NOVEL COMBINATION TREATMENT WITH TRAIL FOR OSTEOSARCOMA THERAPY: THE USE OF INHIBITORS OF THE MEVALONATE PATHWAY.

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Submitted in total fulfilment of the requirements
for the degree of Doctor of Philosophy

May, 2005

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ABSTRACT

Osteosarcoma (OS) is the most common primary malignant tumour of the bone, occurring most frequently in children and young adults. In addition to surgical intervention, the current choice of treatment for OS is chemotherapy however despite significant improvements in patient survival, some patients continue to have a poor prognosis due to problems including tumour resistance to chemotherapy and metastatic spread. Therefore, there is a pressing need to develop better and safe alternative approaches for the treatment of OS. Investigating combinations of agents that alone have little toxicity, but which have additive anti-tumour activity is appealing because of the possibility of using lower drug doses with reduced toxic side effects.

This thesis investigates potential new agents for the treatment of OS. These include TRAIL and various inhibitors of the mevalonate pathway (IMP), including the nitrogen-containing bisphosphonate, zoledronic acid (ZOL), the statins, lovastatin (LOV) and mevastatin (MEV) and the prenyl transferase inhibitors (PTIs), geranylgeranyl transferase inhibitor (GGTI) and farnesyl transferase inhibitor (FTI).

By exploring new combinations of agents that can kill bone tumour cells *in vitro*, the surprising finding was made that ZOL and TRAIL act synergistically to kill bone cancer cells. More importantly, ZOL strongly sensitised TRAIL-resistant cells to the cytotoxic effects of the ligand to an extent comparable with chemotherapeutic agents. The statins and PTIs also resulted in a significantly greater induction of cell death, when used in combination with TRAIL, than either agent alone. Further investigations revealed the importance of prenylation for cell survival. Analysis of cell surface receptors revealed an upregulation of TRAIL death receptors, but not decoy receptors,

in response to ZOL treatment, suggesting one possible mechanism for the observed synergy.

An *in vivo* model of OS was used to examine the anti-tumour and anti-metastatic effects of ZOL and TRAIL. Mice inoculated with K-HOS cells, and left untreated, developed large lesions that invaded the marrow cavity and began to erode the cortical bone, as assessed by radiography, micro-computer tomography and histology. In contrast, animals treated with ZOL, showed remarkable conservation of the tibiae with no evidence of bone destruction and significant increases in cortical thickness and trabecular density. This was reflected in the bone volume, which was significantly increased in ZOL treated animals. As both the ZOL treated and combination ZOL and TRAIL treated animals showed no osteolysis, it was not possible to establish whether the combination treatment was any more beneficial than ZOL treatment alone. The area of tumour growth was lowest in TRAIL treated mice suggesting that TRAIL may have an effect on the extra-osseous tumour. ZOL alone or ZOL and TRAIL in combination failed to inhibit metastases to lungs. Although no definitive relationship between ZOL and TRAIL could be determined *in vivo*, the use of ZOL alone exhibited powerful anticancer properties.

The use of statins and PTIs may prove very beneficial as alternative agents to ZOL, as they do not have a high affinity for bone, thus their widespread systemic distribution may be used for the treatment of other non-osseous malignancies including lung metastases, often associated with increased mortality in OS. Most importantly, the lack of effect on normal bone cells by the IMP make them very desirable anti-cancer agents however further investigation is required to fully understand the mechanism of augmentation with TRAIL. By understanding the pathways involved and exploiting

their use, there is exciting potential for a targeted therapeutic approach in the management of OS clinically.

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in a University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Agatha Labrinidis

ACKNOWLEDGMENTS

My biggest thankyou is to my mentor, Dr Andreas Evdokiou whom I greatly admire and respect and whose enquiring mind and passion for research makes me want to be a better scientist. Thankyou for giving me the opportunity to become part of your lab. I have learnt so much and really appreciate your time and effort over the years.

Professor David Findlay, your endless knowledge has been invaluable, your encouragement has been inspirational and your excitement regarding results contagious. Your support throughout the years has been greatly appreciated. God bless you.

A special thanks to Shelley Hay, for her expertise in the lab and for putting up with my never-ending questions without losing the smile on her face…you are a gem! Thankyou to Gerald Atkins, Katie Welldon, Steve Bouralexis, Vasilios Liapis, Thai Le Minh and Tina Vincent for making the lab a great place to work in!! To all the wonderful friends I have made at the IMVS, especially Peter, Amanda, Angela, Fiona, Penny, Rosa, Sally, Andrea, Chris, Debbie and Anne-Marie. Thanks for the morning teas, coffees, lunches and good times that I will never forget……friends for life!!

Thankyou to Dr Peter Choong, for providing the KRIB cells and for his expertise in intra-tibial injections; Dr Brendan Coventry for his collaboration with our lab regarding the mouse work. Thankyou to Dr Peter Self for his wonderful explanations and help during micro-CT analysis and to Dr Rob Moore for use of the Faxitron for radiograph analysis. A special thankyou to Justin Lokhorst from Health Sector consulting, Faculty of Health Sciences, University of Adelaide for his invaluable statistical support. I extend my appreciation to the Anti-Cancer foundation of SA and the Australian Research Council for supporting this work.

My biggest thankyou is to my wonderful family, for their support and encouragement over the years!! Mum and dad I love you more than life itself...you give me energy!! Stav, Nick and Paul, you guys are the best and I'm proud to be your sister!!! Thanks for putting up with me for so long! Finally, a huge thankyou to all my friends (old and new) and relatives (especially my precious thitsa), who believed in me and supported me through this time. You are all in my heart!

PUBLICATIONS ARISING DURING CANDIDATURE

Evdokiou A, Labrinidis A, Bouralexis S, Hay S, Findlay DM. (2003). Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis. *Bone* **33**: 216-228

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CONFERENCE PRESENTATIONS:

Labrinidis A., Nguyen O., Bouralexis S., Hay S., Findlay DM. and Evdokiou A. 2003 Inhibitors of the mevalonate pathway sensitise osteosarcoma cells to TRAIL induced cell death. International Conference on Metabolic Bone Disease, Coolum, Qld. Oral presentation.

Labrinidis A., Nguyen O., Bouralexis S., Hay S., Findlay DM. and Evdokiou A. 2003 Inhibitors of the mevalonate pathway sensitise osteosarcoma cells to TRAIL induced cell death. Australian Society for Medical Research (ASMR) SA scientific meeting, Adelaide, SA. Oral Presentation.

Labrinidis A. Bouralexis S., Hay S., Findlay DM. and Evdokiou A. 2002 The nitrogen-containing bisphosphonate, zoledronic acid sensitises osteogenic sarcoma cells to

TRAIL-induced cell death. Australian Society for Medical Research (ASMR) SA scientific meeting,, Adelaide, SA. Oral Presentation.

Evdokiou A., **Labrinidis A.**, Bouralexis S., Hay S., Atkins GJ. and Findlay DM. 2002 Zoledronic acid enhances TRAIL cytotoxicity of human osteosarcoma cells: A novel non-toxic approach to bone cancer therapy. The 9th International Congress on TNF-related cytokines, San Diego, California, USA. Oral presentation.

Bouralexis S., Findlay D.M., Atkins G.J., **Labrinidis A.,** Hay S, and Evdokiou A. 2002 Chemotherapy Sensitises Resistant BTK-143 Osteogenic Sarcoma cells to TRAIL-Induced Apoptosis. Australian Society for Medical Research (ASMR) SA scientific meeting, Adelaide, SA. Oral Presentation.

Labrinidis A, Bouralexis S., Hay S., and Evdokiou A. 2002 The nitrogen-containing bisphosphonate, zoledronate sensitises bone cancer cells to TRAIL-induced cell death. 14th Lorne Cancer Conference, Lorne, Vic. Poster Presentation.

Bouralexis S., Findlay D.M., Atkins G.J., **Labrinidis A.,** Hay S., and Evdokiou A. 2002 Progressive resistance of BTK-143 Osteogenic Sarcoma cells to TRAIL-Induced Apoptosis is Mediated by Acquisition DcR2. 14th Lorne Cancer Conference, Lorne, Vic. Poster Presentation.

Labrinidis A, Bouralexis S., Hay S., and Evdokiou A. 2002 The nitrogen-containing bisphosphonate, zoledronate sensitises bone cancer cells to TRAIL-induced cell death. Australian and New Zealand Bone and Mineral Society 12th Annual Scientific Meeting, Adelaide, SA.. Poster Presentation.

AWARDS RECEIVED:

1. Roger Melick Young Investigator award, received at the International Conference on Metabolic Bone disease, Qld, Australia in June 2003.

2. BioRad New Investigator Award for best oral presentation received at the Australian Society for Medical Research Adelaide Meeting in May 2003.

3. Research abroad scholarship awarded by the University of Adelaide to attend the 4th International Conference on Cancer-Induced Bone Diseases in San Antonio, Texas, USA, December 2003.

4. Travel grant awarded to attend the International Conference on Metabolic Bone disease, Qld, Australia, June 2003.

5. Travel grant awarded to attend the 13th Lorne Cancer Conference, Vic, Australia, February 2002.

LIST OF ABBREVIATIONS

αMEM	α-Minimum Essential Media
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
Apo2L	apoptosis-2 ligand
aa	amino acid
AIF	apoptosis inducing factor
Apaf-1	apoptosis protease-activating factor-1
ATCC	American Type Culture Collection
BH	Bcl-2 homology domain
BIR	Baculovirus IAP repeat
BMD	bone mineral density
BMP	bone morphogenetic protein
bp	base pairs
BPs	bisphosphonates
BSA	bovine serum albumin
CAD	caspase-activated deoxyribonuclease
CARD	caspase-activating recruitment domain
CDK	cyclin dependent kinase
CRD	cysteine-rich domains
cDNA	complementary deoxyribonucleic acid
c-FLIP	cellular FLICE inhibitory protein
CMV	cytomegalovirus
CPM	cyclophosphamide
Crm A	Cytokine response modifier A

DAPI	4',6-diamidine-2'-phenylindole dihydrochloride
DcR1	decoy receptor 1
DcR2	decoy receptor 2
DD	death domain
DED	death effector domain
DNA	deoxyribonucleic acid
DIABLO	direct IAP binding protein with low pI
DISC	death-inducing signalling complex
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethyl sulphoxide
DR3	death receptor 3
DR4	death receptor 4
DR5	death receptor 5
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
Erk	extracellular signal-regulating kinase
FADD	Fas-associated death domain
FAK	focal adhesion kinase
FCS	foetal calf serum
FITC	fluorescein
FLICE	FADD-like ICE
FLIP	FLICE inhibitory protein
FTI	farnesyl transferase inhibitor

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GGTI	geranylgeranyl transferase inhibitor
GPI	glycosyl-phosphatidylinositol
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
Hsp	heat shock protein
Htr2A	High temperature requirement-A2
IAP	inhibitor of apoptosis protein
IBM	IAP-binding motif
ICAD	inhibitor of caspase-activated deoxyribonuclease
ICE	interleukin-1-beta-converting enzyme
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of κB
IL	interleukin
IMP	inhibitors of the mevalonate pathway
IMVS	Institute of Medical and Veterinary Science
iv	intravenous
JNK	c-Jun N-terminal kinase
kD	kilodalton
LDL	low density lipoprotein
LOV	lovostatin
LT-α	lymphotoxin-α
Mab	monoclonal antibody
MAPK	mitogen activated protein kinase
MEV	mevastatin

