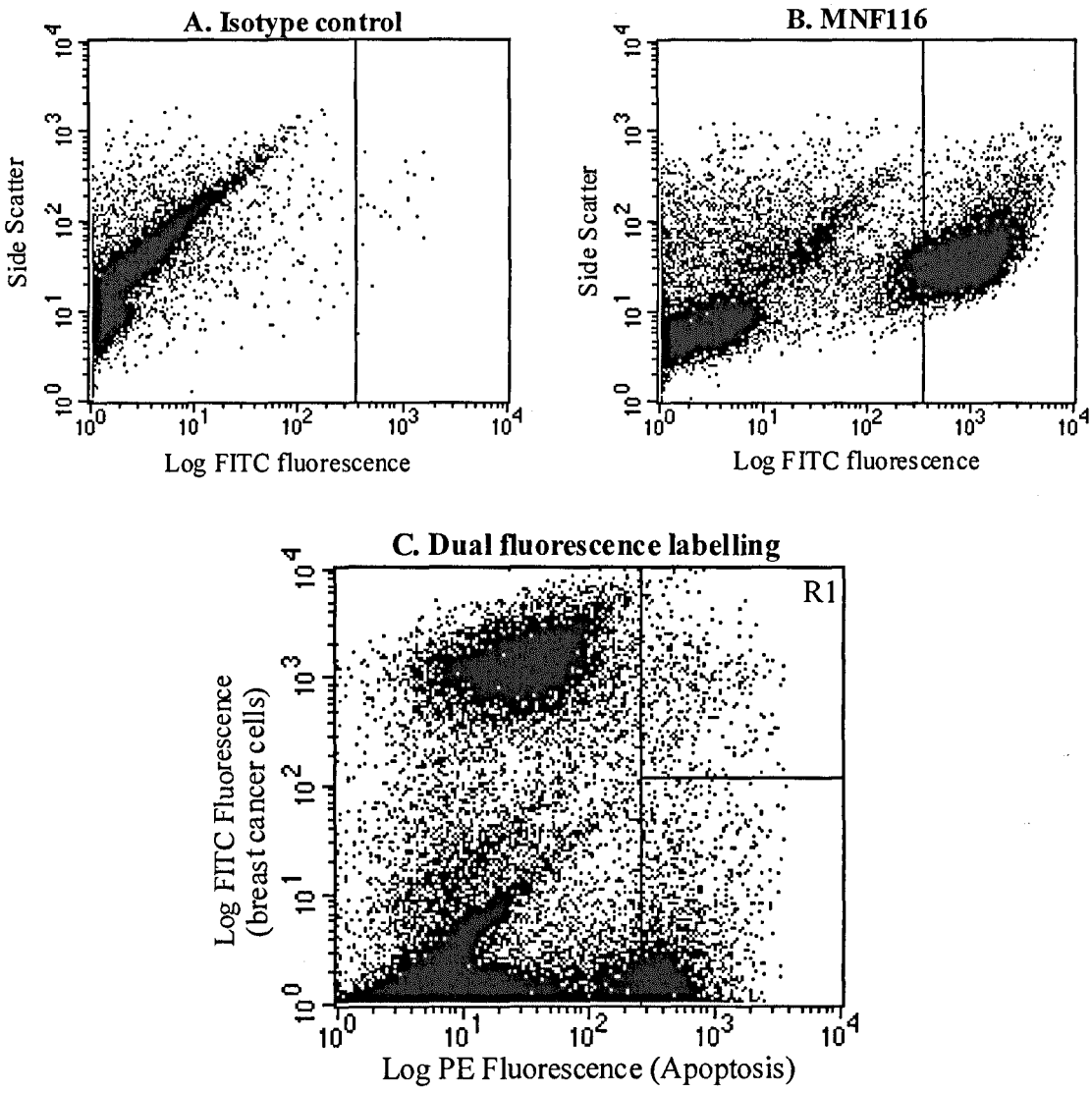


Figure 5.7. Flow cytometry analysis of bone marrow cells from patient 8 (pre-treatment sample). Cells were labeled with isotype control (A) to identify background staining and MNF116 (B) to identify tumour cells (evident above the second log decade for FITC fluorescence). The dual fluorescence labeling assay identified the proportion of cells that were positive for both labels, i.e., apoptotic tumour cells (region R1). Results are expressed as log PE fluorescence against log FITC fluorescence.

The levels of apoptosis in the whole sample population increased after bisphosphonate treatment in four out of nine patients (44.4%) (Table 5.3). The remaining five patient samples revealed a reduction in overall apoptosis. Antibody-positive breast cancer cells were detected in three out of nine new patients analysed (patients 8, 9 and 14). Of these, patients 8 and 14 demonstrated a reduction in tumour cell apoptosis in the post-treatment sample compared with pre-treatment whilst patient 9 showed no change. When the level of apoptosis within all cell populations was examined in all 14 patients, there was no significant difference between pre and post treatment total cell apoptosis ($p=0.08$). Overall, tumour cells were detected in 5 of 12 patients (41.6%) analysed with the MNF116 antibody. The number of patients in the study was too small to draw any conclusions regarding the induction of breast cancer cell apoptosis after intravenous bisphosphonate treatment.

Table 5.3 Analysis of paired patient bone marrow samples with the modified dual fluorescence labelling technique.

Patient	Treatment	Overall apoptosis (%)	Tumour cells (%)	Apoptotic tumour cells (%)
1	Pre	0.08	NA	
	Post	11.44		
2	Pre	0.86	NA	
	Post	2.91		
3	Pre	2.57	*5.08*[a]	
	Post	8.01	*18.99*[a]	
4	Pre	24.49	*X*[a]	
	Post	15.67	*X*[a]	
5	Pre	6.90	*3.22*[a]	
	Post	12.92	*20.08*[a]	
6	Pre	1.4	X	-
	Post	0.55	X	-
7	Pre	0.24	X	-
	Post	0.05	X	-
8	Pre	19.13	31.64	11.47
	Post	6.78	52.01	2.96
9	Pre	0.66	13.37	0
	Post	2.61	42.12	0
10	Pre	1.31	X	-
	Post	0.16	X	-
11	Pre	5.28	X	-
	Post	7.96	X	-
12	Pre	0.82	X	-
	Post	1.79	2.69	9.15
13	Pre	1.06	X	-
	Post	0.89	X	-
14	Pre	0.3	X	-
	Post	0.99	X	-

NA Not analysed with MNF116 antibody

[a] Analysed for presence of tumour cells using the MNF116 antibody subsequent to initial dual labelling with the BerEP4 antibody

5.5 DISCUSSION

Recent clinical studies have suggested that the use of bisphosphonates in the management of patients with cancer-induced bone disease may confer a survival advantage (Berenson J, 1996; Diel IJ, 1998) raising the possibility that these compounds may have direct anti-tumour effects in addition to inhibiting osteoclast-mediated bone resorption. The data presented in Chapters 3 and 4 support the hypothesis that these compounds have anti-tumour effects on breast cancer cells *in vitro*. The important question is whether these effects are apparent in breast cancer cells present at or adjacent to the sites of bone metastasis.

The first step in addressing this question was to develop a technique that would allow the identification of apoptotic breast cancer cells in a mixed cell population present in the bone marrow. Analysis by flow cytometry proved to be very useful as it allowed the simultaneous fluorescence labelling of samples for the presence of breast cancer cells and apoptotic cells. The long and detailed development of the dual fluorescence labelling assay was undertaken in order to optimise the detection of apoptotic breast cancer cells in the bone marrow. The tumour cell label initially used was the FITC-conjugated BerEP4 antibody. This antibody was raised against the human epithelial antigen, present on the surface and in the cytoplasm of epithelial cells, and has been shown to be useful in identifying cells of epithelial origin such as breast cancer cells (Latza U, 1990). This was combined with the *in situ* nick translation assay for apoptosis and cells positive for both fluorescent labels were identified as apoptotic breast cancer cells. The dual labelling method proved to be effective in identifying apoptotic MCF-7 cells loaded in to peripheral blood. Interestingly, when paired patient samples were analysed using this assay, no antibody-positive cells were identified. The assay did however demonstrate an overall increase in apoptosis after bisphosphonate treatment in all the bone marrow cell populations in 4 out of 5

patients. The reasons for the lack of detection of antibody positive cells were unclear. It is possible that the bone marrow aspirates were taken from sites that were not directly related to a site of metastasis and therefore no tumour cells were present in the sample. Alternatively, the BerEP4 antibody may not have been sensitive enough to identify very low numbers of breast cancer cells in the bone marrow. It is also possible that surface antigens may have been lost from the breast cancer cells during apoptosis and it was therefore not possible to identify apoptotic tumour cells with this antibody.

To address the potential concerns about the labelling of tumour cells, the BerEP4 antibody was substituted in the antibody labelling protocol and the dual fluorescence labelling protocol with the FITC-conjugated MNF116 antibody. This antibody has been raised to a panel of cytokeratins (5, 6, 8, 17, 19) (Moll R, 1982) and has been widely used in the identification of tumours cells of epithelial origin. The flow cytometric analysis of peripheral blood spiked with MCF-7 cells showed that this antibody was able to detect breast cancer cells in a mixed cell population. The assay was able to distinguish tumour cells from other cell populations with a sensitivity of approximately 1%. When patient samples that had been examined with the previous assay were re-analysed using the MNF116 antibody, there were clear increases in the proportions of antibody positive cells in 2 out of 3 patients. This suggests that the BerEP4 antibody was not as effective at detecting tumour cells as the MNF116 antibody and that further samples should be analysed by a dual fluorescence labelling assay that incorporated the latter rather than the former antibody.

The analysis of 9 further paired patient bone marrow samples with the modified dual labelling assay demonstrated that, firstly, antibody positive cells were identifiable in 33% of patient samples. Although all bone marrow aspirates were taken from the posterior iliac crests, the absolute proportions of tumour cells varied greatly between samples and between patients. This

observation possibly reflected the fact that the aspirate sites did not necessarily correspond to sites of bone lesions apparent on radiographs and may also be related to the varying degrees of metastatic tumour burden in the skeleton of individual patients.

The results from the first five paired patient samples analysed with the dual labelling technique demonstrated an overall increase in apoptosis in all cell populations after intravenous bisphosphonate treatment in 80% of patients (4/5). Intriguingly, this observation was not supported by analysis of subsequent paired samples, which showed little or no change in apoptosis between pre-and post-treatment samples. The differences between these two sets of samples may be accounted for by the fact that, in the first five patients, bone marrow aspirates were taken from the same site both pre- and post-treatment. In subsequent patients, aspirates were taken from alternate iliac crests to avoid the influence of local trauma-related changes. Therefore, it is possible that the initial increases seen in apoptosis in the whole bone marrow cell population may have occurred as a result of a response to local trauma caused by the pre-treatment biopsy.

The comparison of the proportions of apoptotic breast cancer cells in the bone marrow of breast cancer patients did not reveal an increase in tumour cell death following bisphosphonate treatment. There may be several reasons for this. Firstly, the timing of the post-treatment bone marrow aspirate at 72 hours after treatment may mean that any apoptotic breast cancer cells present in the bone marrow microenvironment may have already been phagocytosed and disposed of by local macrophages. Alternatively, as mentioned previously, the biopsy site may not coincide with a metastatic site and therefore sufficient numbers of tumour cells to be detected by the assay may not have been sampled. There are methods available to concentrate tumour cells into a smaller volume and hence aid detection, such as immunomagnetic techniques (Diel IJ & RJ., 2000). These generally involve the use of microspheres coated with antibodies that recognise

tumour cell antigens and bind to them. The tumour cell-microsphere complexes are then separated using a magnet. These techniques have been used in purging bone marrow stem cell grafts of tumour cells (Franklin WA *et al.*, 1996; Ross AA, 1993) and have also proven effective in allowing the sensitive detection and characterisation of carcinoma cells in peripheral blood (Racila E, 1998). Other methods of tumour cell detection include immunhistochemistry and molecular biological techniques, such as DNA- and RT-PCR. The latter techniques have proven to be highly sensitive but can give false positive results due to factors such as contamination and detection of specific genes in non-specific cells (Ghossein RA, 1999). Quantitative PCR methods may allow for a 'background' level of expression to be identified and therefore may alleviate these problems somewhat (Slade MJ, 1999). Immunohistochemistry techniques can also very sensitively detect tumour cells using specific antibodies. Again, the problems of cross-reactivity and false positives remain. However these techniques have the added advantage of enabling morphological characterisation of antibody-positive cells. Indeed, new automated techniques, such as the Chromovision ACIS (automated cellular imaging system), are highly sensitive and can detect approximately 1 epithelial tumour cell per 1 million mononuclear cells (Osborne MP, 1991).

In any case, it is likely that the effects of bisphosphonate treatment on the bone marrow may be far more subtle than outright cell death. It is possible that the effects of bisphosphonates such as inhibition of breast cancer cell adhesion and invasion (Boissier, 1997; Magnetto S, 1999; Van der Pluijm G, 1996; (Boissier S, 2000) may be more prominent than the induction of tumour cell apoptosis. In addition, there may be subtle changes induced by bisphosphonates in the bone marrow microenvironment that may play a part in inhibiting breast cancer cell growth and proliferation.

In summary, the dual fluorescence labelling technique described in this chapter is a useful tool for the identification of apoptotic breast cancer cells in bone marrow aspirate samples. The analysis of bone marrow samples from patients with breast cancer-induced bone disease did not demonstrate a clear anti-tumour effect of bisphosphonate treatment *in vivo,* as sample numbers were very small. However, intravenous bisphosphonate therapy may have more subtle effects on the bone marrow microenvironment and it would be pertinent to study these *in vivo* changes more closely.

CHAPTER 6

THE EFFECTS OF INTRAVENOUS BISPHOSPHONATE TREATMENT ON CYTOKINES AND GROWTH FACTORS IN PATIENTS WITH BREAST CANCER AND BONE METASTASES.