MLS	mitochondrial localisation sequence
MMP	mitochondrial membrane permeabilization
MMTV	mouse mammary tumour virus
MMR	mismatch-repair
mRNA	messenger ribonucleic acid
N-BPs	nitrogen containing bisphosphonates
NF-κB	nuclear factor kappa beta
NH_2	amino group
NK	natural killer
NSAIDs	non-steroidal anti-inflammatory drugs
OH	hydroxyl group
OPG	osteoprotegerin
OS	osteogenic sarcoma (osteosarcoma)
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCD	programmed cell death
PE	phycoerythrin
PI	propidium iodide
PI-3-kinase	phosphatidylinositol 3-kinase
PKC	protein kinase C
PMSF	phenylmethylsulphanyl fluoride
PTI	prenyl transferase inhibitors
RANKL	receptor activator NF-κB ligand
Rb	retinoblastoma protein
RIP	receptor-interaction protein

rpm	revolutions per minute
sc	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
Smac	second mitochondria-derived activator of caspase
SRE	skeletal-related event
tBID	truncated Bid
TBS	Tris buffered saline
THD	TNF homology domain
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAIL	TNF related apoptosis inducing ligand
TRAIL-R1	TRAIL receptor 1 (also known as DR4)
TRAIL-R2	TRAIL receptor 2 (also known as DR5)
TRAIL-R3	TRAIL receptor 3 (also known as DcR1)
TRAIL-R4	TRAIL receptor 4 (also known as DcR2)
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
Tween 20	Polyxyethylene Sorbitan Monolaerate
UV	ultraviolet
v-FLIP	viral FLICE inhibitory proteins
XIAP	X-linked inhibitor of apoptosis protein
ZOL	zoledronic acid

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CHAPTER ONE

INTRODUCTION

1.1 OSTEOGENIC SARCOMA

1.1.1 Definition, classification, epidemiology and localisation

Osteogenic sarcoma commonly referred to as osteosarcoma (OS), is the most common non-haematopoietic primary malignancy of the skeleton [Pringle 1999]. Osteosarcoma is defined as a spindle cell neoplasm with proliferating malignant cells that produce osteoid (unmineralised bone) or bone [Campanacci 1999]. The production of osteoid is central to the diagnosis of OS but it may be focal or minimal and therefore not obvious [Pringle 1999; Unni 1996]. A diagnosis of OS may be reasonable provided the neoplasm exhibits other typical features even though the tumour has other predominant elements such as chondroid or fibromatoid differentiation [Unni 1996]. There are consequently several varieties of OS that are not distinct enough to warrant a separate classification, as well as several that possess special features (summarised in **Table 1.1**).

Between 1100 and 1500 new cases of OS are reported in the United States yearly [Whyte 1999]. Males are 1.5 times more likely to develop OS than females. The majority of cases occur before the age of 30 with peak incidence in the second decade of life. The cause of most OS is unknown, however several cases seen in later life, particularly the fifth decade, are usually secondary to Paget's disease, radiation, chondrosarcoma, fibrous dysplasia, bone infarct or chronic osteomyelitis [Campanacci 1999; Unni 1996]. Rare cases of siblings presenting with OS have also been reported [Harmon and Norton 1966].

Three quarters of all cases fall into the "classic" OS category, which is considered a high-grade malignant bone tumour and includes osteoblastic, fibroblastic and chondroblastic OS. The remaining 25 percent are classified as "variants" on the basis of clinical, morphological characteristics and location [Pringle 1999]. A representative radiograph image of an OS is shown in **Figure 1.1** [Campanacci 1999].

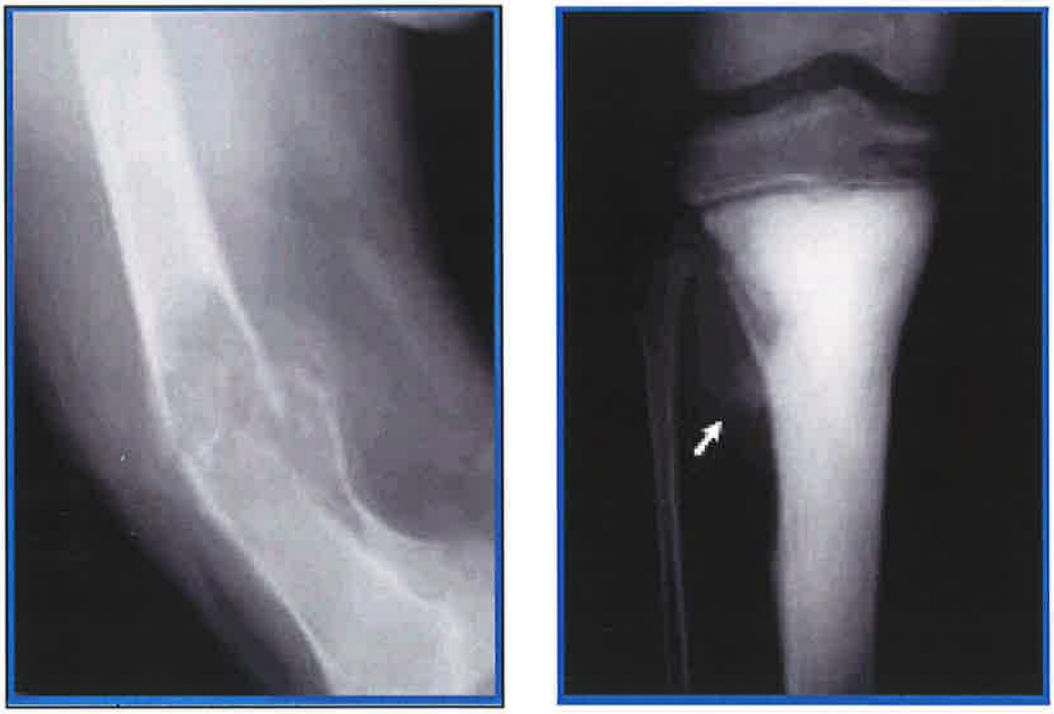


Figure 1.1 Radiological images of an osteosarcoma. The image on the left represents an osteosarcoma located in the distal femur and the image on the right shows the location at the proximal tibia.

High Grade Malignancy

- Classic osteosarcoma (osteoblastic, fibroblastic, chondroblastic)
- Telangiectatic osteosarcoma
- Osteosarcoma of the jaws
- Small cell osteosarcoma
- Osteosarcoma of the bone surface (Peripheral conventional osteosarcoma)
- Intracortical osteosarcoma
- Multifocal osteosarcoma
- Secondary osteosarcoma to disease
- Secondary osteosarcoma to radiation

Low Grade Malignancy

- Parosteal (juxtacortical) osteosarcoma
- Periosteal osteosarcoma
- Low-grade central osteosarcoma

Table 1.1 Osteosarcoma are divided into two major categories with various types within each category. These are based on histology and location of the tumour.

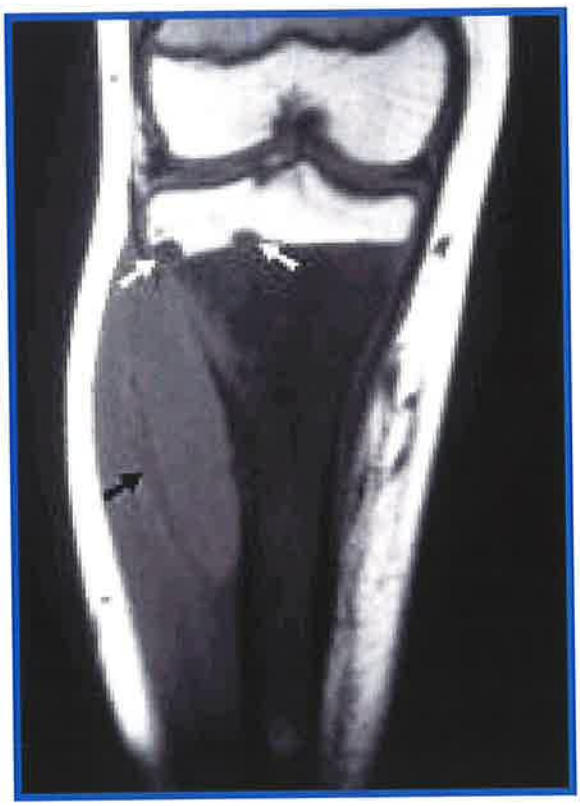


Figure 1.2 Visualisation of an osteosarcoma within the proximal tibia using magnetic resonance imaging. Lytic lesions in the bone are indicated by white arrows and soft tissue tumour is indicated by a black arrow.

Osteosarcoma is predominantly an intramedullary tumour but in rare cases may originate at the bone surface in the case of peripheral conventional OS and parosteal OS or intracortically (intracortical OS) [Campanacci 1999]. Localisation is often found in the metaphyseal part of the long bones with common sites involving the distal femur, proximal tibia and proximal humerus [Campanacci 1999]. Almost 70 percent of all OSs are located in close proximity to the knee or the shoulder with symptoms including pain and swelling of the affected area [Unni 1996]. Tumours are evaluated further using numerous visual methods such as radiography, angiography, bone scanning, computer tomography and magnetic resonance imaging [Whyte 1999]. Histopathology studies also help determine tumour type and staging, critical for choosing the most beneficial treatment and determining prognosis [Whyte 1999]. Metastatic spread to the lungs is common to 20% of presenting patients, with lymph nodes and other sites of the skeleton less common sites of metastases [Saeter *et al.* 1997; Saeter *et al.* 1997].

1.1.2 Diagnosis of osteosarcoma

The diagnosis of OS depends on a combination of clinical, imaging and histological data thus requiring multiple methods of investigation. To base diagnosis exclusively on one method may be misleading as sampling size of a biopsy alone may not identify all cell types, or imaging alone may not be characteristic for that tumour type [Huvos 1991; Campanacci 1999].

Clinical tools or important information include the age of a patient, which will help identify possible tumour types and the growth rate of the lesion, which can differentiate between high grade (rapid growth), or low grade (slow growth) malignancy. Symptoms such as pain and fever also give clues to diagnosing the neoplasm. The most important information regarding the type of tumour is the site and

localisation and this can be investigated in detail using a range of imaging methods [Huvos 1991; Campanacci 1999]. These include:

1. Conventional radiography- is an essential element in the diagnosis of bone tumours. It provides a negative image of the tumour and reveals the extent of damage to the host bone. Radiographs can often reveal a combination of radiolucency due to osteolysis and radiodensity due to a predominantly osteoblastic phenotype. Substantial osteolysis with undefined borders usually indicates a rapidly aggressive tumour whereas a defined osteogenic border indicates a slow growing tumour [Campanacci 1999]. Radiographs also aid in localising biopsy site for further investigations. Occasionally the presence of an OS is not apparent on a radiograph and further analysis is required [Doney and Vilke 2004].

2. Radioactive isotope bone scans, which identify sites of increased bone turnover by isotope uptake, are useful in exploring the entire skeleton from the skull to the toes, revealing all tumours localised to the bone as well as adjacent soft tissue tumours that may affect the bone.