6.1 SUMMARY

The data presented so far in this thesis suggest that bisphosphonates may have direct anti-tumour actions *in vitro* and possibly *in vivo*. However, as potent inhibitors of osteoclast-mediated bone resorption, bisphosphonates may also modulate the bone marrow microenvironment by inhibiting the release of growth factors from the cells of bone and also bone matrix. The aims of the study presented in this chapter were, firstly, to identify the differences in growth factor levels between patients with early breast cancer and those with bone metastases and, secondly, to investigate the effect of intravenous bisphosphonate treatment on cytokines and growth factors present in the local bone marrow microenvironment.

19 patients with breast cancer and bone metastases were recruited to the study along with 13 patients with primary breast cancer. Samples of serum, urine and bone marrow were taken from the advanced group before and three days after intravenous treatment with pamidronate (90mg) or zoledronic acid (4mg and 8mg) and from the primary breast cancer group on a single occasion. Serum and bone marrow samples were assayed for the presence of a panel of cytokines and growth factors that may be produced by the cells of bone or present in the bone matrix, including TGFβ-1, IGF-1, FGF-2, VEGF, IL-6 and soluble IL-6 receptor (sIL-6R). Urine samples were assayed for the marker of bone resorption, N-telopeptide (NTX).

Samples of bone marrow plasma from patients with advanced breast cancer had significantly higher levels of TGFβ–1, with higher serum levels of IL-6 and soluble IL-6 receptor than those from patients with primary breast cancer ($p<0.05$). In the advanced breast cancer group, no changes were seen in IGF-1, IL-6 or TGFβ-1 levels 3 days after bisphosphonate treatment. However, treatment did result in significantly lowered levels of serum FGF-2 and bone marrow VEGF ($p<0.05$).

There appear to be differences in certain cytokines and growth factors between patients with advanced and primary breast cancer. The beneficial effects of bisphosphonates experienced by patients with breast cancer and bone metastases may be due to alterations in cytokines and growth factors, such as FGF-2 and VEGF that are important in bone.

6.2 INTRODUCTION

Bone resorption by osteoclasts is a complex process that is influenced by many factors, including cytokines and cellular products, to maintain normal bone homeostasis (Mundy, 1997, Guise T, 1998). The arrival of metastatic breast cancer cells in the bone appears to have profound effects on this process, ultimately resulting in highly accelerated bone breakdown. Complex interactions seem to occur between the tumour cells and the bone microenvironment. Breast cancer cells are known to express and produce soluble factors, such as PTHrP (Ogata, 2000; Powell G, 1991), (Birch MA, 1995; Carron J, 1997) which are potent stimulators of osteoclast maturation and activity (Guise T, 1996; Iezzoni J, 1998). There may also be direct cell-cell interactions between the breast cancer cell and the osteoclast which augment bone resorption. A possible candidate for this is the RANK-RANKL-OPG system. In any case, the increased rate of bone breakdown in metastatic breast cancer may cause the release of bone matrix products, such as TGF-β, bFGF and IGF's, into the bone microenvironment. These factors have been shown in experimental studies to influence the growth and proliferation of breast cancer cells or to stimulate the production of further soluble mediators that enhance osteoclast activity (reviewed by (Guise T, 1998)). In this way, a loop forms between tumour-derived factors which stimulate increased osteoclast activity and the accelerated release of cytokines and growth factors from the bone matrix which serve to enhance tumour cell proliferation.

Bisphosphonates are potent inhibitors of osteoclast-mediated bone resorption. The work presented so far in this thesis demonstrates that these compounds can have direct anti-tumour effects on breast cancer cells *in vitro* although it is less clear whether this occurs

in vivo. Another potential consequence of bisphosphonate-induced inhibition of bone resorption is a reduction in bone-derived growth factors available to tumour cells and alterations in the bone marrow microenvironment that may inhibit tumour cell proliferation. The aim of the work presented in this chapter is to investigate the effects of a single treatment with intravenous bisphosphonate on cytokines and growth factors in the bone marrow microenvironment of patients with advanced breast cancer. In addition, the differences in these factors between patients with early breast cancer and those with advanced breast cancer and bone metastases were investigated.

6.3 EXPERIMENTAL DESIGN

6.3.1 Patients

19 postmenopausal women with breast cancer-induced bone metastases attending the Outpatients Department at Weston Park Hospital, Sheffield, UK were recruited for this study. There was no significant difference in ages between the two groups. The only concurrent treatment allowed was long-term hormone therapy and these patients were bisphosphonate-naïve. A group of postmenopausal patients with primary breast cancer were recruited from the Department of Surgery, Royal Hallamshire Hospital, Sheffield. The characteristics of the patients in the study are shown in Table 6.1. Ethics approval was gained from the South Sheffield Ethics Committee and all patients provided written informed consent.

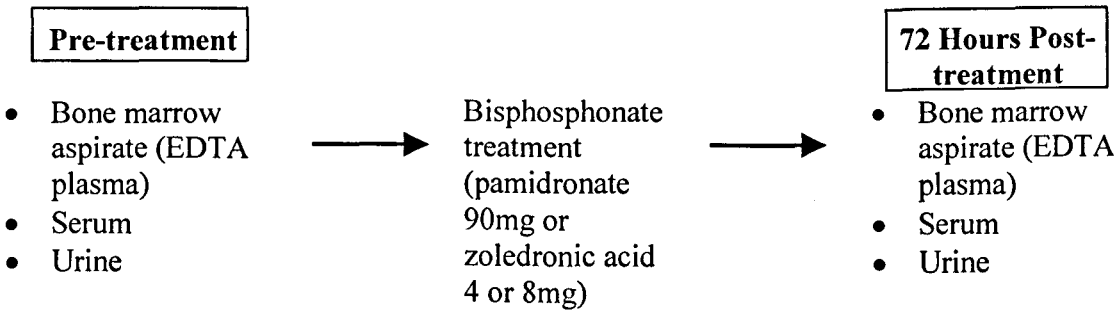
	Group 1 (primary breast cancer) n=13	**Group 2 (advanced breast cancer)** n=19
Mean Age ± SD	59.3 ± 13 years	51.7 ± 8 years
Previous Chemotherapy	-	2/19 (10.5%)
Previous endocrine therapy	-	12/19 (63.1%)
Bisphosphonate received:	-	
Pamidronate		11/19 (57.8%)
Zoledronic acid		8/19 (42.2%)
Baseline NTX (nM/mM of creatinine)	50.52 + 4.3	164.44 ± 20.3

Table 6.1 Baseline characteristics of patients entered into the study.

6.3.2 Study design

Samples were taken from patients according to the following study design:

ADVANCED BREAST CANCER PATIENTS



PRIMARY BREAST CANCER PATIENTS

- Bone marrow aspirate (EDTA plasma)
- Serum
- Urine

] On a single occasion

6.3.3 Sample preparation

Peripheral blood clotted samples were centrifuged at 2000 x g for 10 minutes, the serum collected and frozen at -80^0C for later analysis. Bone marrow samples were processed as described in section 5.3.5.4.

6.3.4 Analysis of cytokines/growth factors.

Bone marrow plasma samples for 5 patients were sent for analysis of PTHrP levels (data not shown) and were therefore not available to be assayed for any other growth factors. In two further patients, bone marrow sampling yielded a dry aspirate in the pre-treatment sample hence a total of 12 patients were analysed for the presence of bone marrow factors

listed in table 6.2. Serum and bone marrow plasma samples were analysed by the enzyme-linked immunosorbant assay (ELISA) technique for a panel of cytokines and growth factors (Table 6.2). Urine was analysed using an automated chemi-luminescence assay (Vitros ECI analyser), at the Northern General Hospital, Sheffield, UK for the presence of N-telopeptide molecules (NTX) as markers of bone resorption.

Cytokine/ Growth Factor	Assay Kit/ Source	Assay Range (pg/ml)	Measured in serum	Measured in bone marrow plasma
TGF-β1	R&D Systems, Oxon, UK	7-2000	✓	✓
IGF-1	Immunodiagnostic Systems (IDS), UK	10-1000	✓	✓
bFGF (FGF-2)	R&D Systems, Oxon, UK	0.2-64	✓	✓
VEGF	R&D Systems, Oxon, UK	5-1000	✓	-
IL-6	Amersham Pharmacia, UK	0.1-20	✓	-
sIL-6R	Sigma Chemical Co, UK[a]	15-2000	✓	-
sgp130	Diaclone, Cedex, France	15-1800	✓	-
IFN-γ	Diaclone, Cedex, France	5-400	✓	-

Table 6.2 Sources and assay range of ELISA kits used for the analysis of patient serum and bone marrow samples.
[a] Sandwich ELISA assay developed in house.

6.3.5 Statistical analysis

Statistical analysis was performed using SPSS Version 10.00. As the patient numbers were relatively small, the data for each parameter were tested for normality. The data for IL-6 and b-FGF were found to be non-parametric. Therefore the differences between the primary and advanced breast cancer groups and the pre- and post-treatment levels were analysed using the Mann-Whitney-U and the Wilcoxon Matched Pairs tests, respectively. The data for all the other parameters were found to be normally distributed. In these cases, the differences in cytokine levels between advanced and primary breast cancer groups were analysed using the independent samples t-test and differences between pre- and post-treatment cytokine levels in the advanced breast cancer group were analysed using paired samples t-test.

6.4 RESULTS

6.4.1 Differences in serum levels of cytokines and growth factors between patients with primary and advanced breast cancer.

The analysis of serum samples from patients with primary and advanced breast cancer revealed differences in levels of certain cytokines and growth factors (Table 6.3). Patients with bone metastases had higher levels of TGFβ–1 (5.14 ± 0.9 pg/ml), and IGF-1 (115.45 ± 12.12 pg/ml) compared with primary breast cancer patients (2.91 ± 0.9 pg/ml and 110.50 ± 7.4 pg/ml respectively) although these differences did not achieve statistical significance. Similarly, in patients with advanced breast cancer, the levels of b-FGF (7.83 ± 4.27 pg/ml), VEGF (165.63 + 39.81 pg/ml), and sgp-130 (219.70 ± 36.0 μg/ml) were elevated in comparison to the primary breast cancer group (3.80 ± 2.5 pg/ml, 184.91 + 35.9 pg/ml, and 186.97 ± 19.5 μg/ml) although, again, these differences were not statistically significant. Importantly, however, there were significant differences in the levels of IL-6 and sIL-6R between the two groups. IL-6 levels were significantly higher in the advanced breast cancer group (4.72 ± 1.2 pg/ml) than in patients with primary breast cancer (1.31 ± 0.6 pg/ml) ($p<0.05$). There were also markedly higher levels of sIL-6R in patients with bone metastases (260.61 ± 24.8 pg/ml) than in those with early breast cancer (153.08 ± 16.5 pg/ml) ($p<0.005$). No interferon gamma (IFN-γ) was detected in these samples.

6.4.2 Differences in bone marrow levels of growth factors between patients with primary and advanced breast cancer.

Patients with bone metastases had significantly higher levels of TGFβ–1 than those with primary breast cancer (6.85 ± 0.7 pg/ml and 3.56 ± 0.9 respectively) ($p<0.05$) (Table 6.3). They also had higher levels of IGF-1 and b-FGF than the primary breast cancer group (82.88 ± 14.55 pg/ml and 126.49 ± 12.5 pg/ml compared with 56.19 ± 6.27 pg/ml and 110.17 ± 4.4 pg/ml respectively) although these differences were not statistically significant. Furthermore, levels of VEGF in the advanced breast cancer group were significantly higher than those in patients with primary breast cancer (217.3 ± 10.62 vs. 175.56 ± 38.4 pg/ml respectively) ($p<0.01$).

	Group 1 (primary breast cancer)		Group 2 (advanced breast cancer)	
	Serum	**Bone marrow plasma**	**Serum**	**Bone marrow plasma**
TGF-β1	2.91 ± 0.9	3.56 ± 0.9	5.14 ± 0.9	6.85 ± 0.7†
IGF-1	110.50 ± 7.4	56.19 ± 6.27	115.45 ± 12.12	82.88 ± 14.55
bFGF (FGF-2)	3.80 ± 2.5	110.17 ± 4.4	7.83 ± 4.27	126.49 ± 12.5
VEGF	184.91 + 35.9	175.56 ± 38.4	165.63 ± 39.81	217.3 ± 10.62†† [a]
IL-6	1.31 ± 0.6	X	4.72 ± 1.2*	X
sIL-6R	153.08 ± 16.5	X	260.61 ± 24.8**	X
sgp130 (μg/ml)	186.97 ± 19.5	X	219.70 ± 36.0	X
IFN-γ	X	X	X	X

Table 6.3 Serum and bone marrow cytokine and growth factor levels (pg/ml) in patients with primary (Group 1) and advanced (Group 2) breast cancer.
* $p<0.05$ when group 1 compared with group 2 (serum)
** $p<0.005$ when group 1 compared with group 2 (serum)
† $p<0.05$ when group 1 compared with group 2 (bone marrow)
†† $p<0.005$ when group 1 compared with group 2 (bone marrow)
[a] n=4
X Not detected

6.4.3 Effect of intravenous bisphosphonate treatment on serum levels of cytokines and growth factors in patients with advanced breast cancer.

Serum samples taken before and three days after intravenous bisphosphonate treatment were analysed for the presence of cytokines and growth factors (figure 6.1). Bisphosphonate treatment resulted in significantly lower levels of b-FGF in post-treatment samples compared with pre- treatment (42.82 $\pm$ 14.4% of baseline) ($p<0.05$). Mean post-treatment serum levels of b-FGF were lower than those in the primary breast cancer group (1.59 + 0.3 vs. 3.8 + 2.5 pg/ml respectively). VEGF levels were also lower in post–treatment samples (95.04 $\pm$ 19.2 % of baseline), although not significantly. Levels of IGF-1 and TGFβ-1 appeared to increase with bisphosphonate treatment (114.74 $\pm$ 13.7 and 205.60 $\pm$ 80.57 % of baseline respectively) although statistical significance was not achieved. Bisphosphonate treatment did not result in significant changes in serum levels of IL-6 and sIL-6R, (116.45 $\pm$ 65.3% of baseline and 94.51 $\pm$ 6.0 % baseline respectively). In contrast, sgp-130 levels showed a tendency to increase after treatment (480.85 $\pm$ 344.17 % of baseline).