3. Magnetic Resonance Imaging (MRI)- can distinguish between different tissue types and therefore give superior analysis of soft tissues and bone marrow [Exner *et al.* 1990; Dousset and Buthiau 1998]. The advantages include lack of ionising radiation so it is safe for pregnant women. As OSs are hypervascular, the use of MR angiography is useful in identifying vessels within and around the tumour [Dousset and Buthiau 1998]. A representative MRI image of an OS is shown in **Figure 1.2** [Campanacci 1999].

4. Computerised Tomography (CT) is by far the most sensitive method of tumour detection of the bone. In bone tumours it reveals: the extent of tumour in the bone, bone marrow spaces, soft tissue and extra-compartmental spaces; the involvement with the bone cortex if any; tumour involvement of the joint; and relationship with nearby neurovascular structures [Murphey *et al.* 2003; Dousset and Buthiau 1998]. CT is also

used to reveal pulmonary metastases, as it is more sensitive than standard radiography. The presence of metal such as plates and screws inhibits the generation of good quality images but this is improving with new technological advances such as new generation CT instruments and metal artefact reduction [Dousset and Buthiau 1998; Looney *et al.* 2002; Puri *et al.* 2002; Schwarz *et al.* 2003; Stamenkov *et al.* 2003].

After visualisation, a small biopsy, to minimise spreading the tumour, is carefully taken from the site and sent for histological testing [Mankin *et al.* 1982]. The tumours are staged using the histological data, in combination with extension of the tumour into other compartments, as well as metastases. The reason for staging tumours is to try and classify them into groups with a similar behaviour and prognosis and as a guide for treatment [Huvos 1991]. Prognosis is related to the stage, with a higher survival rate observed in early stage patients that those diagnosed at a later stage.

1.1.3 Treatment of osteosarcoma

The treatment of OS has undergone considerable changes over the past 20 years with the efficacy of chemotherapy significantly improving long-term survival to between 55-70%. This is compared to less than 20% survival after 2 years with surgery alone [Link *et al.* 1986; Provisor *et al.* 1997].

Current treatment consists of preoperative chemotherapy (neoadjuvant), to improve the stage of the tumour, followed by surgery (conducted in over 80 percent of OS patients), followed by postoperative (adjuvant) chemotherapy, to prevent local recurrences and metastases [Provisor *et al.* 1997; Kumta *et al.* 2002]. Response to chemotherapy depends on the type and combination of drugs used, the doses given and sensitivity/resistance of the tumour cells. Chemotherapeutic agents used include doxorubicin, etoposide, vincristine, methotrexate and many more, all which act to arrest the growth and induce necrosis in tumour cells.

The surgical procedure in practice today is termed limb-sparing surgery and has replaced the former preference for amputation [DiCaprio and Friedlaender 2003]. This has been made possible with the use of chemotherapy, which improves tumour burden preoperatively therefore making tumour removal more successful [Rougraff *et al.* 1994; DiCaprio and Friedlaender 2003]. Comparison studies of limb salvage versus amputation in OS patients found no significant difference in the rate of survival between the two groups. There was a slightly higher local recurrence and an additional operation on the limb was necessary more often for patients who had limb-sparing surgery [Rougraff *et al.* 1994]. Comparisons also revealed little difference in the quality of life between these two groups [Refaat *et al.* 2002].

The most important factor to prognosis for long-term survival is response to chemotherapy. The development of drug resistance to chemotherapeutic agents is evident in the clinic and remains a problem [Chan *et al.* 1997]. Drug resistance can vary significantly and recent studies suggest the use of gene expression profiles for the prediction of tumour response to adjuvant chemotherapy [Ochi *et al.* 2004; Desai *et al.* 2003]. This prediction would prove very beneficial in identifying tumours with poor responsiveness to chemotherapy so a more intense treatment regime can be administered. Pharmacogenomic studies are also proving to be important by investigating the inherited basis of variable drug response in patients, based on variations in drug metabolising enzymes and drug transporters [Desai *et al.* 2003]. This knowledge will advance treatment outcome, however has yet to become standard procedure prior to the commencement of chemotherapy. Additional improvements to survival might be obtained with new agents, which have differing mechanisms of action, and these may also be used in combination to increase their effectiveness in the clinic.

1.2 THE APOPTOTIC PATHWAY

1.2.1 Introduction

While the phenomenon of programmed cell death had been accepted for many years within the scientific community, it was not until 1972 that the term 'apoptosis', was first proposed, by Kerr and colleagues. Apoptosis is commonly identified in single cells usually surrounded by healthy-looking neighbours and defined as a series of morphological events that are distinct from necrosis. Apoptosis has been observed in a diverse range of cells and tissues undergoing cell death. Programmed cell death is essential in controlling cell turnover and together with cell proliferation controls the homeostasis of tissues [Jacobson *et al.* 1997]. Increased levels of apoptosis have been implicated in a number of diseases, including autoimmune disease and degenerative conditions. In contrast a failure to initiate apoptosis can be detrimental leading to tumour development and growth [Johnstone *et al.* 2002; Degli-Esposti 1999]. Apoptosis also has an integral role in the programmed destruction of cells in many physiological and pathological events. This includes: the destruction of cells during embryogenesis and metamorphosis, hormone-dependent atrophy, cell deletion in proliferating cell populations, cell death in tumours and in immune cells, cell death induced by cytotoxic T cells and cell death due to injurious stimuli including virally infected and damaged cells [Kerr and Harmon 1991; Degli-Esposti 1999; Nagata 1997]. Therefore, understanding the mechanism of apoptosis may prove to be beneficial in identifying the causes and developing treatment strategies for countless diseases.

Another type of cell death was also identified known as necrosis, usually occurring due to physical or chemical trauma. Necrosis is phenotypically characterised by cytoplasmic swelling and eventual bursting of the cell, releasing its contents into the extra-cellular space and causing an inflammatory response [Trump *et al.* 1965; Nagata

1997]. Since 1972, apoptosis and necrosis have been the two general processes used to describe cell death.

1.2.2 The history of apoptosis

Naturally occurring cell death was first reported in 1842 by Carl Vogt, who noticed this phenomenon in the notochord and adjacent cartilage of metamorphic toads [cited by Vaux 2002]. This led to other landmark discoveries, including the description of cell death by Weismann in 1864, chondrocyte death during endochondral ossification by Steida in 1872 and phagocytosis associated cell death in the muscles of metamorphic toads by Metschnikoff in 1883 [cited by Clarke and Clarke 1996]. In 1889, Beard discovered that an entire population of neurons is lost in fish embryos, and Felix reported scattered cell death in developing mammalian muscle. These reports triggered many papers on the subject but it was Flemming in 1885, who gave the first morphological description of apoptosis, in which he termed "chromatolysis", in a study of naturally regressing ovarian follicles [cited by Clarke and Clarke 1996]. In the early 20th century the popularity of the concept of naturally occurring cell death declined, largely due to the decreasing number of German speaking scientists unable to read early reports. It was not until 1951, when Glucksmann's review re-discovered this phenomena, that a fresh surge of interest was generated. The term 'programmed cell death' was first used in 1965 by Lockshin and Williams to describe developmental cell deaths in insect systems [Lockshin and Williams 1965].

In 1972, Kerr and colleagues used the term 'apoptosis' to describe programmed cell death after realising that the morphology of dying cells was similar to that described by Glucksmann, noting a common morphological pattern that was likely not accidental. Following Kerr's description of apoptosis came two exciting discoveries that carried apoptotic research through to the 21st century. Firstly came the observation that DNA

was degraded into nucleosomal-sized fragments during cell death, which was later reported in 1997 to be due to an endogenous nuclease activated in response to apoptotic stimuli [Liu and Zou 1997; Wyllie 1980]. Secondly, the use of nematode worm *Caenorhabditis elegans* was invaluable in studying apoptosis during development, with the observation that exactly 131 of the 1090 somatic cells generated during development underwent apoptosis [Sulston and Horvitz 1977]. Mutants of worm *C. elegans* were used to identify 12 genes involved in programmed cell death [Hedgecock *et al.* 1983; Hengartner *et al.* 1992; Ellis and Horvitz 1986].

It was not until the late 1980's that the explosion of interest came with an insight into the identification of the biochemical and genetic processes involved in apoptosis, beginning with Bcl-2 and its anti-apoptotic capabilities [Vaux *et al.* 1988].

1.2.3 Morphological and biochemical changes of apoptosis

In contrast to necrosis, the controlled process of apoptosis is characterised by the cell and its nucleus condensing itself for engulfment by macrophages or neighbouring cells without any leakage of cytosolic components that will initiate an inflammatory response [Kerr *et al.* 1972]. Using electron microscopy, the morphological changes associated with apoptosis were identified. These begin with cell shrinkage including a dense cytoplasm and tightly packed organelles. This is followed by chromatin condensation, the most characteristic feature of apoptosis, where the chromatin clumps peripherally under the nuclear membrane with occasional fragmentation of the nucleus. This leads to cell surface blebbing and fragmentation and the formation of membrane bound apoptotic cells or bodies. The apoptotic bodies are phagocytosed by adjacent healthy cells or macrophages and degraded within lysosomes [Searle *et al.* 1975; Kerr *et al.* 1972; Wyllie *et al.* 1981].

Distinct biochemical changes, specifically the activation of a group of cysteine proteases, cause most of the morphological changes observed during apoptosis. This group of cysteine proteases are homologous to each other and are known as caspases [Alnemri *et al.* 1996]. Caspases are highly conserved across different species and over a dozen have been identified in humans [Earnshaw *et al.* 1999]. All caspases possess an active-site cysteine, which cleaves substrates C-terminal to aspartate residues, with the distinct substrate specificity determined by four residues amino terminal to the cleavage site [Thornberry *et al.* 1997]. Activation of caspases leads to the selective cleavage of target proteins resulting in either their subsequent activation or inactivation.

Markers of apoptosis are often used to identify apoptosis. This includes the hallmark 'DNA ladder' due to cleavage of nuclear DNA by endonucleases, which are activated by caspase-3-mediated cleavage [Sakahira *et al.* 1998], generating fragments of 180-200 base pair lengths representing nicks at the linker regions between nucleosomes [Walker *et al.* 1997; Wyllie 1987]. This differs from the random fragmentation of DNA seen in necrosis. It has been shown that apoptotic cell death can occur in the absence of DNA fragmentation [Cohen *et al.* 1992].

The cleavage of the enzyme poly(ADP-ribose) polymerase (PARP-1) is another marker of apoptosis [Kaufmann *et al.* 1993]. PARP-1 is involved in DNA repair, replication and transcription, therefore cleavage of the protein may be necessary for cell death to occur [Jeggo 1998; D'Amours *et al.* 1999]. The enzymes caspase-3 and caspase-7, are able to cleave PARP-1, producing an 89 kDa C-terminal fragment and a 24 kDa fragment, thus preventing DNA repair, vital for survival [Kaufmann *et al.* 1993]. It has been reported that liver apoptotic cells do not show PARP-1 cleavage and PARP-1 gene knockout studies in mice have not shown any apoptotic defects [Jones *et al.* 1999; Wang *et al.* 1995]. Alternatively, possible PARP-1 cleavage has also been

detected in non-apoptotic cells, suggesting that there may be exceptions to the rule [Budihardjo *et al.* 1998].