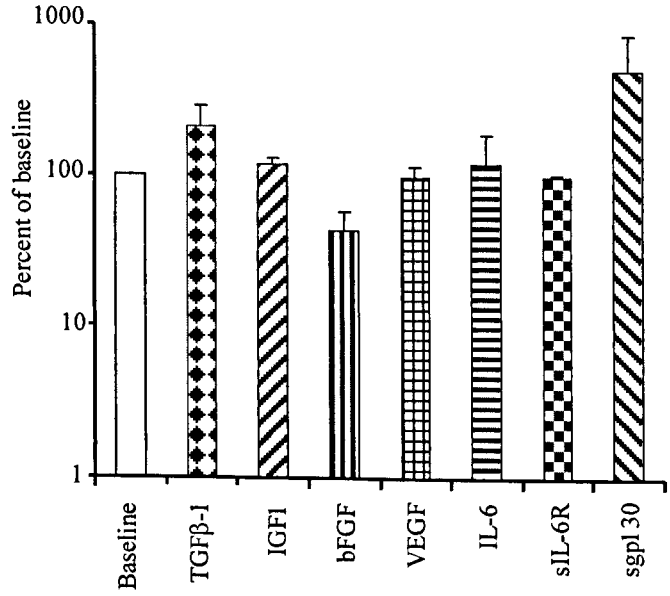


Figure 6.1 Levels of a panel of cytokines and growth factors in serum of patients with advanced breast cancer and bone metastases. Samples were taken before and three days after intravenous treatment with either pamidronate (90mg) or zoledronic acid (4mg or 8mg) and analysed using the ELISA technique. Results are expressed as percent of baseline (pre-treatment level) for each parameter and are shown as the mean $\pm$ SEM. * $p<0.05$

The values for b-FGF detected in the serum of individual patients are shown in figure 6.2 below.

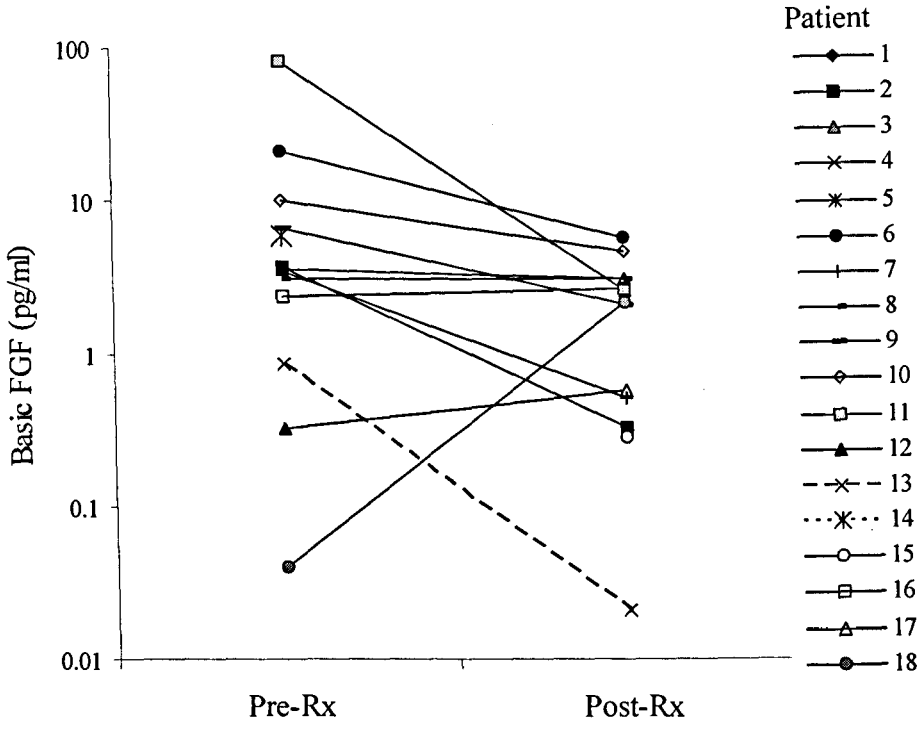


Figure 6.2 Serum levels of b-FGF (FGF-2) in individual patients 1-18 with breast cancer bone metastases before and 3 days after treatment with either pamidronate (90mg) or zoledronic acid (4mg or 8mg). Samples were analysed using the ELISA technique. Results are expressed as pg/ml of b-FGF pre- and post-treatment.

6.4.4 Changes in growth factor levels in bone marrow plasma from patients receiving intravenous bisphosphonate treatment.

Samples of bone marrow plasma were taken from patients with advanced breast cancer before and three days after intravenous bisphosphonate treatment and analysed for the presence of growth factors (figure 6.3). Levels of TGF-β1 and IGF-1 showed a tendency to increase after therapy with bisphosphonates (136.41 $\pm$ 43.4% and 114.73 + 14.8 % of baseline respectively). In contrast, b-FGF appeared to decrease following treatment with bisphosphonates (84.98 + 14.7 % of baseline) as did VEGF (71.77 + 23.2 % of baseline, ($n=4$, $p<0.05$).

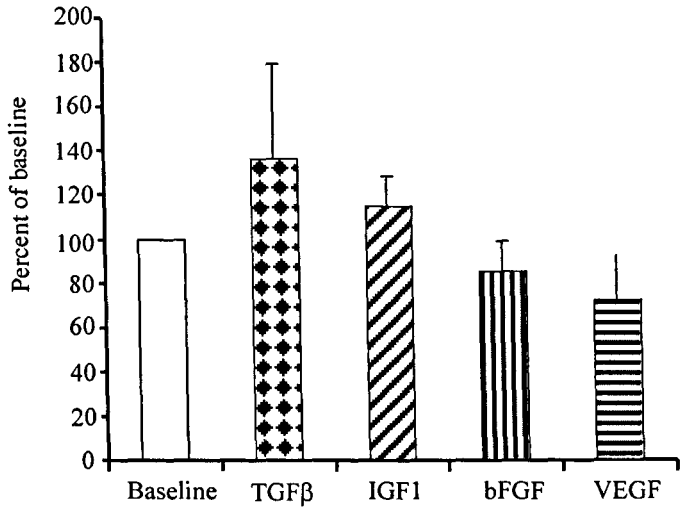


Figure 6.3 Levels of a panel of cytokines and growth factors in bone marrow plasma of patients with advanced breast cancer and bone metastases. Samples were taken before and three days after intravenous treatment with either pamidronate (90mg) or zoledronic acid (4mg or 8mg) and analysed using the ELISA technique. Results are expressed as percent of baseline (pre-treatment level) for each parameter and are shown as the mean ± SEM.
* $p<0.05$

The values for VEGF detected in the bone marrow of individual patients (n=4) are shown in figure 6.4 below.

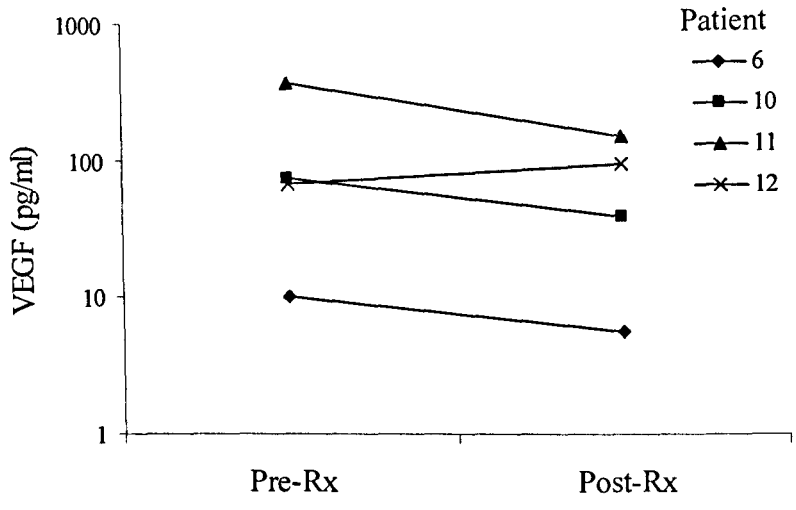


Figure 6.4 Bone marrow plasma levels of VEGF in individual patients 6, 10, 11 and 12 with breast cancer bone metastases before and 3 days after treatment with either pamidronate (90mg) or zoledronic acid (4mg or 8mg). Samples were analysed using the ELISA technique. Results are expressed as pg/ml of VEGF pre- and post-treatment.

6.4.5 Differences in clinical picture between patients with detectable tumour cells and those without.

The work outlined in chapter 5 demonstrated that tumour cells were detectable in 33% of patients. The presence of breast cancer cells in the bone marrow as detected by the dual labelling method described in Chapter 5 appeared to relate to the clinical picture (Table 6.5). 100% of patients in whom tumour cells were detectable experienced bone pain on presentation compared with 77.7% of patients (7/9) without detectable tumour cells. Approximately equal proportions of patients in each group experienced a symptomatic response to bisphosphonate treatment (60% vs 55.5%). Interestingly, a higher proportion

of patients with detectable breast cancer cells experienced skeletal related events (40%) compared with those in whom breast cancer cells were not detected (22.2%).

Breast cancer cells detected	Bone pain at presentation	Symptomatic response	Skeletal related events
Yes (n=5)	100%	60%	40%
No (n=9)	77.7%	55.5%	22.2%

Table 6.5 Relationship between the presence of breast cancer cells detectable by the dual labelling assay and clinical features.

6.5 DISCUSSION

The present study set out to identify firstly whether there were differences in serum and bone marrow cytokines and growth factors between patients with breast cancer induced bone metastases and those with early breast cancer but no evidence of spread. Secondly, we investigated the effects of intravenous treatment with either pamidronate or zoledronic acid on cytokines and growth factors in the bone marrow microenvironment.

There was no significant difference in age between patients with primary breast cancer and those with advanced breast cancer and bone metastases. Predictably, patients with bone metastases had much higher concentrations of urinary N-telopeptide than those with early breast cancer, indicating accelerated bone resorption due to the presence of bone secondaries. Of the advanced breast cancer group, 73.6% had received previous treatment for their breast cancer either in the form of chemotherapy or endocrine therapy. In this group, 57.8% received intravenous pamidronate (90mg) and 42.2% received zoledronic acid (4mg or 8mg). There was no significant difference in cytokine response between patients that had received pamidronate and those that had received zoledronic acid therefore the data from these two groups were pooled.

The study demonstrated that all of the bone-derived factors were increased in the bone marrow plasma in patients with advanced breast cancer and bone metastases although the differences were only significant in the cases of TGFβ-1 and VEGF. TGFβ-1, produced predominately by osteoblasts, (Bonewald LF & GR, 1990) is present in abundance in the bone matrix and is released on osteoclast-mediated bone resorption (Hauschka P, 1986;

Pfeilschifter J, 1987). Recent studies suggest that tumour cells themselves may secrete TGFβ (Pederson L, 1999) and this growth factor, along with related peptides, has been shown to influence a range of cellular processes including proliferation, differentiation, motility and death (reviewed by Massague) (Massague, 1998). Although there is evidence to suggest that TGFβ–1 inhibits growth of mammary carcinoma cells (Pierce DF, 1995; (Sun L, 1994) it has been postulated that TGFβ may also stimulate breast cancer cell growth (Arteaga CL, 1996)and may even induce a more metastastic phenotype (Diudenvoorden W, 1999; Farina A, 1998; Yin J, 1999). Furthermore, in metastatic bone disease, the release of TGFβ into the bone microenvironment by the accelerated activity of osteoclasts may further enhance the evolution of the metastasis by promoting the production of PTHrP by the carcinoma cells (Guise T, 1996; Guise T, 1993; Yin J, 1999). This in turn potently stimulates osteoclast maturation and activity (Guise T, 1996; Iezzoni J, 1998) and bone destruction is further enhanced. The findings from our study that bone marrow levels of TGFβ are significantly elevated in patients with bone metastases, when compared to those without, supports the hypothesis that accelerated bone resorption influenced by breast cancer cells results in the release of bone matrix products into the microenvironment to perpetuate this cycle. Alternatively, TGFβ may be produced by the tumour cells themselves in the bone microenvironment.

Patients in this study with advanced breast cancer and bone metastases were found to have significantly higher serum levels of IL-6 and sIL-6R than those with early breast cancer. This supports previous observations of elevated serum levels of IL-6 and sIL-6R (Jablonska, 1998)in breast cancer patients compared with normal controls and the

correlation with more aggressive disease (Haverty A, 1997; Jiang X, 2000; Yokoe T, 1997). IL-6 has been shown to be secreted by mammary carcinoma cells (Chiu J, 1996; Pederson L, 1999) as well as other tumour types, particularly multiple myeloma (reviewed by Guise and Mundy (Guise T, 1998) and Klein et al (Klein B, 1995) and normal bone marrow stromal cells (Boyce B, 1999; Mohan S, 1991). In multiple myeloma, this cytokine acts as important growth regulator in its ability to support myeloma cell proliferation (Kawano M, 1988) and indeed the stage of advancement of the disease has been shown to correlate with patient serum and bone marrow IL-6 concentrations (Bataille R *et al.*, 1992; Bataille R, 1989). In contrast, IL-6 has been shown to result in growth inhibition in mammary carcinoma cells (Novick D, 1992) and it has also been suggested that this cytokine may regulate the growth of normal and transformed human mammary epithelial cells differentially (Chiu J, 1996; Lai L, 1994). Interestingly, IL-6 has been implicated in the stimulation of osteoclast precursors, possibly through the activity of other factors such as PTHrP, which in turn would accelerate bone resorption (de la Mata J, 1995). The soluble form of the Il-6 receptor (sIL-6R) binds IL-6 and the complex interacts with the membrane-bound receptor, gp130, to stimulate signalling and enhance IL-6 effects. Bisphosphonate treatment did not significantly alter serum concentrations of either IL-6 or sIL-6R, suggesting that inhibition of bone resorption, or direct bisphosphonate action, did not affect release of these cytokines from cells such as monocytes, macrophages and granulocytes.