Over 100 caspase substrates have been identified, including cleavage of the nuclear lamins during apoptosis, which results in nuclear shrinking and budding, and the cleavage of fodrin and gelsolin, disrupting cytoskeletal integrity [Nicholson 1999; Rao *et al.* 1996; Kothakota *et al.* 1997].

1.2.4 Anoikis

Cell death can be triggered by numerous factors including the loss of cell anchorage. In 1994, Frisch and Francis first used the term anoikis, derived from the Greek language meaning state of homelessness, to describe this form of apoptosis. Since then, the association of cell anchorage and cell survival has been studied in numerous adherent cell types and found to play an important role in tissue homeostasis *in vivo*. The role of anoikis in the pathogenesis of disease is of particular importance in trying to understand how cells are rendered resistant to loss of cell anchorage such as tumour metastasis [Ruoslahti and Reed 1994]. Overexpression of oncogenes such as ras, raf and rac has been shown to render cells resistant to anoikis and these oncogenes are implicated in neoplasms that are highly metastatic [McFall *et al.* 2001; Rytomaa *et al.* 2000; Coniglio *et al.* 2001].

Cell anchorage is an extremely complex process involving many adhesion molecules such as integrins and cadherins, that are capable of interacting with varying structural components, including the extracellular matrix (cell-matrix anchorage) and other cells (cell-cell anchorage) for review, [Giancotti and Ruoslahti 1999; Aberle *et al.* 1996]. The process of loss of anchorage alters anchorage-dependent survival signals ultimately leading to the cell's demise [Khwaja and Downward 1997; Cardone *et al.* 1997; Barberis *et al.* 2000].

1.3 THE CASPASE CASCADE

1.3.1 Caspase structure and function

Caspases are fundamental mediators of apoptosis that are highly conserved across phyla and essential for the normal development of higher organisms. Thornberry and Molinequx identified the first caspase, ICE (interleukin-1β converting enzyme; caspase-1), in 1992, by using sequence homology studies based on the *C. elegans* death gene, *ced-3*, essential for all 131 programmed cell deaths that occur during hermaphrodite development. The *ced-3* gene product was found to be homologous to mammalian ICE, implicating a similar cell death pathway in mammals [Yuan *et al.* 1993]. More importantly, this information led to the realisation that specific proteolysis is an essential role in apoptotic cell death. Caspase substrates were also identified, shedding light on the biochemical complexity involved following caspase cleavage.

The caspase family now consists of at least 14 members, of which 11 human enzymes are known, with similarities in amino acid sequence, structure and substrate specificity [Nicholson and Thornberry 1997]. All members are cysteine proteases capable of cleaving proteins C-terminal to an aspartic acid residue [Thornberry *et al.* 1997]. They are produced as inactive zymogens containing an amino terminal pro-domain, a large subunit domain (~20kD) and a small subunit domain (~10kD) [Thornberry and Lazebnik 1998]. Phylogenetic analysis reveals two sub-families related to either ICE (caspase-1), important for inflammation, or the mammalian equivalent of CED-3, important for apoptosis (**Figure 1.3**). Further subdivisions can be made depending on the size of the prodomain [Nicholson 1999]. The first group, such as caspases-1, -2, -4, -5, -9, -11 and –12, are the initiator caspases and contain a long prodomain (>10kDa) and a N-terminal caspase-activating recruitment domain (CARD). Caspase-8 and –10 have a N-terminal death effector domain (DED). Caspase –1, -4, -5, -11 and –12 are mainly responsible for processes other than apoptosis. The other group

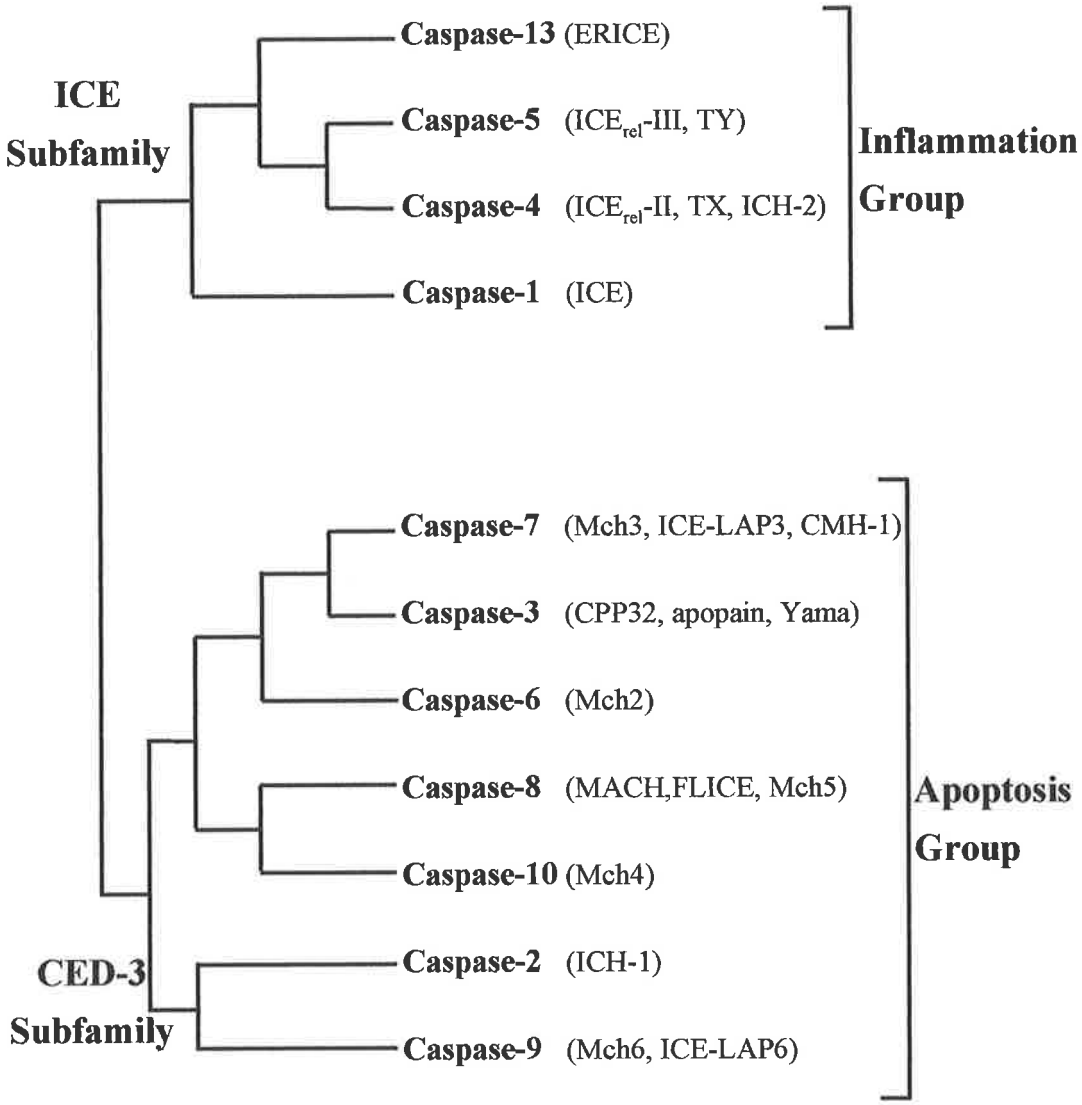


Figure 1.3 The human caspase gene family. Two major subfamilies exist in the caspase family related to either ICE or CED-3. Further subdivisions can be made based on their proteolytic specificities (not shown). Each subfamily has a role in either inflammation or apoptosis.

consists of the effector caspases, such as caspases-3, -6, -7, which contain a short prodomain (<30 aa) and lack the N-terminal enzymatic domain. These differences lead to different modes of activation between the two groups.

A pro-apoptotic signal can lead to either death receptor- or mitochondrial-mediated activation of initiator caspases, capable of triggering the proteolytic caspase cascade during apoptosis [Medema *et al.* 1997; Green 1998].

1.3.2 Caspase activation

At least three mechanisms of caspase activation exist in mammalian cells; (1) recruitment-activation, (2) trans-activation and (3) auto-activation. In the first case, recruitment of the proenzyme to an oligomeric activation complex using an adaptor molecule leads to activation, such as, recruitment of pro-caspase-8 by FADD, after ligand binding to CD95/Apo-1/Fas/TNF death receptors. This leads to the formation of a death inducing signalling complex (DISC) and subsequent caspase activation (will be discussed in detail in section 1.5.4), [Bodmer *et al.* 2000; Medema *et al.* 1997]. Another example of recruitment activation involves the cytosolic protein apoptosis protease activating factor-1 (Apaf-1), which remains latent until the release of cytochrome *c* from the mitochondria during apoptosis [Li *et al.* 1997; Green and Reed 1998]. Cytochrome *c* forms a complex with Apaf-1 that is able to enhance autoactivation of caspase-9 in the presence of dATP. Activated caspase-9 acts as the initiator caspase activating pro-caspase-3, resulting in activation of the caspase cascade. Initiator caspases -8 and -9 represent the starting points for caspase activation either *via* death receptor binding or mitochondrial participation, respectively.

Secondly, trans-activation occurs when an active caspase is able to induce proteolytic activation by coming into very close proximity to the pro-caspase, with the help of Hsp60 activity [Buckley *et al.* 1999]. The pro-domain is removed by

autocatalysis and the large and small subunit domains then associate to form heterodimers, which then associate with other heterodimers to form tetramers, containing two independent catalytic sites. Both subunits contribute to form the catalytic sites that are highly specific, only cleaving after an aspartic acid with a minimum of four NH_2-terminal amino acids to the cleavage site for effective proteolysis [Walker *et al.* 1998]. This is a common mechanism, by which initiator caspases activate effector caspases in sequential order and recognition of this sequential activation has led to the common term 'caspase cascade' [Walker *et al.* 1998].

Finally, caspases can also undergo autocatalytic-activation to ensure a rapid response to apoptotic stimuli [Buckley *et al.* 1999].