Intriguingly, we demonstrated that serum concentrations of b-FGF were significantly reduced from baseline after bisphosphonate treatment. Serum VEGF levels showed a

similar change with treatment. Likewise, concentrations of VEGF and b-FGF in bone marrow plasma showed similar trends with a reduction after treatment with intravenous bisphosphonate. Both of these factors have been implicated in tumour dissemination and angiogenesis and VEGF is constitutively produced by many tumours, including breast cancer cells as well as osteoblasts (reviewed by Van Der Pluijm) (Van Der Pluijm G, 2000). FGF-2 (b-FGF) is produced by osteoblastic cells, stored in the bone matrix, and released on bone resorption therefore the inhibition of bone resorption with bisphosphonate treatment may well result in a reduction in serum and bone marrow levels. In addition, there is evidence to suggest that b-FGF may dose-dependently induce the expression of VEGF by osteoblasts indicating that the activity of the two factors may be closely linked (Saadeh P, 2000). Also, the potent bisphosphonate zoledronic acid has been shown to inhibit endothelial cell proliferation *in vitro* and b-FGF–induced angiogenesis *in vivo* (Wood J, 2002), suggesting this as an alternative mechanism for the beneficial effects of bisphosphonates in patients with solid tumour bone metastases.

Overall, the data presented here suggests that there are differences in the bone marrow microenvironment between patients with early breast cancer and those with advanced breast cancer and bone metastases that relate to accelerated bone resorption stimulated by breast cancer cells. In patients with bone metastases, intravenous bisphosphonate treatment may modulate factors that are important in tumour cell migration and angiogenesis. These observations are made at a single point in time, 3 days after bisphosphonate treatment and further important information about the effects of these compounds on the bone microenvironment may be gained by repeating these studies at

later time points. However, these data may provide some insight into the mechanism via which bisphosphonates may exert effects in breast cancer-induced metastatic bone disease.

CHAPTER 7

GENERAL DISCUSSION

Bisphosphonates have become important agents in the management of bone metastases in breast cancer. The clear clinical benefits on skeletal morbidity have been highlighted by several studies and their use has become well established for breast cancer-induced metastatic bone disease as well as in multiple myeloma (Berenson JR *et al.*, 1998; Diel IJ *et al.*, 1998; Hortobagyi GN *et al.*, 1996; McCloskey *et al.*, 1998; Paterson AHG *et al.*, 1993; Theriault RL *et al.*, 1999). Breast cancer cells are found in close proximity to sites of active bone resorption and may well be exposed to high concentrations of bisphosphonates. The inhibition of bone resorption by these compounds may result in indirect effects on breast cancer cells due to reduced release of growth factors into the bone microenvironment. However, the observation that bisphosphonate treatment may also confer a survival benefit in breast cancer patients, (Diel IJ *et al.*, 1998) as well as influencing skeletal events, raises the intriguing possibility that these compounds may have direct anti-tumour effects. This is further hinted at in studies using animal models of breast cancer in which apoptosis of mammary carcinoma cells occurs after administration of bisphosphonate treatment. The aim of this PhD was to determine whether bisphosphonates have direct anti-tumour effects on breast cancer cells.

The initial aim of the work presented here was to determine whether zoledronic acid had anti-tumour effects on breast cancer cells *in vitro*. We demonstrated that treatment with zoledronic acid resulted in a time-dependent and concentration dependent reduction in cell number and induction of apoptosis in two mammary carcinoma cell lines (Chapter 3). These observations are supported by recent reports showing similar effects of nitrogen-containing bisphosphonates on breast cancer cell proliferation and apoptosis (Fromigue

O, 2000; Hiraga T, 2001; Senaratne SG, 2000) as well as on multiple myeloma cells (Aparicio A *et al.*, 1998; Shipman CM *et al.*, 1997). Our studies showed that the effects on cell number and apoptosis were as marked after short term exposure (2-24 hours) to zoledronic acid as after longer term exposure up to 72 hours (Chapter 3). This is an important observation in view of the fact that bisphosphonates have a very high affinity for mineralised bone and localise to bone rapidly, thus in a clinical setting it is likely that primary breast cancer cells or those in visceral metastases may be exposed to these compounds for shorter periods. Indeed, peak serum concentrations are likely to be in the range of 1-3μM and maintained for only a few hours (Berenson JR *et al.*, 2000). This is consistent with observations from a study in J774 macrophages which demonstrated a time-dependant increase in caspase-3-like enzyme activity, associated with apoptosis, when acutely exposed to nitrogen-containing bisphosphonates (Coxon FP *et al.*, 1998)

The mechanism of action of nitrogen-containing bisphosphonates has only recently been elucidated. Studies have shown that these compounds, in contrast to clodronate, induce apoptosis by inhibiting enzymes of the mevalonate pathway in cells such as macrophages, (Luckman SP *et al.*, 1998) osteoclasts (Fisher JE, 1999) and multiple myeloma cells (Shipman CM *et al.*, 1998) and hence prevent protein prenylation. The exact target of amino-bisphosphonates has recently been identified as the enzyme, farnesyl pyrophosphate synthase (Van Beek E, 1999a). A further aim of the studies presented in Chapter 3 was to determine whether zoledronic acid acted on the same molecular target in breast cancer cells. We demonstrated that breast cancer cells could be rescued from zoledronic acid-induced apoptosis by the addition of GGOH, a precursor of GGPP, which

is an intermediate of the mevalonate pathway. This indicated that zoledronic acid did indeed interfere with this pathway in breast cancer cells. In contrast to observations in macrophages (Luckman SP *et al.*, 1998) and multiple myeloma cells, (Shipman CM *et al.*, 1998) the addition of FOH (a precursor of FPP) did not significantly rescue breast cancer cells from zoledronic acid-induced apoptosis. This may indicate that, in this particular cell type, farnesylated proteins may play a less important part in cellular function than geranyl geranylated proteins. Indeed, similar observations have been made in osteoclasts, where geranyl geranylated proteins such as Rac and Rho may be essential for functions such as maintenance of the cytoskeleton and integrity of the ruffled border (Fisher JE, 1999).

Bisphosphonates are often used in combination with chemotherapy and endocrine treatment in everyday clinical practice. There are data suggesting that bisphosphonates may act synergistically with anti-neoplastic agents to affect tumour cells and survival, both in animal models (Yoneda T, 1999) and in clinical studies (Berenson JR *et al.*, 1998; Diel IJ *et al.*, 1998). The aim of the work presented in Chapter 4 was to investigate whether zoledronic acid and paclitaxel, a spindle toxin, had synergistic effects on breast cancer cells when combined. Our studies demonstrated that paclitaxel and zoledronic acid acted synergistically on breast cancer cells to induce apoptosis (Chapter 4). Previous studies have shown that bisphosphonates and taxanes have synergistic effects on breast cancer cells in other ways. Magnetto *et al* found that the combination of ibandronate with taxotere or taxol caused loss of breast cancer cell adhesion and invasion in an additive fashion (Magnetto S *et al.*, 1999). Interestingly, no additive effect was seen on apoptosis

at the low concentrations of bisphosphonates used in these studies. In contrast, studies by Yoneda *et al* in animal models of breast cancer have shown a greater reduction in both bony and visceral metastases when ibandronate was combined with a chemotherapy agent (Tegafur, an oral prodrug of 5-fluorouracil) than with either drug alone (Yoneda T, 1999).

The next aim of this PhD was to determine whether the anti-tumour effects observed in *vitro* could be translated to the *in vivo* patient setting. The first step in doing this was to develop an assay that could simultaneously detect breast cancer cells and measure apoptosis in these cells. This could then be used to identify the proportions of apoptotic cells in samples of bone marrow taken before and after intravenous bisphosphonate treatment from patient with breast cancer-induced bone metastases. We developed a flow cytometry-based assay, the dual fluorescence labelling assay, which combined the MNF116 antibody as a tumour cell label with the *in situ* nick translation assay to detect apoptosis (Chapter 5). Bone marrow samples taken before and three days after treatment with intravenous pamidronate or zoledronic acid were analysed using this technique. The assay positively identified breast cancer cells in 33% of patient samples. Interestingly, the patients in whom tumour cells were detected also appeared to have more clinically advanced disease and less alteration in serum and bone marrow cytokines and growth factors with bisphosphonate treatment. Unfortunately, the numbers of patients in whom breast cancer cells were detected in both pre- and post-treatment samples were too small to draw any conclusions about the effect of bisphosphonate treatment on *in vivo* tumour cell apoptosis. Similar studies in multiple myeloma patients have an increase in the

proportions of apoptotic myeloma cells in the bone marrow after intravenous pamidronate treatment (Gordon S, 2002). In contrast to haematological malignancies such as multiple myeloma, the proportions of breast cancer cells may in bone marrow samples may be very small unless a metastasis is directly sampled. The yield of positive tumour cells may be improved in several ways. One possibility would be to sample sites that are known radiographically to contain metastatic lesions. However, this approach raises ethical issues as these sites are often where the patient may experience pain. From a technical point of view, the method may be improved by using magnetic bead separation or similar techniques to isolate and concentrate breast cancer cells before labelling them. This has been used to good effect in previous studies looking at circulating or bone marrow solid tumour cells (Racila E, 1998). Other methods to allow detection of tumour cells include immunohistochemistry and PCR.

Bisphosphonates have been shown unequivocally to inhibit osteoclast-mediated bone resorption. In so doing, they may inhibit the release of important factors from the bone matrix and even influence the production of tumour derived factors. The final aim of this PhD was to determine the effect of intravenous bisphosphonate treatment on the bone microenvironment and circulating growth factors and cytokines *in vivo*. We found that there were significant differences between patients with primary breast cancer and those with bone metastases (Chapter 6). Samples from patients with bone metastases had higher levels of IL-6 and sIL-6R. This may reflect tumour cell burden and activity (Jablonska, 1998; Jiang X, 2000). In addition, patients with advanced disease had significantly higher concentrations of TGFβ-1, which may be an indication of accelerated release of this

factor as a result of bone breakdown. Interestingly, VEGF levels in the bone marrow plasma of patients with bone disease were significantly higher than in patients with primary breast cancer. Clinical studies have reported elevated serum concentrations in both primary breast cancer patients and those with metastatic disease. The increase in IGF-1, b-FGF and VEGF may reflect bone-derived factor production.

This clinical study also demonstrated that bisphosphonates appear to have selective effects on bone marrow and circulating factors (Chapter 6). We demonstrated that bisphosphonates result in a significant reduction in circulating bFGF and bone marrow VEGF levels (Chapter 6). Intriguingly, there has been recent evidence to suggest that aminobisphosphonates are potent inhibitors of angiogenesis both *in vivo* and *in vitro* (Wood J, 2002). It is possible therefore that these compounds may have a selective effect on the release of these factors in to the bone marrow microenvironment.

The observations that bisphosphonate treatment results in a reduction in tumour burden in animal models (Hiraga T, 1998) and a possible survival benefit in patients with breast cancer (Diel IJ *et al.*, 1998) raise the possibility that bisphosphonates have direct anti-tumour effects in breast cancer. The work presented in this thesis demonstrates that zoledronic acid does have anti-tumour effects on breast cancer cells *in vitro,* an effect that is mediated via the same molecular target as in macrophages, osteoclasts and multiple myeloma cells. There are also synergistic anti-tumour effects when zoledronic acid is combined with paclitaxel. The technique described in Chapter 5 is currently being used to evaluate the induction of tumour cell apoptosis in patient bone marrow samples before

and after intravenous bisphosphonate treatment. These continuing studies may provide valuable insight into whether bisphosphonates have direct anti-tumour effects on breast cancer cells *in vivo*.

The observations of direct anti-tumour effects *in vitro* and possibly *in vivo* have clear implications for the clinical use of bisphosphonates, particularly aminobisphosphonates. At the moment these compounds are being used mainly in the palliative setting in breast cancer but they may also be of benefit in the adjuvant setting, particularly if a survival benefit can be confirmed. The studies conducted to date using adjuvant clodronate in patients with primary breast cancer and high risk of recurrence show conflicting results in terms of the clinical benefits (Diel IJ *et al.*, 1998; Paterson AHG *et al.*, 1993; Saarto T *et al.*, 2001, Powles T *et al* 2002). Larger scale trials are needed to confirm the role of bisphosphonates in the adjuvant treatment of patients with primary breast cancer and are currently being planned.

The benefits of bisphosphonate treatment observed in patients with metastatic bone disease may be due to the indirect effects on the bone microenvironment demonstrated in Chapter 6, as well as the inhibition of osteoclast-mediated bone destruction. These indirect effects are likely to make a significant contribution to the overall efficacy of bisphosphonate treatment and may even be more influential in halting bone disease progression than any direct anti-tumour effects. It is anticipated that the ongoing clinical studies in patients with breast cancer-associated bone metastases will provide valuable insight into the action and future use of bisphosphonates in this disease.

REFERENCES

Case Records of the Massachusetts General Hospital (case no 27461), (1941). *N Engl J Med*, **22,** 789-791.

Abou-Samra AB, Juppner H., Force T, Freeman MW, Kong XF, Schipani E, Urena P, Richards J, Bonventra JV, Potts Jr JT, Kronenburg HM, Segre GV. (1992). Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci, USA*, **89,** 2732-2736.

Amin D, Cornell S., Gustafson S, Needle S, Ullrich J, Bilder G, Perrone M. (1992). Bisphosphonates used for the treatment of bone disorders inhibit squalene synthase and cholesterol biosynthesis. *J Lipid Res*, **33,** 1657-1663.