1.3.3 Inhibition of caspase activation

The endogenous inhibitors of caspases in humans are members of the inhibitor of apoptosis (IAP) family. They act to counteract cell death by directly inhibiting active caspases and blocking caspase activation [Holcik and Korneluk 2001; Deveraux and Reed 1999]. All IAP family members contain up to three baculovirus IAP repeat (BIR) domains of ~70 amino acids, essential for their inhibitory function. Proteins containing BIR domains have been identified in a wide range of species including yeast, insects, birds and mammals [Deveraux and Reed 1999]. The apoptotic program is unclear in yeast, suggesting that proteins containing BIR domains may be involved in other processes [Fraser and James 1998]. Eight members of the IAP family have been identified in humans including, XIAP/hILP, c-IAP/HIAP-2, c-IAP/HIAP-1, NAIP, Survivin, BRUCE, MLIAP and ILP-2 as shown in **Figure 1.4** [LeBlanc 2003]. The first member of the IAP family identified in humans, X-linked IAP (XIAP), is a potent inhibitor of caspases-3, -7 and -9 [Deveraux *et al.* 1997; Deveraux and Reed 1999]. More specifically, the second BIR domain (BIR2) targets caspases 3 and 7 and the third

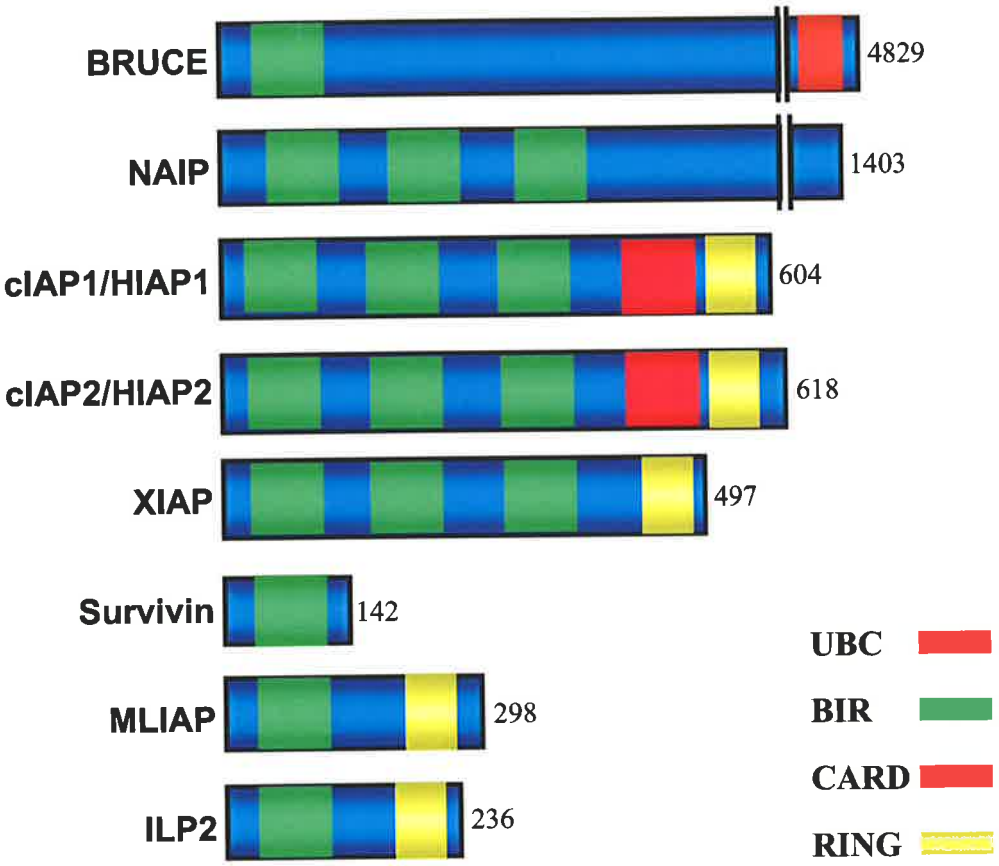


Figure 1.4 Structural representation of the IAP family. Eight human IAPs have been identified and are represented above. The amino acid length is indicated to the right of the IAP and the location of the Baculovirus IAP repeat (BIR), caspase recruitment domain (CARD), really interesting new gene (RING) and ubiquitin conjugating (UBC) domains are indicated.

BIR domain (BIR3) targets caspase 9, indicating that a single BIR domain can be sufficient to inhibit caspases [Chai *et al.* 2001; Huang *et al.* 2001; Riedl *et al.* 2001]. Other family members have also been shown to act as caspase inhibitors including c-IAP/HIAP-2, c-IAP/HIAP-1, ML-IAP and ILP-2 [Salvesen and Duckett 2002]. In addition to regulation of apoptosis, IAP members have also been implicated in other processes for example the involvement of the protein survivin during mitosis [Reed and Bischoff 2000].

IAPs do not bind or inhibit caspase-8, the initiator caspase in CD95/Apo-1/Fas/TNF death receptor-induced apoptosis, instead they protect the cell by binding to its substrate, caspase-3 and inhibit further caspase activation [Deveraux *et al.* 1997]. Conversely, activation of the mitochondrially dependent pro-caspase-9 is directly inhibited by IAPs, preventing activation by the cytochrome *c*/Apaf-1 complex. IAPs also inhibit the next substrate in the cascade, caspase-3, preventing activation of pro-caspase-9 *via* a feedback loop mechanism [Srinivasula *et al.* 1998]. Over-expression studies have confirmed that IAPs are able to protect against anti-apoptotic proteins of the Bcl-2-family such as Bax, which are able to induce cytochrome *c* release from the mitochondria [Deveraux *et al.* 1997; Jurgensmeier *et al.* 1998]. Over-expression studies of pro-caspases 3, 7, or 9 leads to apoptosis but co-transfection of IAPs prevents activation of caspases suggesting that the ratio of caspase to IAPs is critical in determining cell life or death [Deveraux *et al.* 1997].

The activity of IAPs is controlled at various levels. At the transcriptional level, NFκB is capable of stimulating expression of c-IAP/HIAP-1, c-IAP/HIAP-2 and XIAP [Deveraux and Reed 1999]. In contrast, caspase-mediated cleavage is regulated by the release of the proteins Smac/Diablo and HtrA2/Omi from the mitochondria, which bind and inhibit IAP, hence promoting apoptosis [Holcik and Korneluk 2001].

1.3.4 Importance of caspase activity

The caspase connection to apoptosis was first revealed in the nematode *C. elegans* when a *ced-3* gene mutation led to the survival of cells normally destined to die [Ellis and Horvitz 1986]. Genetic studies in mammals have also revealed the importance of caspases in normal development. Gene deletion experiments have provided much information regarding the importance of caspases in development, however the significance of these deleted genes may be underestimated, as alternative caspases may compensate for the loss of a particular gene, therefore double knockouts may be more informative in defining their roles. Mammalian caspase mutants often display tissue-specific defects in apoptosis probably due to the large number of caspases that exist.

The first knockout studies to show developmental defects due to insufficient apoptosis were observed in caspase-$3^{-/-}$ knockout mice, which led to excess brain tissue and eventual postnatal death [Kuida *et al.* 1998]. No abnormalities were seen in any other organs. Caspase-$9^{-/-}$ knockout mice exhibited similar but less extensive abnormalities, suggesting that caspase-3 plays a critical role in the development of the mammalian brain where it depends on caspase-9 for activation [Hakem *et al.* 1998]. Caspase-$6^{-/-}$ mice develop normally, with minor defects in B cell development [Zheng and Flavell 2000]. Conversely, knockout of caspase-7 leads to embryonic death, indicating that compensation by other caspases exist for caspase-6 but not caspase-7. Premature animal death seen in caspase-$7^{-/-}$ knockouts, as opposed to other caspase knockout mice, might be due to differences in the localisation and onset of expression [Sadowski-Debbing *et al.* 2002].

Caspase-1 and –11 knockout mice revealed normal development but immune deficiencies. Caspase-1 deficient animals had problems with the production of IL-1α and IL-1β, TNF- α, IL-6 and interferon γ production indicating a vital role in the immune response [Kuida *et al.* 1995]. Mice deficient in caspase-11 were also found to

have problems with the production of IL-1α and IL-1β and have fewer apoptotic cells due to a defect in caspase-3 activation, suggesting that caspase-11 may act as an initiator caspase under certain conditions [Kang *et al.* 2000]. Both caspase-1 and –11 play an important role in inflammation but not necessarily apoptosis. In humans, mutations in caspase-10, leads to defective apoptosis of lymphocytes and is associated with type II auto-immune lymphoproliferative syndrome [Wang *et al.* 1999].

Caspase-2 and -12 knockout mice exhibit no marked abnormalities except excess numbers of female germ cells in the caspase-2 deficient mice [Bergeron *et al.* 1998].

Caspase-8 is an important caspase in death receptor signal-mediated apoptosis. Cell lines deficient in caspase-8 are completely resistant to death receptor induced apoptosis and partially resistant to apoptosis induced by UV-irradiation, adriamycin and etoposide [Juo *et al.* 1998]. In mice, caspase-8 deficiency leads to death *in utero,* due to abnormal heart development, abdominal haemorrhage and erythrocytosis in many organs, demonstrating a role in haematopoeisis and a non-redundant role in development [Varfolomeev *et al.* 1998].

Knockout studies have unveiled the role of caspases in apoptosis and normal development as well as providing clues into the specific participation of caspases in other systems such as cytokine maturation, immunity and haematopoeisis.

1.4 THE INTRINSIC PATHWAY OF APOPTOSIS

1.4.1 The Bcl-2 family

The Bcl-2 family of proteins are key regulators of apoptosis capable of responding to death and survival signals generated both inside and outside the cell [Strasser *et al.* 2000]. The Bcl-2 family of proteins are involved in apoptosis during embryogenesis and continue to play an important role in haematopoiesis and tissue homeostasis after birth

[Jacobson *et al.* 1997]. More importantly, the Bcl-2 protein family regulates apoptosis induced by cytokine withdrawal or cytotoxic stress conditions, such as those elicited by anti-cancer drugs [Adams and Cory 1998].

C. elegans encodes only two members of the Bcl-2 family, CED-9 survival factor and EGL-1 death factor. The human Bcl-2 family includes more than 30 anti- and pro-apoptotic molecules that bind to each other to form homo- and heterodimers [Tsujimoto 2003]. The Bcl-2 family is subdivided into 3 categories: (i) (CED-9-like) anti-apoptotic *Bcl-2-like survival factors* such as Bcl-2, Bcl-x_L, Mcl-l, Al/Bfl-l, NR-13, Boo/Diva/Bcl2-L-10 and Bcl-B; (ii) pro-apoptotic, *Bax-like death factors* such as Bax, Bak, Bok/Mtd, Bcl-xs; and (iii) EGL-1-like pro-apoptotic, *BH3-only death factors* such as Bik/Nbk, Blk, Hrk/DP5, BNIP3, Bim_L/Bod Bad, Bid, Noxa, PUMA/Bbc3 and Bmf [Huang and Strasser 2000]. Interactions between a pro-apoptotic and anti-apoptotic factor affects the threshold, which determines the fate of the cell.

The *Bcl-2-like survival factors* contain three to four Bcl-2 homology domains (BH1-BH4), capable of mediating interactions with other proteins and essential to their survival function [Farrow and Brown 1996; Hanada *et al.* 1995; Kelekar and Thompson 1998]. These proteins are commonly bound to intracellular membranes such as the mitochondria and nuclear/ER membranes, and act as scavenger molecules for *BH3-only* and *Bax-like death factors* thereby inhibiting their apoptotic function. They have been shown to localise to other regions within the cell and still act as survival factors, but to a lesser extent [Borner *et al.* 1994; Borner 2003].

As the name suggests, the *BH3-only death factors* share only the short BH3 domain [Huang and Strasser 2000]. The levels of *BH3-only death factors* in mammalian cells are controlled at the transcriptional and post-translational level, and are activated in response to an apoptotic signal *via* several mechanisms [Puthalakath and Strasser 2002]. These include (i) transcriptional induction- PUMA/Bbc3 and NOXA are induced

by a p53-dependent apoptotic signal, (ii) post-translational dephosphorylation- BAD is normally phosphorylated and sequestered in the cytoplasm by binding to scaffold proteins until an apoptotic stimulus leads to its dephosphorylation and dissociation so that it can bind to Bcl-2 survival proteins (iii) proteolysis- the inactive cytosolic form of BID is cleaved by caspase-8 into a truncated form that translocates to the mitochondria, (vi) cytoskeletal sequestration- Bmf interacts with the actin cytoskeleton and is released in response to anoikis stress signals. Thus *BH3-only death factors* act as sensors and are activated in response to an apoptotic signal.

Bax-like death factors are converted into pro-apoptotic proteins after removal of the N-terminal BH4 domain by proteolysis [Cheng *et al.* 1997]. Modified *Bax-like death factors* contain three BH domains and undergo a conformational change that favours apoptosis [Adams and Cory 1998]. This cannot entirely explain the difference between *Bcl-2-like survival* and *Bax-like* death *factors* as some *Bcl-2-like* lack a BH4 domain and the addition of BH4 to Bax is insufficient to convert it then to survival factors, suggesting that other protein domains may be involved [Adams and Cory 1998; Borner 2003]. Activation of the *Bax-like death factors* leads to their stable insertion into the mitochondrial membrane to form a pore, thus increasing permeability of the outer membrane [Antonsson 2001]. How activation occurs at the mitochondrial membrane in response to apoptotic stimuli remains unknown.

1.4.1 The role of the Bcl-2 family in cancer

Bcl-2 and its homologues have been associated with numerous types of cancer including human follicular centre lymphoma in which up to 85% of cases contain a translocation of the Bcl-2 gene leading to overexpression of the protein and increased survival of the tumour cell [Tsujimoto *et al.* 1988]. Overexpression of Bcl-2 has also been reported in prostate, breast and colon carcinomas and glioblastomas [Bettaieb *et al.*

2003]. Another *Bcl-2-like survival factor*, Mcl-l, is often overexpressed in relapsed AML resulting in poorer prognosis. Similarly loss of function mutations in pro-apoptotic proteins such as Bax have also been implicated in certain cancers leading to changes in the pro- and anti-apoptotic protein ratio [Bettaieb *et al.* 2003].

As part of its role in the cell, the tumour suppressor gene p53 can induce cell cycle arrest or apoptosis in response to DNA damage. Mutations in p53 are found in almost half of all human cancers and mutations in other genes involved in the p53 signalling pathway, such as arf and mdm2, have also been identified despite the presence of a wild-type p53 [Levine 1997]. Defective p53 signalling also affects the induction of the *BH3-only death factors,* PUMA/Bbc3 and NOXA effectively tipping the balance of anti- and pro-apoptotic proteins towards tumour cell survival [Oda *et al.* 2000; Nakano and Vousden 2001].

Since overexpression of anti-apoptotic proteins promotes tumourigenesis, then pro-apoptotic proteins may serve as tumour suppressors such as the induction of the *BH-3-only death factor* Bik/Nbk in response to an overexpressing adenovirus protein [Mathai *et al.* 2002].

It still remains controversial as to whether the outcome of cancer treatment is determined by apoptosis sensitivity or a result of the irreparable damage sustained by the cell [Brown and Wouters 1999].

1.4.3 The mitochondrial intrinsic pathway

The first critical step in the intrinsic apoptotic pathway is the release of proteins from the mitochondrial membrane, which ultimately leads to caspase activation. This is regulated by numerous factors which leads to mitochondrial membrane permeabilization (MMP), and can be induced by pro-apoptotic second messengers including Ca^{2+}, reactive oxygen species, certain lipids such as ceramide and stress

kinases [Zamzami and Kroemer 2003]. As mentioned earlier, pro-apoptotic Bcl-2 proteins also promote MMP, whereas anti-apoptotic Bcl-2 proteins act to inhibit MMP.

An apoptotic signal can lead to cell death *via* activation of either initiator caspase –8 or –9, which are capable of mediating two distinct sets of signals. Activation of caspase-8, *via* death receptor-mediated apoptosis (discussed in detail in section 1.5.4), leads to the cleavage of the Bcl-2 protein Bid [Luo *et al.* 1998]. Evidence suggests that truncated Bid (tBid) translocates from the cytosol to the mitochondrial membranes, where it induces MMP together with either Bax or Bak (Bcl-2 anti-apoptotic proteins), which are able to insert into the membrane and cause permeabilization. Translocation of Bid specifically to the mitochondria is due to the membrane lipid cardiolipin, which is not present in any other organelles [Lutter *et al.* 2000]. Anti-apoptotic Bcl-2 proteins bound to the mitochondrial membrane inhibit apoptosis by antagonizing Bax or Bak. Thus the ratio of pro- and anti-apoptotic proteins will determine MMP and thus the fate of the cell [Letai *et al.* 2002].

Similarly apoptotic signals without the involvement of the death receptors and caspase-8, still leads to cell death *via* Bcl-2 proteins. Activation of pro-apoptotic proteins disrupts the Bcl-2 protein ratio and as a consequence mitochondrial membrane integrity is lost, leading to permeabilization and the release of toxic proteins from the mitochondria involved in both caspase-dependent and -independent apoptotic pathways [Saelens *et al.* 2004].

MMP leads to the release of cytochrome *c* into the cytosol where it binds to the C-terminal region of Apaf-1. Apaf-1 is a cytosolic protein with an N-terminal caspase-recruitment domain (CARD), a nucleotide-binding domain with homology *to C. elegans* CED-4, and a C-terminal domain containing 12-13 WD-40 repeats [Zou *et al.* 1997]. The WD-40 repeats facilitate binding to cytochrome *c* and through association of dATP with Apaf-1 leads to the exposure and subsequent oligomerization of the N-terminal

CARD, allowing the recruitment of pro-caspase-9, and activation through a CARD-CARD interaction [Adrain *et al.* 1999; Jiang and Wang 2000]. The cytochrome *c*/Apaf-1/procaspase-9 complex is termed the apoptosome and activated caspase-9 in turn cleaves and activates caspase-3, initiating the caspase cascade as shown in **Figure 1.5** [Li *et al.* 1997; Wang 2001].

1.4.4 Regulation of the mitochondrial intrinsic pathway

Procaspase activation is the critical mechanism within the apoptotic pathway and is regulated by complex processes at various levels including caspase recruitment and procaspase oligomerisation of initiator caspases. Inhibitors of apoptosis proteins (IAP) directly inhibit caspase activation (as mentioned previously in 1.3.3). Regulation also occurs prior to caspase/adaptor interactions with the involvement of Bcl-2 anti-apoptotic proteins such as Bcl-X_L and Boo, which bind to Apaf-1 and procaspase-9 to form a complex that inhibits caspase-9 activation as shown in **Figure 1.6** [Pan *et al.* 1998; Song *et al.* 1999]. Other anti-apoptotic proteins include Bcl-2, which functions to block the release of cytochrome *c* from the mitochondria, thereby preventing Apaf-1 mediated activation of caspase-9 [Green and Reed 1998]. Conversely, Bid acts to promote cytochrome *c* release [Luo *et al.* 1998; Jiang and Wang 2000]. Other anti-apoptosis proteins include, catalytically inactive molecules, which mimic caspases and possess an intact CARD or DED. A variant of caspase-9 has been identified, which interferes with the formation of a functional Apaf-1/caspase-9 activating complex [Seol and Billiar 1999]. Caspase-8-like molecules such as FLIP, contain DED but lack catalytic function and act by blocking caspase-8 recruitment to FADD [Nicholson and Thornberry 1997].

To add complexity to the regulation process, IAP antagonists also exist to overcome IAP inhibition of caspase activation. Numerous proteins exist in *Drosophila* including Hid, Grim, Reaper, Sickle and Jafrac2, and at least two mitochondrial proteins

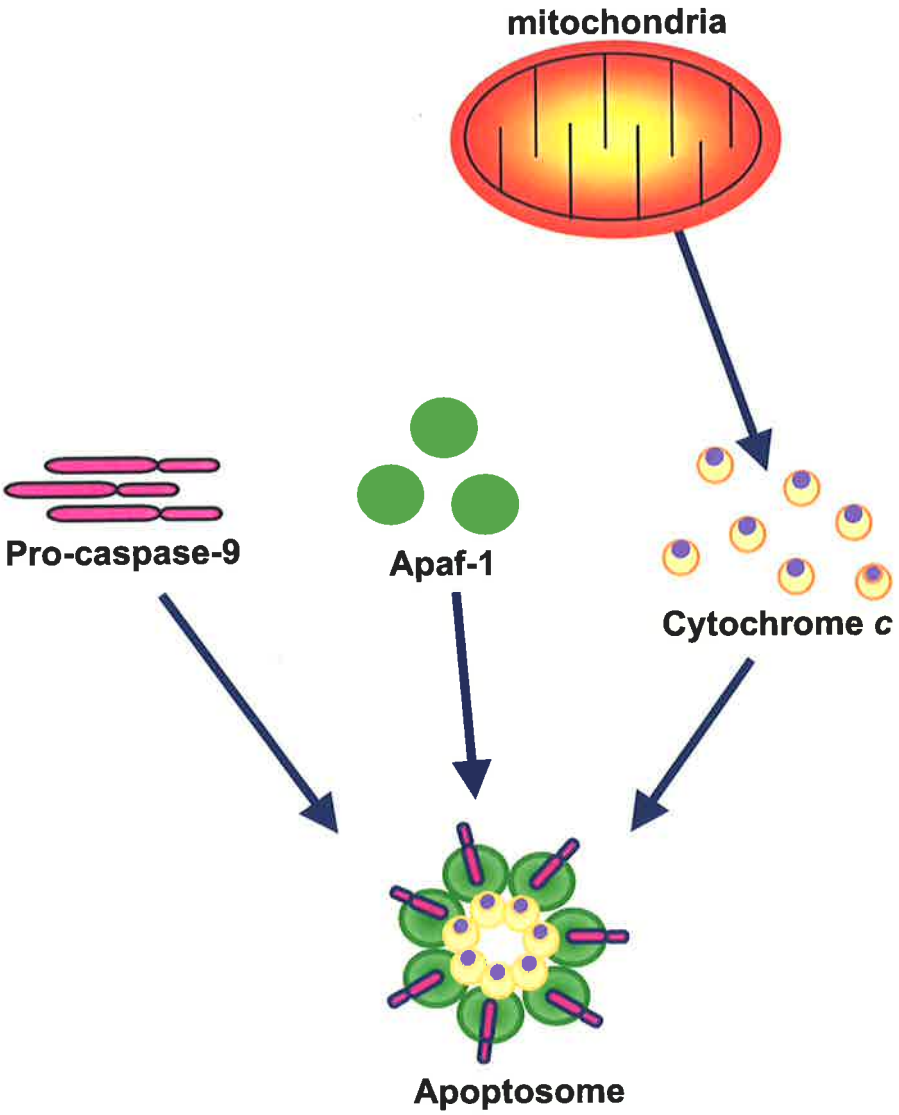


Figure 1.5 Formation of the apoptosome adapted from Cain *et al.*, 2002.