Amin D, Cornell S., Perrone MH, Bilder GE. (1996). 1-hydroxy-3-(methylpentylamino)-propylidene-1, 1-bisphosphonic acid as a potential inhibitor of squalene synthase. *Drug Res*, **46,** 759-762.

Anderson DM, Marakovsky E., Billingsley WL, Dougall WC, Tometski ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L. (1997). A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic cell function. *Nature*, **390,** 175-179.

Aparicio A, Gardner A, Tu Y, Savage A, Berenson J & A., L. (1998). *In vitro* cytoreductive effects on multiple myeloma cells induced by bisphosphonates. *Leukaemia*, **12,** 220-229.

Arends MJ, Morris RG, Wyllie AH. (1990). Apoptosis: the role of endonuclease. *Am J Pathol*, **136,** 593-608.

Arteaga CL, Dugger T, Hurd SD. (1996). The multifunctional role of transforming growth factor (TGF)-βs on mammary epithelial cell biology. *Breast Cancer Res Treat*, **38,** 49-56.

Aslakson C, Miller F. (1992). Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumour. *Cancer Res*, **52,** 1399-1405.

Badache A, Hynes N. (2001). Interleukin 6 inhibits proliferation and, in cooperation with an epidermal growth factor autocrine loop, increases migration of T47D breast cancer cells. *Cancer Res*, **61,** 383-391.

Baron, R. (1989). Molecular mechanism of bone resorption by the osteoclast. *Anatom Rec*, **224,** 317-324.

Bataille R, Chappard D & Klein B. (1992). The critical role of interleukin 6, interleukin-1β and macrophoage colony-stimulating factor in the pathogenesis of bone lesions in multiple myeloma. *Int J Clin lab Res*, **21**, 283-287.

Bataille R, Jourdan M., Zhang XG. (1989). Serum levels of interleukin-6, a potent myeloma cell growth factor, as a reflect of dyscrasias. *J Clin Invest*, **84**, 2008-2011.

Batson, O. (1940). The function of the vertebral veins and their role in the spread of metastases. *Ann Surg*, **112**, 138-149.

Behrens, J. (1993). The role of cell adhesion molecules in cancer invasion and metastasis. *Breast Cancer Res Treat*, **24**, 175-184.

Behrens J, Mareel M., Van Roy FM, Birchmeier W. (1989). Dissecting tumour cell invasion: epithelial cells acquire ivasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J Cell Biol*, **108**, 2435-2447.

Belch AR, Bergsagel D., Wilson K, O'Reilly S, Wilson J, Sutton D, Pater J, Johnston D, Zee B. (1991). Effect of daily etidronate on the osteolysis of multiple myeloma. *J Clin Onc*, **9**, 1397-1402.

Bellahcene A, Menard S., Bufalino R, Moreau L, Castronovo V. (1998). Expression of bone sialoprotein in primary human breast cancer is associated with poor survival. *Int J Cancer*, **69**, 350-353.

Berenbaum, M. (1989). What is synergy? *Pharmacol Rev*, **41**, 93-141.

Berenson J, Lichtenstein A, Porter L, Dimopoulos M, Bordoni R, George S, Lipton A, Keller A, Ballester O, Kovacs M, Blacklock H, Bell R, Simeone J, Reitsma D, Heffernan M, Seaman J & Knight R. (1996). Efficacy of pamidronate in reducing skeletal events in patients with advanced multiple myeloma. *New Engl J Med*, **334**, 488-493.

Berenson JR, Lichtenstein A, Porter L, Dimopoulos MA, Bordoni R, George S, Lipton A, Keller A, Ballester O, Kovacs M, BlacklockH, Bell R, Simeone JF, Reitsma DJ, Heffernan M, Seaman J & Knight RD. (1998). Long term pamidronate treatment of advanced multiple myeloma patients reduces skeletal events. *J Clin Oncol*, **16**, 593-602.

Berenson JR, Ravera C, Ma P, Deckert F, Sasaki Y, Saeki T, Takashima S, LoRusso P, Goodin S, Seaman J, Schran H & Zhou H. (2000). Population Pharmacokinetics (PK) of Zometa. *Proc Am Soc Clin Oncol.*

Birch MA, Carron J., Scott M, Fraser WD, Gallagher JA. (1995). Parathyroid hormone (PTH) / PTH-related protein (PTHrP) receptor expression and mitogenic responses in human breast cancer cell lines. *Br J Cancer*, **72**, 90-95.

Blagosklonny MV, Fojo T. (1999). Molecular effects of paclitaxel: myths and reality (a critical review). *Int J Cancer*, **83**, 151-156.

Blavier L, Delaisse J. (1995). Matirx metalloproteinases are obligatory for the migration of preosteoclasts to the developing marrow cavity if primitive long bones. *J Cell Sci*, **108,** 3649-3659.

Body JJ, Vandershueren B, Alexiou J, Piccart M, Dumont JC. (1995). Clinical and Radiological effects of pamidronate in breast cancer-induced osteolysis. *Support Care Cancer*, **3,** 355 (Abstr 80).

Boissier S, Ferreras M., Peyruchaud O, Magnetto S, Ebetino F, Colombel M, Delmas P, Delaisse J-M, Clezardin P. (2000). Bisphosphonates inhibit breast and prostate carcinoma invasion, an early event in the formation of bone metastases. *Cancer Res*, **60,** 2949-2954.

Bonewald LF & Mundy GR. (1990). Role of transforming growth factor-beta in bone remodelling. *Clin Orthop Rel Res*, **250,** 261-76.

Bonneterre J, Thurlimann B, Robertson JFR, Krzakowski H, Mauriac L, Karalewski P, Vergote I, Webster A, Steinberg M, von Euler M. (2000) Anastrazole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the Tamoxifen or Arimidex Randomised Group Efficacy and Tolerability study. *J. Clin .Onc* **18**: 3748-3757

Boonekamp P, Van der Wee-Pals L., Van Wijk-van Lennep M, Thesing C, Bijvoet O. (1986). Two modes of action of bisphosphonates on osteoclastic resorption of mineralised matrix. *Bone Miner*, **1,** 27-39.

Boyce B, Yoneda T., Guise T. (1999). Factors regulating the growth of metastatic cancer in bone. *Endocrine-related cancer*, **6,** 333-347.

Brooks S, Saunders D., Singhokowinta A. (1980). Relation of tumour content of estrogen and progesterone receptors with response of patients to endocrine therapy. *Cancer*, **46,** 2775.

Brufman, G. (1993). Prognostic factors affecting response to aminoglutethamide in advanced breast cancer. *Anticancer Res*, **13,** 1235.

Bundred NJ, Ratcliffe W., Walker RA, Coley S, Morrison JM, Ratcliffe JG. (1991). Parathyroid hormone-related protein and hypercalcaemia in breast cancer. *Br Med J*, **303,** 1506-1509.

Burtis WJ, Wu T, Bunch C, Wysolmerski JJ, Insogna KL, Weir EC, Broadus AE, Stewart AF. (1987). Indentification of a novel 17,000- dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumour associated with humoral hypercalcaemia of malignancy. *J Biol Chem*, **262,** 7151-7156.

Cailleau R, Young R, Olive M & Reeves WJ. (1974). Breast tumour cell lines from pleural effusions. *J Natl Cancer Inst*, **53,** 661-674.

Carano A, Teitelbaum S, Kosek J, Sclesinger P, Blair H. (1990). Bisphosphonates directly inhibit the bone resorption activity of isolated avian osteoclasts. *J Clin Invest*, **85,** 456-461.

Carron J, Fraser W, Gallagher J. (1997). PTHrP and the PTH/PTHrP receptor are co-expressed in human breast and colon tumours. *Br J Cancer*, **76,** 1095-1098.

Centrella M, Horowitz M., Wozney JM, McCarthy TL. (1994). Transforming growth factor β gene family members and bone. *Endocr Rev*, **15,** 27-39.

Chaplin DJ, Trotter D, Dougherty GJ. (1997). Microregional tumour blood flow: heterogeneity and therapeutic significance. In *Tumour Angiogensis*, Bicknell R, L.C., Ferrara N. (ed) pp. 61-71. Oxford University Press: Oxford.

Chiu J, Sgagias M., Cowan K. (1996). Interleukin 6 acts as a paracrine growth factor in human mammary carcinoma cell lines. *Clin Cancer Res*, **2,** 215-221.

Church MW, Dintcheff B., Gessner PK. (1988). The interactive effects of alcohol and cocaine on maternal and fetal toxicity in the Long-Evans rat. *Neurotoxicol Teratol*, **10,** 355-361.

Coleman, R. (1998). Mangement of bone metastases. *ASCO Fall Educational Book*, 16-26.

Coleman RE, Houston S., Purohit OP, Rubens RD, Kandra A, Ford J. (1998). A randomised phase II study of oral pamidronate for the treatment of bone metastases from breast cancer. *Eur J Cancer*, **34,** 820-824.

Coleman RE, Purohit O., Black C (1999). Doule-blind, randomised, placebo-controlled study of oral ibandronate in patients with metastatic bone disease. *Ann Oncol*, **10,** 311-316.

Coleman RE, Rubens R. (1987). The clinical course of bone metastases fron breast cancer. *Br J Cancer*, **55,** 61-66.

Coleman RE, Smith P., Rubens RD. (1988a). Clinical course and prognostic factors following recurrence from breast cancer. *Br J Cancer*, **17,** 336-340.

Coleman RE, Woll P., Miles M, Scrivener W, Rubens RD. (1988b). Treatment of bone metastases from breast cancer with (3-amino-1-hydroxypropydilene)-1, 1-bisphosphonate (APD). *Br J Cancer*, **58,** 621-625.

Conte PF, Mauriac L., Calabresi F (1996). Delay in progression of bone metastases treated with intravenous pamidronate: Results from a multicentre randomised controlled trial. *J Clin Onc*, **14,** 2552-2559.

Coxon FP, Benford HL, Russell RG & Rogers MJ. (1998). Protein synthesis is required for caspase activation and induction of apoptosis by bisphosphonate drugs. *Mol Pharmacol*, **54,** 631-638.

Dao T, Nemoto T. (1980). Steroid receptors and response to endocrine ablations in women with metastastic cancer of the breast. *Cancer*, **46,** 2779.

David P, Nguyen H., Barbier A, Baron R. (1996). THe bisphosphonate tiludronate is a potent inhibitor of osteoclast vacuolar H^+-ATPase. *J Bone Miner Res*, **11,** 1498-1507.

de la Mata J, Uy H., Guise T, Story B, Boyce B, Mundy G. (1995). Interleukin-6 enhances hypercalcaemia and bone resorption mediated by parathyroid hormone-related protein in vivo. *J Clin Invest*, **95,** 2846-2852.

Degenshein Q, Bloom N, Tobin E. (1980). The value of progesterone receptor assays in the management of advanced breast cancer. *Cancer*, **46,** 2789.

Derynk, R. (1994). TGF-β-receptor-mediated signaling. *Trends Biochem Sci*, **19,** 548-553.

Diel IJ & Cote RJ. (2000). Bone marrow and lymph node assessment for minimal residual disease in patients with breast cancer. *Cancer Treat Rev*, **26,** 53-65.

Diel IJ, Solomayer EF, Costa SD, Gollan C, Goerner R, Wallwiener D, Kaufmann M & Bastert G. (1998). Reduction in new metastases in breast cancer with adjuvant clodronate treatment. *New Engl J Med*, **339,** 357-363.

Diudenvoorden W, Hirte H, Singh G. (1999). Transforming growth factor β1 acts as an inducer of amtrix metalloproteinase expression and activity in human bone-metastasizing cancer cells. *Clin Exp Med*, **17,** 27-34.

Dodds PR, Caride V., Lytton B. (1981). The role of the vertebral veins in the dissemination of prostate carcinoma. *J Urol*, **126,** 753-755.

Duffy MJ, Reilly D., O'Sullivan C, O'Higgins N, Fennelly JJ, Andreason P. (1990). Urokinase-plasminogen activator, a new and independent prognostic marker in breast cancer. *Cancer Res*, **50,** 6827-6829.

Dvorak HF, Brown L., Detmar M, Dvorak AM. (1995). Vascular permeability factor/ vascular endothelial growth factor, microvascular hyperpermeability and angiogenesis. *Am J Pathol*, **146,** 1029-1039.

Endo N, Rutledge J, Opas E, Vogel R, Rodan G, Scmidt A. (1996). Human protein tyrosine phosphatase-u; Alternative splicing and inhibition by bisphosphonates. *J Bone Miner Res*, **11,** 535-543.

Farina A, Coppa A., Tiberio A, Tacconelli A, Turco A, Colletta G, Gulino A, Mackay A. (1998). Transforming growth factor-β1 enhances the invasiveness of human MDA-MB-231 breast cancer cells by up-regulating urokinase activity. *Int J Cancer*, **75,** 721-730.

Fedarco NS, Fisher L. (1999). Bone sialoprotein in serum is elevated in osteotrophic cancer patient sera. *Proceedings of the Second North American Symposium on Skeletal Complicatons of Malignancy, Montreal, Canada.*, 14-17.

Ferrara N, Davis-Smyth T. (1997). The biology of vascular endothelial growth factor. *Endocrine Rev*, **18,** 4-25.

Firek A, Jennings J, Tabuenca A, Caulfield M, Garberoglio C, Linkhart T. (1994). TGFb stimulates PTHrP release from human mammary cancer. *Program of the 76th Annual Meeting of the Endocrine Society, Annaheim,CA.*, 395 (Abstract 769).

Fisher JE, Rogers M., Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, Wesolowski G, Russell RGG, Rodan GA, Reszka A. (1999). Alendronate mechanism of action: Geranylgeraniol reverses inhibition of osteoclast formation, bone resorption and kinase activation *in vitro*. *Pron Natl Acad Sci USA*, **96,** 133-138.