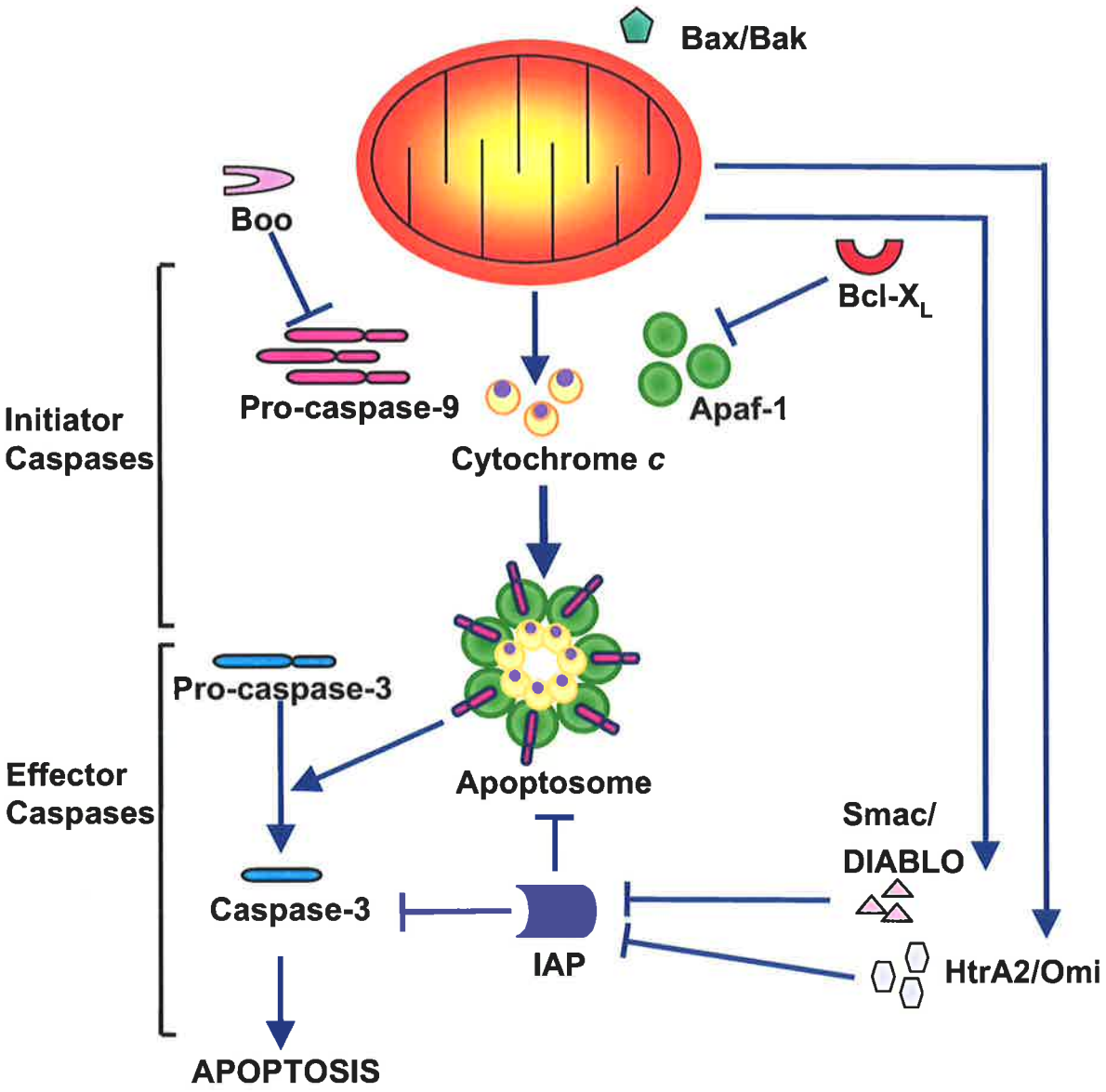


Figure 1.6 The mitochondrial intrinsic pathway.

in mammals, Smac/DIABLO and Omi/HtrA2 [Srinivasula *et al.* 2002; Vaux and Silke 2003]. All these proteins share a conserved four residue IAP-binding motif (IBM) at their N-terminus, which allows them to bind IAPs.

The Smac/DIABLO protein was independently identified by two groups. Smac was found to stimulate caspase-3 activation in humans and DIABLO was found in mice [Verhagen *et al.* 2000; Du *et al.* 2000]. The nuclear encoded Smac/DIABLO protein is expressed in most human tissues and is synthesized as a precursor protein with a mitochondrial localisation sequence (MLS) required for import into the mitochondria. Once there it is modified by proteolysis to remove the MLS and to expose the IBM of the mature protein, which exists as a dimer [Srinivasula *et al.* 2001]. The IBM of the Smac/DIABLO is able to bind XIAP, cIAP1, cIAP2 and surviving (**Figure 1.6**). Each Smac/DIABLO within the dimer is able to bind to BIR2 and BIR3 (baculovirus IAP repeat), which is essential for IAP activity. BIR3 normally binds to caspase-9 and prevents activation, however the binding of IBM of Smac/DIABLO displaces caspase-9 from XIAP and promotes caspase activity [Srinivasula *et al.* 2001].

High temperature requirement-A2 (Htr2A/Omi) is so named due to its upregulation of both expression and proteolytic activity, following heat shock [Gray *et al.* 2000]. Like Smac/DIABLO, Htr2A/Omi protein is imported into the mitochondria with the help of the MLS, where it is also modified to expose the IBM of the mature 35 kD protein, that exist as a trimer [Suzuki *et al.* 2001; Hegde *et al.* 2002]. Apoptotic signals lead to the release of Htr2A/Omi from the mitochondria into the cytosol, where it contributes to caspase-dependent apoptosis by binding to and inhibiting IAP proteins and caspase-independent cell death by signalling pathways *via* its protease activity [Hegde *et al.* 2002; Blink *et al.* 2004]. Htr2A/Omi function resembles Smac/DIABLO by competing with caspase-3, -7 and –9 for XIAP, cIAP1 and cIAP2 binding, thus promoting caspase activity [Suzuki *et al.* 2001; Verhagen *et al.* 2002]. Unlike

Smac/DIABLO, Htr2A/Omi preferentially binds to the BIR2 domain of XIAP and not BIR3 [Verhagen *et al.* 2002]. Following binding to its IBM, Htr2A/Omi then cleaves the IAPs, rendering them irreversibly inactive [Srinivasula *et al.* 2003; Yang *et al.* 2003].

It has been reported by several groups that procaspases are localised within the mitochondria and may promote apoptosis after their release and subsequent activation in the cytosol [Mancini *et al.* 1998; Susin *et al.* 1999; Zhivotovsky *et al.* 1999]. Exactly how the procaspases are imported into the mitochondria remains to be identified, as they all lack a MLS except for procaspase-2. Van Loo and colleagues were unable to identify the presence of caspases within the mitochondria therefore caspase localisation within the cell remains controversial [van Loo *et al.* 2002].

1.4.5 Caspase-independent cell death

Bax-like death factors are directly involved in mitochondrial permeabilisation and therefore provoke the release of pro-apoptotic proteins, including cytochrome *c* and Smac/DIABLO, which have their own specific functions. Up to 30 different proteins were released into the cytoplasm after mitochondrial perforation in Bid- treated mitochondria. These proteins were found to be involved in both caspase-dependent and caspase-independent pathways [van Loo *et al.* 2002].

Endonuclease G is a 33 kD protein, which contains a mitochondrial targeting sequence required for import into the organelle. Endonuclease G is able to induce caspase-independent cell death, by translocating to the nucleus after mitochondrial release, where it degrades DNA [Van Loo *et al.* 2001]. DNA fragmentation occurs in a sequential manner with the cleavage of genomic DNA into high molecular weight sizes followed by nucleosome-sized fragments and is independent of caspases [Widlak *et al.* 2001; Li *et al.* 2001].

Apoptosis-inducing factor (AIF) is a 57 kD flavoprotein, with NADH oxidase activity required for protection against oxidative stress [Miramar *et al.* 2001]. This function contrasts with AIFs ability to also contribute to apoptosis. AIF is released into the cytosol where it translocates to the nucleus following an apoptotic stimulus. In the nucleus AIF contributes to large-scale (50 kb) DNA fragmentation and condensation [Lorenzo *et al.* 1999]. Studies have revealed that AIF itself cannot cleave DNA and may recruit or activate an endonuclease to facilitate DNA fragmentation and condensation [Susin *et al.* 1999; Ye *et al.* 2002]. Specific inhibition of AIF translocation was shown to inhibit nuclear apoptosis in fibroblasts, confirming the importance of AIF nuclear translocation in the execution of cell death [Susin *et al.* 1999]. Mice deficient in AIF show developmental defects including lack of blastocysts, suggesting that caspase-independent cell death is a crucial part of normal development [Joza *et al.* 2001].

Although the activity of Endonuclease G and AIF is caspase-independent, their release into the cytosol may be both caspase dependent and independent, subject to the apoptotic stimulus.

Other non-caspase mediators of programmed cell death are the calpains. The calpains are cysteine proteases and are localised in the cytosol in an inactive form. Activation requires an increase in calcium levels and leads to proteolytic cleavage of the zymogen followed by association with membrane phospholipids. They participate in apoptotic signalling both upstream and downstream of caspases [Nakagawa and Yuan 2000; Waterhouse *et al.* 1998].

The cathepsin protease family have also been implicated in caspase-independent cell death, more specifically cysteine cathepsins B and L and aspartate cathepsin D [Johnson 2000]. Cathepsins are activated in the endosomal/lysosomal compartment by autoproteolysis under low pH, or proteolysis by other cathepsins, and translocate to the

nucleus [Mathiasen and Jaatela 2002]. Cathepsin participation occurs in both caspase dependent and independent cell death and can be triggered by various stimuli.

Studies on the serine protease proteins granzyme A and B, demonstrated that granzyme B is able to cleave and directly activate caspases in caspase-mediated apoptosis only, whereas granzyme A is able to induce death by cleaving DNA even in the presence of caspase inhibitors [Talanian *et al.* 1997; Beresford *et al.* 2001].

Interestingly, calpains, cathepsins and granzymes can also trigger MMP and may be able to activate pro-apoptotic Bcl-2 proteins [Roberg *et al.* 1999; Trapani *et al.* 2000; Waterhouse *et al.* 1998].

1.5 THE EXTRINSIC PATHWAY OF APOPTOSIS

1.5.1 Introduction

The TNF superfamily consists of the TNF receptor (TNFR) family and the corresponding TNF ligand family and is involved in proliferation, survival, cell differentiation and apoptosis [Mallett and Barclay 1991; Smith *et al.* 1994]. To date, 19 different ligand members that signal through 29 receptors of the TNF superfamily have been identified. Many of them have been found to associate with a wide variety of diseases including cancer, septic shock, viral replication, bone resorption, rheumatoid arthritis, diabetes, allergy, atherosclerosis, myocardial infarction, and acquired immune deficiency disease. All the cytokines of the TNF superfamily mediate their effects through the activation of the transcription factors NF-kappaB and c-Jun N-terminal kinase, apoptosis, and proliferation [Gaur and Aggarwal 2003].