Flanagan AM, Chambers T. (1989). Dichloromethylenebisphosphonate (Cl_2MBP) inhibits bone resorption through injury to osteoclasts that resorb Cl_2MBP-coated bone. *Bone Miner*, **6,** 33-43.

Flanagan AM, Chambers T. (1991). Inhibition of bone resorption by bisphosphonates. *Calcified Tiss Int*, **49,** 407-415.

Fleisch, H. (2000). *Bone and Mineral Metabolism.* Academic Press: London.

Fleisch H, Russell R., Francis MD. (1969). Diphosphonates inhibit hydroxyapatite dissolution *in vitro* and bone resorption in tissue culture i*n vivo*. *Science*, **165,** 1262-1264.

Folkman, J. (1984). Angiogenesis: initiation and modulation. In *Cancer invasion and metastasis: biologic and therapeutic aspects.*, Nicholson GL, M.L. (ed) pp. 201-209. Raven Press: New York.

Franklin WA, Shpall EJ, Archer P, Johnston CS, Garza-Williams S, Hami L, Bitter MA, Bast RC & Jones RB. (1996). Immunocytochemical detection of breast cancer cells in marrow and peripheral blood of patients undergoing high dose chemotherapy with autologous stem cell support. *Breast Cancer Res Treat*, **41,** 1-13.

Frith JC, Monkkonen J, Blackburn GM, Russell RG & Rogers MJ. (1997). Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5'-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells in vitro. *J Bone Miner Res*, **12,** 1358-67.

Frith JC, Coxon F., Auriola S, Monkkonen J, Yu X, Sceven BA, Russell RGG, Rogers MJ. (1998). The molecular mechanism of action of clodronate in osteoclasts. *Bone*, **23,** S551 (Abstract).

Fromigue O, Lagneaux L, Body JJ. (2000). Bisphosphonates induce breast cancer cell death in vitro. *J Bone Miner Res*, **15,** 2211-2221.

Frost, H. (1964). Dynamics of bone remodeling. In *Bone Biodynamics.*, Little (ed) pp. 315. Brown: Boston.

Fuller K, Wong, Fox S, Choi Y, Chambers T. (1998). TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J Exp Med*, **188,** 997-1001.

Geddes AD, Da Souza S., Ebetino FH, Ibbotson KJ. (1994). Bisphosphonates: structure-activity relationships and therapeutic implications. *J Bone Miner Res*, **8**, 265-306.

Gessner, P. (1988). A straightforward method for the study of drug interactions: an isobolgraphic primer. *J Am Coll Toxicol*, **7**, 987-1012.

Ghossein RA, Battacharya S., Rosai J. (1999). Molecular detection of micrometastases and circulating tumour cells in solid tumours. *Clin Cancer Res*, **5**, 1950-1960.

Gibbs JF, Oliff A. (1997). The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. *Ann Rev Pharmacol Toxicol*, **37**, 143-166.

Goldhirsch, A. (1997). Breast Cancer. In *Textbook of Medical Oncology*, Cavalli F, H.H.a.S.K. (ed) pp. 53-86. Martin Dunitz: London.

Gorczyca W, Melamed M, Darzynkiewicz Z. (1993). Apoptosis of S-phase HL-60 cells induced by DNA topoisomerase inhibitors: Detection of DNA strand breaks by flow cytometry using the in situ nick translation assay. *Toxicology Lett*, **67**, 249-258.

Gordon S, Helfrich M, Sati H, Greaves M, Ralston S, Culligan D, Soutar R, Rogers M. (2002) Pamidronate causes apoptosis of plasma cells *in vivo* in patients with multiple myeloma. *Br J Haematology*, **119**, 475.

Greco WR, Bravo G., Parsons JC. (1995). The search for synergy: a critical review from a response surface perspective. *Pharmacol Reviews*, **47**, 331-385.

Green JR, Muller K, Jaeggi KA. (1994). Preclinical pharmacology of CGP42'446, a new potent heterocyclic bisphosphonate compound. *J Bone Miner Res*, **9**, 745-751.

Guise T, Mundy G. (1998). Cancer and bone. *Endocrine Rev*, **19**, 18-54.

Guise T, Yin J, Taylor S, Kumagai Y, Dallas M, Boyce B. (1996). Evidence for a causal role of parathyroid hormone- related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest*, **98**, 1544-1549.

Guise T, Yoneda T., Yates J, Mundy G. (1993). The combined effect of tumour-produced parathyroid hormone-related protein and transforming growth factor-alpha enhance hypercalcaemia in vivo and bone resorption in vitro. *J Clin Endo Metab*, **77**, 40-45.

Harris JR, Lippman M, Veronesi U, Willett W. (1992). Medical Progress: Breast Cancer (three parts). *N Eng J Med*, **327**, 319-28; 390-98;473-80.

Hauschka P, Mavrakos A, Iafrati M, Doleman S. (1986). Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-sepharose. *J Biol Chem*, **261**, 12665-12674.

Haverty A, Harmey J., Redmond H, Bouchier-Hayes D. (1997). Interleukin-6 upregulates GP96 expression in breast cancer. *J Surg Res*, **69**, 145-149.

Hearing VJ, Law L., Corti A, Appella E, Blasi F. (1998). Modulation of metastatic potential by cell surface urokinase of murine melanoma cells. *Cancer Res*, **48,** 1270-1278.

Hiraga T, Tanaka S., Ikegame M, Koizumi M, Iguchi H, Nakajima T, Ozawa H. (1998). Morphology of bone metastases. *Eur J Cancer*, **34,** 230-239.

Hiraga T, Williams P., Mundy G, Yoneda T. (2001). The bisphosphonate ibandronate promotes apoptosis in MDA-MB-231 breast cancer cells in bone metastases. *Cancer Res*, **61,** 4418-4424.

Horiuchi N, Caulfield M, Fisher JE, Goldman ME, McKee RL, Reagan JE, Levy JJ, Nutt RF, Rodan SB, Schoefield TL, Clemens T, Rosenblatt M. (1987). Similarity of synthetic peptides from human tumour to parathyroid hormone *in vivo* and *in vitro*. *Science*, **238,** 1566-1568 [published erratum appears in Science 1998; 239: 128].

Hortobagyi GN, Theriault RL, Porter L, Blayney D, Lipton A, Sinoff C, Wheeler H, Simeone JF, Seaman J & Knight RD. (1996). Efficacy of pamidronate in reducing skeletal complications in patients with breast cancer and lytic bone metastases. Protocol 19 Aredia Breast Cancer Study Group. *New Engl JMed*, **335,** 1785-91.

Hughes DE, Wright KR, Uy HL, Sasaki A, Yoneda T, Roodman GD, Mundy GR & Boyce BF. (1995). Bisphosphonates promote apotosis in murine osteoclasts in vitro and in vivo. *Journal Bone Miner Res*, **10,** 1478-1487.

Hughes DE, McDonald B., Russell RGG, Gowen M. (1989). Inhibition of osteoclast-like cell function in long-term cultures of human bone marrow. *Journal Clin Invest*, **83,** 1930-1935.

Hultborn R, Ryden S, Gunderson S (1996). Efficacy of pamidronate on skeletal complications from breast cancer metastases. A randomised prospective double blind placebo controlled trial. *Acta Oncol*, **35,** 73-74.

Hynes, R. (1992). Integrins: versatility, modulation and cell adheseion. *Cell*, **69,** 11-25.

Iezzoni J, Bruns M, Frierson H, Scott M, Pence R, Deftos L, Bruns D. (1998). Coexpression of parathyroid hormone-related protein and its receptor in breast cancer: a potential autocrine effector system. *Mod Pathol*, **11,** 265-270.

Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, NAkamura Y, Yamaguchi A, Matsuda T, Hirano T, Kishimoto T, Suda T. (1990). IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol*, **145,** 3297-3303.

Jablonska, E. (1998). Changes in sIL-6R and sTNF-Rs release by PMNs and the serum levels in breast cancer patients at different stages of treatment. *Cytokine*, **10,** 540-543.

Jacob K, Webber M., Benayahu D, Kleinman HK. (1999). Osteonectin promotes prostate cancer cell migration and invasion: A possible mechanism for metastasis to bone. *Cancer Res*, **59,** 4453-4457.

Jagdev SP, Coleman R, Rostami-H A, Croucher PI. (2001). The bisphosphonate, zoledronic acid, induces apoptosis of breast cancer cells: evidence of synergy with paclitaxel. *Br J Cancer*, **84,** 1126-1134.

Janicke F, Schmitt M, Hafter R et al. (1990). Urokinase-type plasminogen activator (uPA) antigen is a predictor of early relapse in breast cancer. *Fibrinolysis*, **4,** 69-78.

Jiang X, Yang D., Elliott R, Head J. (2000). Reduction in serum IL-6 after vaccination of breast cancer patients with tumour-associated antigens is related to eostrogen receptor status. *Cytokine*, **12,** 458-465.

Jones JI, Clemmons D. (1995). Insulin-like growht factors and their binding proteins: biological actions. *Endocr Rev*, **16,** 3-34.

Jung A, Bisaz S., Fleisch H. (1972). The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals. *Calcified Tissue Res*, **11**.

Kanis J, O'Rourke N, McCloskey E. (1994). Consequences of neoplasia induced bone resorption and the use of clodronate. *Int J Oncol*, **5,** 713-731.

Kawano M, Hirano T., Matsuda T, Horii Y, Iwato K, Asaoku H, Tang B, Tanabe O, Tanaka H, Kuramoto A, KishimotoT. (1988). Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. *Nature*, **332,** 83-85.

Kerr JFR, Winterford C., Harmon BV. (1994). Apoptosis: its significance in cancer and cancer therapy. *Cancer*, **73,** 2013-2026.

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis supresses tumour growth *in vivo*. *Nature*, **362,** 841-844.

King RH, Rabbai S. (1996). Overexpression of urokinase receptor in breast cancer cells results in increased tumour invasion, growth and metastasis. *Int J Cancer*, **67,** 423-429.

Klein B, Zhang X, Lu ZY, Bataille R. (1995). Interleukin-6 in human multiple myeloma. *Blood*, **85,** 863-872.

Koeneman KS, Yeung F, Chung LW,. (1999). Osteomimetic properties of prostate cancer cells: A hypothesis supporting the prediliction of prostate cancer metastases and growth in the bone environment. *Prostate*, **39,** 246-261.

Kohno N, Kitazawa S, Fukase M, Sakoda Y, Kanabara Y, Furuya Y, Ohashi O, Ishikawa Y, Saitoh Y. (1994). The expression of parathyroid hormone-related protein in human breast cancer with skeletal metastases. *Surg Today*, **24,** 215-220.

Kong YY, Yoshida H., Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Penninger JM. (1999). OPGL is a key regulator of osteoclastgenesis, lymphocyte development and lymph node organogenesis. *Nature*, **397,** 315-323.

Lacey DL, Timms E., Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliot G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Caparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. (1998). Osteoprotogerin (OPG) ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*, **93,** 165-176.

Lahtinen R, Laakso M, Palva I, Virkkunen P, Elomaa I. (1992). Randomised placebo-controlled multicentre trial of clodronate in multiple myeloma. *Lancet*, **340,** 1049-1052.

Lai L, Kadory S, Siraj A, Lennard T. (1994). Oncostatin M, Interleukin-2, Interleukin-6 and interleukin-8 in breast cyst fluid. *Int J Cancer*, **59,** 369-372.

Latza U, Niedobitek G, Schwarting R, Nekarda H, Stein H. (1990). BerEP4: new monoclonal antibody which distinguishes epithelia from mesothelia. *J Clin Pathol*, **43,** 213-9.

Leonessa F, Jacobson M, Boyle B, Lippman J, McGarvey M, Clarke R. (1994). Effect of tamoxifen on the multidrug-resistant phenotype in human breast cancer cells: isobologram, drug accumulation, and *Mr* 170,000 glycoprotein (gp170) binding sites. *Cancer Res*, **54,** 441-447.

Lowik C, Van der Plujm G, Van der Wee-Pals L, Van Treslong-de-Groot H, Bijvoet O. (1988). Migration and phenotypic transformation of osteoclast precursors into mature osteoclasts: The effect of a bisphosphonate. *J Bone Miner Res*, **3,** 185-192.

Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G & Rogers MJ. (1998). Nitrogen-containing bishosphontes inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. *J Bone MinerRes*, **13,** 581-589.

MacDougall JR, Matrisian L. (1995). Contribution of tumour and stromal matrix metalloproteinases to tumour progression, invasion and metastasis. *Cancer Met Rev*, **14,,** 351-352.

Madigan MP, Zeigler RG, Benichou J, Byrne C, Hoover RN. (1995). Proportion of breast cancer cases in the United States explained by well established risk factors. *J Natl Cancer Inst,* **87,** 1681-1685.

Magnetto S, Boissier S, Delmas PD & Clezardin P. (1999). Additive anti-tumour activities of taxoids in combinaton with the bisphosphonate ibandronate against invasion and adhesion of human breast carcinoma cells to bone. *Intl J Cancer*, **83,** 263-9.

Major P, Lortholary A, Hon J, Abdi E, Mills G, Menssen HD, Yunus F, Bell R, Body J, Quebe-Fehling E, Seaman J. (2001). Zoledronic acid is superior to pamidronate in the treatment of hypercalcaemia of malignancy: a pooled analysis of two randomised trials. *J Clin Onc*, **19,** 558-567.

Mandriota S, Liotta J, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson D, Orci L, Alitalo K, Christoforo G, Pepper M. (2001). Vascular endothelial growth factor-C mediated lymphangiogenesis promotes tumour metastasis. *EMBO J*, **20,** 672-682.

Marshall C. (1993). Protein prenylation: A mediator of protein-protein interactions. *Science*, **259**, 1865-1866.

Marshall MJ, Wilson A, Davie MWJ. (1990). Effects of (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate on mouse osteoclasts. *Journal Bone Miner Res*, **5**, 1246-1250.

Massague, J. (1998). TGFβ signal transduction. *Annu Rav Biochem*, **67**, 753-91.

Mbalaviele G, Dunstan C, Sasaki A, Williams PJ, Mundy GR, Yoneda T. (1996). E-cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in an experimental metastasis model. *Cancer Res*, **56**, 4063-4070.

McCloskey DE, Kaufmann S, Prestigiacomo LJ, Davidson NE. (1996). Paclitaxel induces programmed cell death in MDA-MB-468 human breast cancer cells. *Clin Cancer Res*, **2**, 847-854.

McCloskey EV & MacLennan ICM, D.M., Chapman C, Dunn J, Kanis JA. (1996). Effect of clodronate on morbidity and mortality in myelomatosis. *Annals of Oncol*, **7**, 4120 (Abstract).

McCloskey, E.V., Maclennan, C.M., Drayson, M.T., Chapman, C., Dunn, J. & Kanis, J.A. (1998). A randomized trial of the effect of clodronate on skeletal morbidity in multiple myeloma. *British Journal of Haematology*, **100**, 317-325.

Meyer T, Hart I. (1998). Mechanisms of tumour metastases. *Eur J Cancer*, **34**, 214-221.

Miller SC, Jee W. (1979). The effect of dichloromethylenebisphosphonate, a pyrophsphate analog, on bone and bone cell structure in the growing rat. *The Anatomical Record*, **193**, 439-462.

Mohan S, Baylink D. (1991). Bone growth factors. *Clin Ortho*, **263**, 30-48.

Morton AR, Cantrill J, Pillai GV, mcMahon A, Anderson D, Howell A. (1988). Sclerosis of lytic bone metastases after disodium aminohydroxypopylidene bishponate (APD) in patients with breast carcinoma. *BMJ*, **297**, 772-773.

Mosely JM, Kubota M, Diefenbach-Jagger H, Wettenhall REH, Kemp BE, Suva LJ, Rodda CP, Ebeling PR, HUdson PJ, Zajac JD, Martin TJ. (1987). Parathyroid hormone-related protein purified from a human lung cancer cell line. *Proc Natl Acad Sci, USA*, **84**, 5048-5052.

Mouridsen H, Gershanovich M, Sun Y, Perez-Carrion R, Boni C, Mounier A, Apftelstaedt J, Smith R, Sleeboom HP, Janick F, Pluzanska A, Dank M, Becquart D, Bapsy P, Salminen E, Snyder R, Lassus M, Verbeek J, Staffler B, Chaudri-Ross HA, Dugan H. (2001) Superior efficacy of letrozole versus tamoxifen as first-line therapy for post-menopausal women with advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group. *J Clin Onc*, **19**, 2596-2606.

Mundy, G. (1996) Bone resorbing cells. In *Primer on metabolic bone diseases and disorders of mineral metabolism*, MJ, F. (ed) pp. 16. Lippincott-Raven: Philadelphia.

Mundy, G. (1997). Mechanisms of bone metastases. *Cancer*, **80 (suppl)**, 1546-1556.

Mundy G, Yoneda T. (1996). Mechanisms of bone metastasis. In *Bone Metastasis- Mechanisms and Pathophysiology*, Orr FW, S.G. (ed) pp. 1-66. Chapman and Hall: New York.

Murakami H, Kinosaki M, Sasaki T, Udagawa N, Tanaka S, Nakamura I, Zhang D, Barbier A, Suda T. (1995). A possible mechanism of the specific action of bisphosphonates on osteoclasts: Tiludronate preferentially affects polarised osteoclast having ruffled borders. *Bone*, **17**.

Muss H, Case L, Richards F (1991). Interrupted versus continuous chemotherapy in patients with metastatic breast cancer. *N Engl J Med*, **325**, 1342.

Nabholtz J, Buzdar A, Pollak M, Harwin W, Burton G, Mangalik A, Steinberg M, Webster A, von Euler M. (2000) Anastrozole is superior to tamoxifen as first-line therapy for advanced breast carcinoma in postmenopausal women: results of a North American multicenter randomised trial. *J Clin Onc*, **18**, 3758-3767.

Nakagawa N, Kinosaki M., Yamaguchi K, Shima N, Yasuda H, Yano K, Morinaga T, Higashio K. (1998). RANK is the essential signalling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem Biophys Res Comm*, **253**, 395-400.

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. (1999). Vascular endothelial growht factor (VEGF) and its receptors. *FASEB*, **13**, 9-22.

Nikishawa M, Akatsu T, Katayama Y, Yasutomo Y, Kado S, Kugal N, et al. (1996). Bisphosphonates act on osteoblastic cells and inhibit osteoclast formation in mouse marrow cultures. *Bone*, **18**, 9-14.

Novick D, Shulman L, Chen L, Revel M. (1992). Enhancement of interleukin 6 cytostatic effect on human breast carcinoma cells by soluble IL-6 receptor from urine and reversion by monoclonal antibody. *Cytokine*, **4**, 6-11.

Ogata, E. (2000). Parathyroid hormone-related protein as a potential target of therapy for cancer-associated morbidity. *Cancer*, **12 (supp)**, 2909-2911.

Okada Y, Naka K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, Sato H, Seiki M. (1995). Localization of matrix metalloproteinase 9 (92-kilo-dalton gelatianase/ type IV collagenase=gelatinase B) in osteoclasts: Implications for bone resorption. *Lab Invest*, **72**, 311-322.

Osborne MP, Wong G, Asina S, Old LJ, Cote RJ, Rosen PP. (1991). Sensitivity of immunocytochemical detection of breast cancer cells in human bone marrow. *Cancer Res*, **51**, 2706-9.

Paget, S. (1889). The distribution of secondary growths in cancer of the breast. *Lancet*, **1,** 571-573.

Paterson AHG, Powles TJ, Kanis JA, McCloskey E, Hanson J & Ashley S. (1993). Double-blind controlled trial of oral clodronate in patients with bone metastases from breast cancer. *J Clin Onc*, **11,** 59-65.

Pederson L, Winding B, Foged NT, Spelsberg T, Oursler M. (1999). Identification of breast cancer cell-line derived paracrine factors that stimulate osteoclast activity. *Cancer Res*, **59,** 5849-5855.

Pepper MS, Ferrara N, Orci L, Montesano R. (1991). Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem Biophys Res Comm*, **181,** 902-906.

Pfeilschifter J, Chenu C, Bird A, Mundy GR, Roodman GD. (1989). Interleukin-1 and tumour necrosis factor stimulate the formation of human osteoclast-like cells *in vitro*. *J Bone Miner Res*, **4,** 113-118.

Pfeilschifter J, Mundy G. (1987). Modulation of transforming growth factor-beta activity in bone cultures by osteotropic hormones. *Proc Natl Acad Sci USA*, **84,** 2024-2028.

Plasmans CMT, Jap P, Kuijpers W, Sloof TJJH. (1980). Influences of a diphosphonate on the cellular aspect of young bone tissue. *Calcified Tiss Res*, **32,** 247-256.

Powell G, Southby J, Danks J, Stillwell R, Hayman J, Henderson M, Bennett R, Martin TJ. (1991). Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared to other sites. *Cancer Res*, **51,** 3059-3061.

Powles T, Patterson S, Kanis J, McCloskey E, Ashley S, Tidy A, Rosenqvist K, Smith I, Ottestad L, Legault S, Pajunen H, Neventous A, Maunisto E, Suovuon A, Atula S, Nevalainen J, Pylkkanen L. (2002) Randomised placebo-controlled trial of clodronate in patients with primary operable breast cancer. *J Clin Onc*, **20,** 3219-3224.

Purohit O, Radstone C, Anthony C, Kanis J, Coleman R. (1995). A randomised double-blind comparison of intravenous pamidronate and clodronate in hypercalcaemia of malignancy. *Br J Cancer*, **72,** 1289-1293.

Puzas, J. (1996). Osteoblast cell biology- lineage and functions. In *Primer on the metabolic bone diseases and disorders of mineral metabolism.*, MJ, F. (ed) pp. 11. Lippincott-Raven: Philadelphia.

Pyke C, Kristensen P, Ralfkiaer E (1991). Urokinase-type plasminogen activator is expressed in stromal cells and it's receptor in cancer cells at invasive foci in human colon adenocarcinomas. *Am J Pathol*, **138,** 1059-1067.

Quinn JE, Buhler K, Brown JM, Bladou E, Lange PH, Vessella RL. (1998). A model for prostate cancer bone metastasis:Direct intra-bone injection of prostate cancer xenografts. *Bone*, **23 (Suppl),** S579.

Racila E, Euhus D, Weiss AJ, Rao C, McConnell J, Terstappen LW, Uhr JW. (1998). Detection and characterisation of carcinoma cells in the blood. *Proc Natl Acad Sci USA*, **95**, 4589-4594.

Ralston, S. (1992). Medical Management of hypercalcaemia. *Br J Cancer*, **75**, 295-300.

Ralston S, Gallacher, Patel U, Dryburgh F, Frazer WD, Cowan RA, Boyle IT. (1989). Comparison of three intravenous bisphosphonates in cancer-associated hypercalcaemia. *Lancet*, **2**, 1180-1182.

Ralston S, Thiebaud D., Hermann Z, Steinhauer E, Thurlimann B, Walls J, Lichinitser M, Rizzoli R, Hagberg H, Huss H, Tubiana-Hulin M, Body J. (1997). Dose-response study of ibandronate in the treatment of cancer-associated hypercalcaemia. *Br J Cancer*, **75**, 295-300.

Riebeling C, Forsea AM, Raisova M, Orfanos CE, Gailen CC. (2002) The bisphosphonate pamidronate induces apoptosis in human melanoma cells in vitro. *Br J Cancer*, **87**, 366-371.

Rogers MJ, Brown R, Hodkin V, Blackburn GM, Russell RGG. (1996a). Bisphosphonates are incorporated into adenine nucleotides by human aminoacyl-tRNA synthetase enzymes. *Biochem Biophys Res Comm*, **224**, 863-869.

Rogers MJ, Chilton K, Coxon FP, Lawry J, Smith MO, Suri S, Russell RGG. (1996b). Bisphosphonates induce apoptosis in mouse macrophages-like cells in vitro by a nitric oxide-independent mechanism. *J Bone Miner Res*, **11**, 1482-1491.

Rogers MJ, Ji X, Russell RGG, Blackburn M, Williamson MP, Bayless AV, Ebetino FH, Watts DJ. (1994a). Incorporation of bisphosphonates into adenine nucleotides by amoebae of the cellular slime mould *Dictyostelium discoideum. Biochem J*, **303**, 303-311.

Rogers MJ, Russell R, Blackburn GM, Williamson MP, Watts DJ. (1992). Metabolism of halogenated bisphosphonates by the cellular slime mould *Dictyostelium discoideum. Biochem Biophys Res Comm*, **189**, 414-423.

Rogers MJ, Watts D, Russell RGG, Ji X, Xiong X, Blackburn GM, Bayless AV, Ebetino FH. (1994b). Inhibitory effects of bisphosphonates on growth of amoebae of the cellular slime mould *Dictyostelium discoideum. J Bone Miner Res*, **9**, 1029-1039.

Rosol, T. (2000). Pathogenesis of bone metastases: role of tumour-related proteins. *J Bone Miner Res*, **15**, 844-850.

Ross AA, Cooper B, Lazarus HM, Mackay W, Moss TJ, Ciobanu N, Tallman MS, Kennedy MJ, Davidson NE, Sweet D, Winter C, Akard L, Jansen J, Copelan E, Meagher RC, Herzig RH, Klumpp TR, Kahn DG, Warner NE. (1993). Detection and variability of tumour cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. *J Clin Oncol*, **9**, 2605-10.

Rowe DJ, Hausmann E. (1976). The alteration of osteoclast morphology by diphosphonates in organ culture. *Calcified Tiss Res*, **20,** 53-60.

Rowe DJ, Hausmann E. (1980). The effects of calcitonin and colchicine on the cellular response to diphosphonate. *Br J Exp Pathol*, **61,** 303-309.

Rowe DJ, Hayes S. (1983). Inhibition of bone resorption by difluoromethylene diphosphonate in organ culture. *Metab Bone Dis Rel Res*, **5,** 13-16.

Rubens, R. (1991). The nature of metastastic bone disease. In *Bone Metastases, Diagnosis and Treatment*, Rubens RD, Fogelman I. (ed) pp. 1-8. Springer-Verlag: London.

Rubens, R. (1999). *Clinical aspects of bone metastases.* Tumour bone diseases and osteoporosis in cancer patients. Marcel Dekker: New York.

Russell RGG, Muhlbauer R, Bisaz S, Williams D, Fleisch H. (1970). The influence of pyrophosphate, condensed phosphates, phosphonates and other phophate compounds on the dissolution of hydroxyapatite in vitro and on bone resorption induced by parathyroid hormone in tissue culture and in thyroparathyroidectomised rats. *Calc Tiss Res*, **6,** 183-196.

Saadeh P, Mehrara B, Steinbrech D, Spector J, Greenwald G, Chin G, Ueno H, Gittes G, Logaker M. (2000). Mechanisms of fibroblast growth factor-2 modulation of vacular endothelial growth factor expression by osteoblastic cells. *Endocrinology*, **141,** 2075-2083.

Saarto T, Blomqvist C, Virkkunen P & Elomaa I. (2001). Adjuvant Clodronate Treatment Does Not Reduce the Frequency of Skeletal Metastases in Node-Positive Breast Cancer Patients: 5-Year Results of a Randomized Controlled Trial. *J Clin Onc*, **19,** 10-17.

Sachdev D, Yee D. (2001). The IGF system and breast cancer. *Endo Rel Cancer*, **8,** 197-209.

Sahni M, Guenter H, Fleisch H, Collin P, Martin TJ. (1993). Bisphosphonates act on rat bone resorption through mediation of osteoblasts. *J Clin Invest.*, **91,** 2004-10.

Salo J, Lehenkari P, Mulari M, Metsikko K, Kalervo Vaananen H. (1997). Removal of osteoclast bone resorption products by transcytosis. *Science*, **276,** 270-273.

Sasaki A, Boyce B, Story B, Wright K, Chapman M, Boyce R, Mundy G, Yoneda T. (1995). Bisphosphonate risedronate reduces metastastic human breast cancer burden in bone in nude mice. *Cancer Res*, **55,** 3551-3557.

Sasaki A, Kitamura K, Alcalde R, Tanaka T, Suzuki A, Etoh Y, Matsumara T. (1998). Effect of newly developed bisphosphonate, YH529, on osteolytic bone metastastes in nude mice. *Int J Cancer*, **77,** 279-285.

Sato M, Grasser W, Endo N, Akins R, Simmons H & Thompson DD. (1991). Bisphosphonate action. Alendronate localisation in rat bone and effects on osteoclast ultrastructure. *Journal of Clinical Investigation*, **88,** 2095-2105.

Schenk R, Merz W, Muhlbauer R, Russell RGG, Fleisch H. (1973). Effect of ethane-1-hydroxyl-1,1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl_2MDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. *Calified Tiss Res*, **11,** 196-214.

Schmidt A, Rutledge S, Endo N, Opras E, Tanaka H, Wesolowski G, Ceu C, Huang Z, Ramachandran C, Rodan S, Rodan G. (1996). Protein-tyrosine phosphatase activity regulates osteoclast formation and function: Inhibition by alendronate. *Proc Nat Acad Sci USA*, **93,** 3069-3073.

Scnitt, S. (1993). Pathologic factors predictors of local recurrence in patients with invasive breast cancer treated with conservative surgery and radiation therapy. In *Non-Disseminated Breast Cancer*, Fletcher GH, L.S. (ed) pp. 93-104. Springer-Verlag: Berlin.

Selander K, Monkonnen J, Karhukorpi E, Harkonnen P, Hannuniemi R, Vaananen HK. (1996). Characteristics of clodronate-induced apoptosis in osteoclasts and macrophages. *Mol Pharmacol*, **50,** 1127-1138.

Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. (2000). Bisphosphonates induce apoptosis in human breast cancer cell lines. *Br J Cancer*, **82,** 1459-2468.

Senaratne SG, Mansi JL, Colston KW. (2002) The bisphosphonate zoledronic acid impairs membrane localisation and induces cytochrome c release in breast cancer cells. *Br J Cancer*, **86,** 1479-1486.

Shinoda H, Adamek G, Felix R, Fleisch H, Schenk R, Hagan P. (1983). Structure -activity relationships of various bisphosphonates. *Calicified Tissue International*, **35,** 87-89.

Shipman CM, Croucher PI, Russell RG, Helfrich MH & Rogers MJ. (1998). The bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway. *Cancer Research*, **58,** 5294-5297.

Shipman CM, Rogers MJ, Apperley JF, Russell RG, Croucher PI. (1997). Bisphosphonates induce apoptosis in human myeloma cell lines: a novel anti-tumour activity. *B J Haematology*, 665-72.

Sietsema WK, Ebetino F, Salvagno AM, Bevan JA. (1989). Anti-resorptive dose-response relationships across three generations of bisphosphonates. *Drugs Exper Clin Res*, **15,** 389-396.

Simonet WS, Lacey D, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle W (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell*, **89,** 309-19.

Skory K, Ly H, Kelly J, Hammond M, Ramachandran C, Huang Z, Gresser M, Wang Q. (1997). How does alendronate inhibit protein-tyrosine phosphatases? *J Biolog Chem*, **272,** 22472-22480.

Slade MJ, Smith B, Sinnett HD, Cross NCP, Coombes RC. (1999). Quantitative polymerase chain reaction for the detection of micrometastases in patients with breast cancer. *J Clin Oncol*, **17,** 870-9.

Sommers CL, Thompson E, Torri JA, Kemler R, Gelmann E, Byers SW. (1991). Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. *Cell Growth and Differ*, **2.**

Sonnemann J, Eckervogt V, Truckenbrod B, Boos J, Winkelmann W, van Valen F. (2001). The bisphosphonate pamidronate is a potent inhibitor of human osteosarcoma cell growth in vitro. *Anticancer Drugs*, **12,** 459-65.

Soule HD, Vazquez J, Long A (1973). A human cell line from a pleural effusion derived from breast carcinoma. *J Natl Cancer Inst*, **51,** 1409-1416.

Stearns ME, Wang M. (1996). Effects of alendronate and taxol on PC-3 ML cell bone metastases in SCID mice. *Invasion Metastasis*, **16,** 116-131.

Strewler GJ, Stern P, Jacobs JW, Eveloff J, Klein RF, Leung SC, Rosenblatt M, Nissenson R. (1987). Parathyroid hormone-like protein from human renal carcinoma cells. Structural and functional homology with parathyroid hormone. *J Clin Invest*, **80,** 1803-1807.

Sun L (1994). Expression of transforming growth factor β type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. *J Biol Chem*, **269,** 26449-26455.

Sung V, Stubbs J, Fisher L, Aaron AD, Thompson EW. (1998). Bone sialoprotein supports breast cancer cell adhesion proliferation and migration through differential usage of the alpha(v)beta5 integrins. *J Cell Physiology*, **176,** 482-494.

Tamm I, Toyoko K, Cardinale I, Kreuger J. (1994). Cell-adhesion-disrupting action of interleukin 6 in human ductal breast cancer cells. *Proc Natl Acad Sci, USA*, **91,** 3329-3333.

Tassone P, Forciniti S, Galea E, Morrone G, Turco MC, Martinelli V, Tagliaferri P, Venuta S. (2000). Growth inhibition and synergistic induction of apoptosis by zoledronate and dexamethasone in human myeloma cell lines. *Leukaemia*, **14,** 841-844.

Theriault RL, Lipton A, Hortobagyi GN, Leff R, Gluck S, Stewart JF, Costello S, Kennedy I, Simeone J, Seaman JJ, Knight RD, Mellars K, Heffernan M & Reitsma D. (1999). Pamidronate reduces skeletal morbidity in women with advanced breast cancer and lytic bone lesions: a randomized, placebo-controlled trial. Protocol 18 Aredia Breast Cancer Study Group. *J Clin Oncy*, **17,** 846-54.

Thiebaud D, Leyvras S, von Fleidner V (1991). Treatment of bone metastases from breast cancer and multiple myeloma with pamidroante. *Eur J Cancer*, **27,** 37-41.

Thomas RJ, Guise T, Yin JJ, Elliott J, Horwood NJ, Martin TJ, Gillespie MT. (1999). Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinology*, **140,** 4451-8.

Unemori EN, Ferrara N, Bauer EA, Amento EP. (1992). Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *J Cell Physiol*, **153,** 557-562.

Van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S. (1999a). Farnesyl pyrophosphate synthase is the molecular target of nitrogen-containing bisphosphonates. *Biochem Biophys Res Commun*, **264,** 108-111.

Van Beek E, Pieterman E, Cohen L, Lowik C, Papapoulos S. (1999b). Nitrogen-containg bisphosphonates inhibit isopently pyrophosphate isomerase/ farnesyl pyrophosphate synthase activity with relative potencies corresponding to their anti-resorptive potencies both *in vitro* and *in vivo*. *Biochem Biophys Res Commun*, **255,** 491-494.

Van Der Pluijm G, Lowik C, Papapoulos S. (2000). Tumour progression and angiogenesis in bone metastases from breast cancer: new approaches to an old problem. *Cance Treat Rev*, **26,** 11-27.

Van Holten-Verzantvoort A, Bijvoet O, Hermans J (1987). Reduced morbidity from skeletal metastases in breast cancer patients diring long-term bisphosphonate (APD) treatment. *Lancet*, **2.**

van Holten-Verzantvoort AT, Kroon HM, Bijvoet OL, Cleton FJ, Beex LV & Blijham G (1993). Palliative pamidronate treatment in patients with bone metastases from breast cancer. *J Clin Onc*, **11,** 491-498.

Vitte C, Fleisch H, Guenther HL. (1996). Bisphosphonates induce osteoblasts to secrete an inhibitor of osteoclast-mediated bone resorption. *Endocrinology*, **137,** 2324-33.

Voigt W, Bulankin A, Muller T, Schoeber C, Grothey A, Hoang-Vu C, Schmoll H-J. (2000). Schedule-dependant antagonism of gemcitabine and cisplatin in human anaplastic thyroid cancer cell lines. *Clin Cancer Res*, **6,** 2087-2093.

Waltregny D, Bellahcene A, De Leval X, Florkin B, Weidle U, Castronovo V. (2000). Increased expression of Bone Sialoprotein in bone metastastes compared with visceral metastases in human breast and prostate cancers. *J Bone Miner Res*, **15,** 834-843.

Weber GF, Ashkar S, Glimcher MJ, Cantor H. (1996). Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science*, **271,** 509-512.

Wyllie R, Kerr J, Currie AR. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol*, **68,** 251-306.

Wingen F, Poole B, Klein P, Klenner T, Schmal D. (1988). Anticancer activity of bisphosphonic acids in methylnitrosurea-induced mammary carcinoma of the rat- benefit of combining bisphosphonates with cytostatic agents. *Invest New Drugs*, **6,** 155-167.

Witty JP, Foster S, Stricklin GP, Matrisian LM, Stem PH. (1996). Parathyroid hormone-induced resorption in fetal rat limb bone is associated with production of the metalloproteinases collagenase and gelatinase B. *J Bone Miner Res*, **11,** 72-78.

Wood J, Bonjean K, Ruetz S, Bellahcene A, Devy L, Foidart JM, Castronovo V, Green JR. (2002). Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. *J Pharmacol Exp Ther*, **302,** 1055-61.

Xuan JW, Hota C, Shigeyama Y, D'Errico JA, Somerman MJ, Chambers AF. (1995). Site-directed mutagenesis of the arginine-glycine aspartic acid sequence in osteopontin destroys cell adhesion and migration functions. *J Cell Biochem*, **57,** 680-690.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano A, Goto M, Murakami A, Tsuda E, Morinaga N, Suda T. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/ osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA*, **95,** 3597-3602.

Yeung TK, Germond C, Chen X, Wang Z. (1999). The mode of action of Taxol: apoptosis at low concentration and necrosis at high concentration. *Biochem Biophys Res Commun*, **263,** 398-404.

Yin J, Selander K, Chirgwin J, Dallas M, Grubbs B, Weiser R, Massague J, Mundy G, Guise T. (1999). TGF-β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest*, **103,** 197-206.

Yokoe T, Iino Y, Takei H, Horiguchi J, Koibuchi Y, Maemura M, Ohwada S, Morishito Y. (1997). Changes of cytokines and thyriod function in patients with recurrent breast cancer. *Anticancer Res*, **17,** 695-699.

Yoneda T, Michigami T, Yi B, Williams PJ, Niewolna M, Hiraga T. (1999). Use of bisphosphonates for the treatment of bone metastases in experimental animal models. *Cancer Treat Rev*, **25,** 293-299.

Yoneda T, Michigami T, Bing Y, Williams P, Niewolna M, Hiraga T. (2000). Actions of bisphosphonate on bone metastases in animal models of breast carcinoma. *Cancer*, **88,** 2979-2987.

Yoneda T, Sasaki A, Dunstan C, Williams P, Bauss F, De Clerk Y, Mundy G. (1997). Inhibition of osteolytic bone metastases of breast cancer by combined treatment with the bisphosphonate ibandronate and tissue inhibitor of the matrix metalloproteinase-2. *J Clin Invest*, **99,** 2509-2517.

Yu X, Scoller J, Foged NT. (1996). Interaction between effects of parathyroid hormone and bisaphosphonate on regulation of osteoclast activity by the osteoblast-like cell line UMR-106. *Bone*, **19,** 339-45.

Zhang FL, Casey P. (1996). Protein prenylation: Molecular mechanisms and functional consequences. *Ann Rev Biochem*, **65,** 241-269.

Zhang GJ, Adachi I. (1999). Serum interleukin-6 levels correlate to tumour progression and prognosis in metastatic breast cancer. *Anticancer Res*, **19,** 1427-32.

Zimolo Z, Wesolowski G, Rodan G. (1995). Acid extrusion is induced by osteoclast attachment to bone; Inhibition by alendronate and calcitonin. *J Clin Invest*, **96,** 2277-2283.

APPENDIX 1

Mathematical model used to determine synergy between paclitaxel and zoledronic acid.

$$E(c) = E\max \frac{\left(\frac{C_P}{EC50_P} + \frac{C_Z}{EC50_Z} + \alpha \times \left(\frac{C_P C_Z}{EC50_P EC50_Z}\right)\right)^{\gamma}}{1 + \left(\frac{C_P}{EC50_P} + \frac{C_Z}{EC50_Z} + \alpha \times \left(\frac{C_P C_Z}{EC50_P EC50_Z}\right)\right)^{\gamma}}$$

$E(c)$	Effect at any given concentration of paclitaxel or zoledronic acid
E max	Maximum achievable effect
C_P	Concentration of paclitaxel
C_Z	Concentration of zoledronic acid
$EC50_P$	Concentration of paclitaxel that produces 50% of E max
$EC50_Z$	Concentration of zoledronic acid that produces 50% of E max
α	Interaction term
γ	Sigmoidicity function

The above model was used for analysis of synergy using the GraFit Version 3.00 software (Erithacus Software Ltd, Surrey, UK). This software allows 3-dimensional analysis so that the data for the zoledronic acid and paclitaxel effects can be analysed simultaneously according to the equation given above. This general model for non-additivity assumed an interaction term α. If $\alpha=0$ then the effect of the combining the two drugs could be considered additive only. In contrast, negative values indicated antagonism and positive values demonstrated synergy. The sigmoidicity function γ indicates the shape of the curve.