1.5.2 The TNF ligand family

The TNF ligands are expressed as type II transmembrane proteins that can have both a membrane embedded as well as cleaved, soluble forms [Idriss and Naismith 2000]. Both forms are processed into biologically active trimers. The TNF ligands share 25-30% sequence homology and are characterised by a conserved C-terminal domain of 150 amino acids, termed the TNF homology domain (THD), which is responsible for trimer assembly but have little sequence similarity in their external surface, which accounts for receptor specificity [Bodmer *et al.* 2002; Locksley *et al.* 2001]. The TNF ligand superfamily consists of TNFα, LT-α, LT-β, CD271, CD30L, CD40L, CD95L, 4-1BBL, OX40L, TRAIL, RANKL, TWEAK and Apo-3 Ligand [Smith *et al.* 1994]. Each member of the TNF ligand family binds at least one receptor from the TNF receptor family, with some ligands such as APO2L/TRAIL, capable of binding several receptors. In addition, many ligands bind the same receptors [Browning *et al.* 1993]. Generally, the TNF ligand family members cause multimerization of the receptors after binding [Banner *et al.* 1993].

1.5.3 The TNF receptor family

The TNFR family members are type 1 transmembrane glycoproteins containing highly conserved cysteine-rich domains (CRDs) involved in ligand binding [Gruss and Dower 1995]. Up to 65% protein sequence homology within the extracellular, amino-terminal CRD region is shared between TNFR family members. The number of CDRs can range from one to six, but is commonly three or four and is responsible for receptor folding resulting in a signalling unit of three receptors often bound by a trimeric ligand molecule [Idriss and Naismith 2000].

The interactions between ligands and their corresponding receptors induce cellular responses such as activation, proliferation, differentiation and apoptosis [French and Tschopp 1999]. The type of response depends on the receptor and the cell type and the final signal received by the cell. Some TNFR family members have the ability to transmit an intracellular death signal capable of mediating apoptosis, these are called death receptors [Locksley *et al.* 2001]. Other receptors do not signal, instead it has been suggested they act as decoys that compete with signalling receptors for the corresponding ligand [Idriss and Naismith 2000]. Signalling members can be divided into two groups based on their cytoplasmic region. One class of receptors contain a cytoplasmic death domain (DD) and are called the death receptors, the other class lack this DD [Ashkenazi and Dixit 1999]. Among the 8 homologous death receptors, at least 6 can stimulate apoptosis through caspase activation [Screaton and Xu 2000]. These include DR3 (WSL-1/Apo-3/TRAMP/LARD), DR6, TNF receptor 1 (TNF-R1/p55-R), Fas (CD95/APO-1/APT1), TRAIL-R1 (Apo2/DR4) and TRAIL-R2 (DR5/TRICK2A/TRICKB/KILLER), [Locksley *et al.* 2001]. The involvement of this receptor class in apoptosis will be discussed in section 1.5.4., below.

1.5.4 The death receptor/extrinsic pathway of apoptosis

The extrinsic pathway is death receptor dependent and requires ligand binding to initiate apoptosis. Stimulation of receptors results in receptor aggregation and recruitment of DD-containing adaptor proteins. These include FADD (Fas-associated death domain/MORT1/MACH) and TRADD (TNFR-associated death domain), which bind to death receptors *via* the DD [Boldin *et al.* 1996; Chinnaiyan *et al.* 1995; Hsu *et al.* 1995]. Caspase-8 is recruited by the adaptor protein to form the death inducing signalling complex (DISC), which results in the subsequent activation of caspase-8 and

initiation of the caspase cascade *via* direct cleavage of downstream effectors [Schulze-Osthoff *et al.* 1998; Walczak and Krammer 2000].

The extrinsic and intrinsic pathway can be interconnected at various levels [Roy and Nicholson 2000]. Activation of caspase-8 *via* death receptor stimulation can result in the cleavage of Bid, a Bcl-2 family member. Initially it was thought that the truncated Bid (tBid) translocates to the mitochondria and triggers cytochrome c release [Luo *et al.* 1998; Li *et al.* 1998]. Recently, Deng and colleagues reported that tBid acts on Bax and Bak to induce mitochondrial release of Smac/DIABLO into the cytosol where it can bind inhibitor of apoptosis proteins, as shown in **Figure 1.7** [Deng *et al.* 2002]. In addition, caspase-6 activation by mitochondria may feed back into the extrinsic pathway by cleaving caspase-8 [Slee *et al.* 1999].

1.6 TRAIL AND ITS RECEPTORS

1.6.1 TRAIL

TNF-related apoptosis-inducing ligand (APO-2 Ligand/TRAIL) was identified independently by two groups, using a conserved TNF member sequence to search the expressed sequence tag (EST) database [Pitti *et al.* 1996; Wiley *et al.* 1995]. For the purpose of this thesis APO2L/TRAIL will be referred to as TRAIL. TRAIL is a type II transmembrane protein with a predicted molecular weight of 32 kD. Human TRAIL is 281 amino acids long, divided into a 243 amino acid residue extracellular region, 21 amino acid transmembrane region and a 17 amino acid cytoplasmic tail [Wiley *et al.* 1995; Pitti *et al.* 1996]. The carboxy-terminal extracellular region can be proteolytically cleaved from the cell surface into a soluble form [Mariani and Krammer 1998]. The carboxy-terminal extracellular region of TRAIL forms homotrimers that bind three receptor molecules [Hymowitz *et al.* 1999]. Unlike other TNF ligand family members,

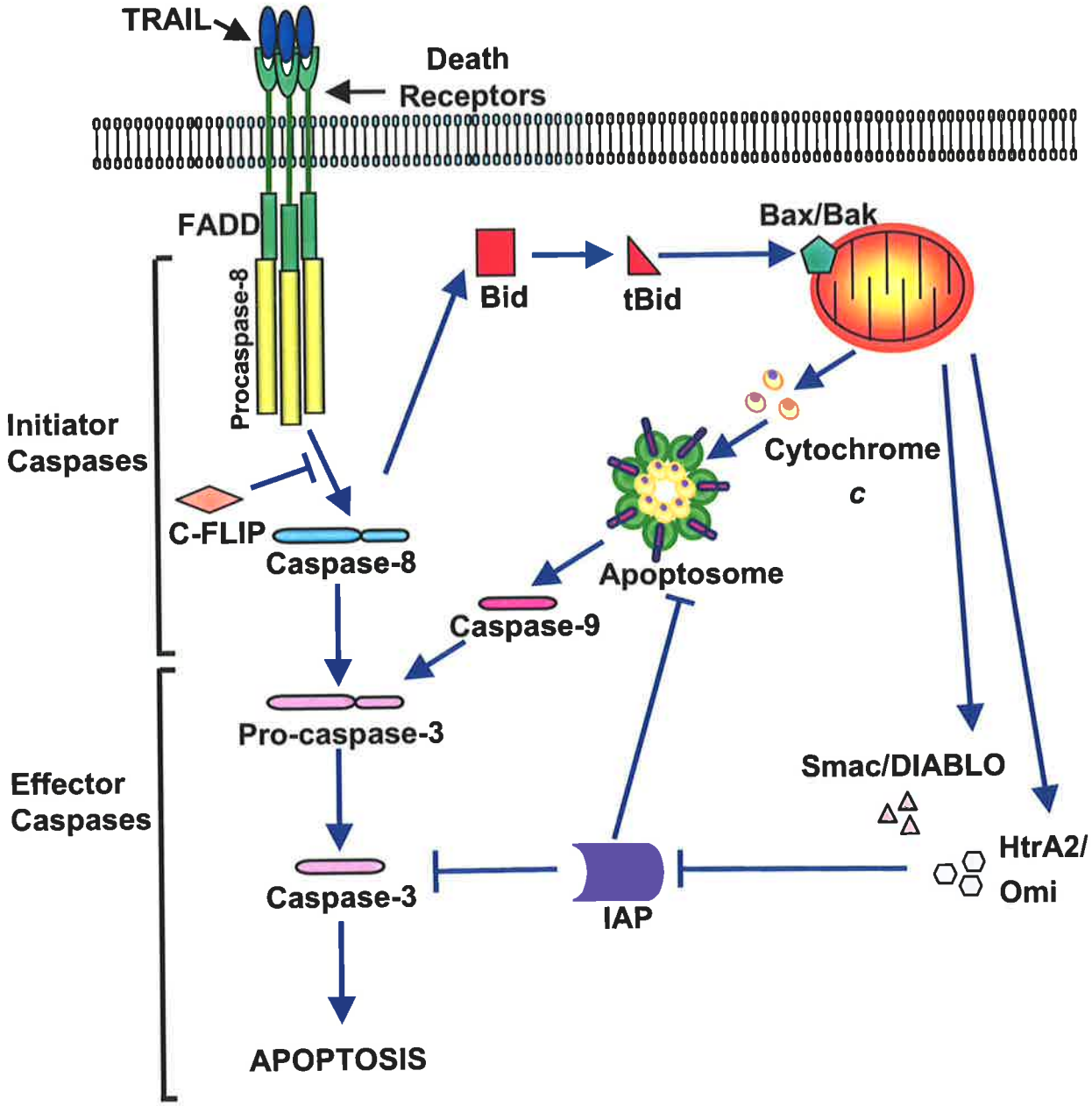


Figure 1.7 The intrinsic and extrinsic pathway

TRAIL contains a zinc ion at the trimer interface held in place by the single unpaired cysteine residue (Cys 230) of each monomer [Mariani and Krammer 1998]. This zinc ion is vital for trimer stability of TRAIL and substituting it leads to reduced apoptotic capability [Bodmer *et al.* 2000; Mariani and Krammer 1998]. Studies have shown that TRAIL has the ability to induce apoptosis in a variety of tumour cells, but not of most normal cells, potentially making it an ideal anticancer agent [El-Deiry 2001; Ashkenazi and Dixit 1999; Jo *et al.* 2000; Walczak *et al.* 1999; Ashkenazi *et al.* 1999; Atkins *et al.* 2002; Evdokiou *et al.* 2002].

TRAIL mRNA expression is widespread in normal tissues and has been reported for peripheral blood leukocytes, bone, brain, colon, kidney, liver, lung, ovary, prostate, spleen, testis and thymus [Wiley *et al.* 1995]. The broad range of tissues expressing TRAIL suggests that TRAIL-induced death may be regulated at the level of the TRAIL receptors, unlike the expression of other TNF family members, which is tightly regulated.

TRAIL induces apoptosis by interacting with its receptors [Wang and El-Deiry 2003]. To date five TRAIL receptors have been identified (see **Figure 1.8**). DR4 and DR5 are known as the death receptors and encode the typical type I transmembrane protein structure, shared with other members of the TNF receptor family. Like several other TNF receptor family members, DR4 and DR5 also possess a sequence within their cytoplasmic domain that is homologous to the death domain [Pan *et al.* 1997; Pan *et al.* 1997; Degli-Esposti *et al.* 1997; Degli-Esposti *et al.* 1997; Pitti *et al.* 1996]. Initially, the discovery of other TRAIL receptors increased the complexity of the TRAIL ligand-receptor system, particularly in regard to the difference in sensitivity of normal and tumour cells to TRAIL-induced apoptosis. However, the receptors known as DcR1 (TRAIL-R3/TRID), DcR2 (TRAIL-R4) and OPG were found to lack apoptotic capability, potentially providing a logical explanation for the protection of normal cells

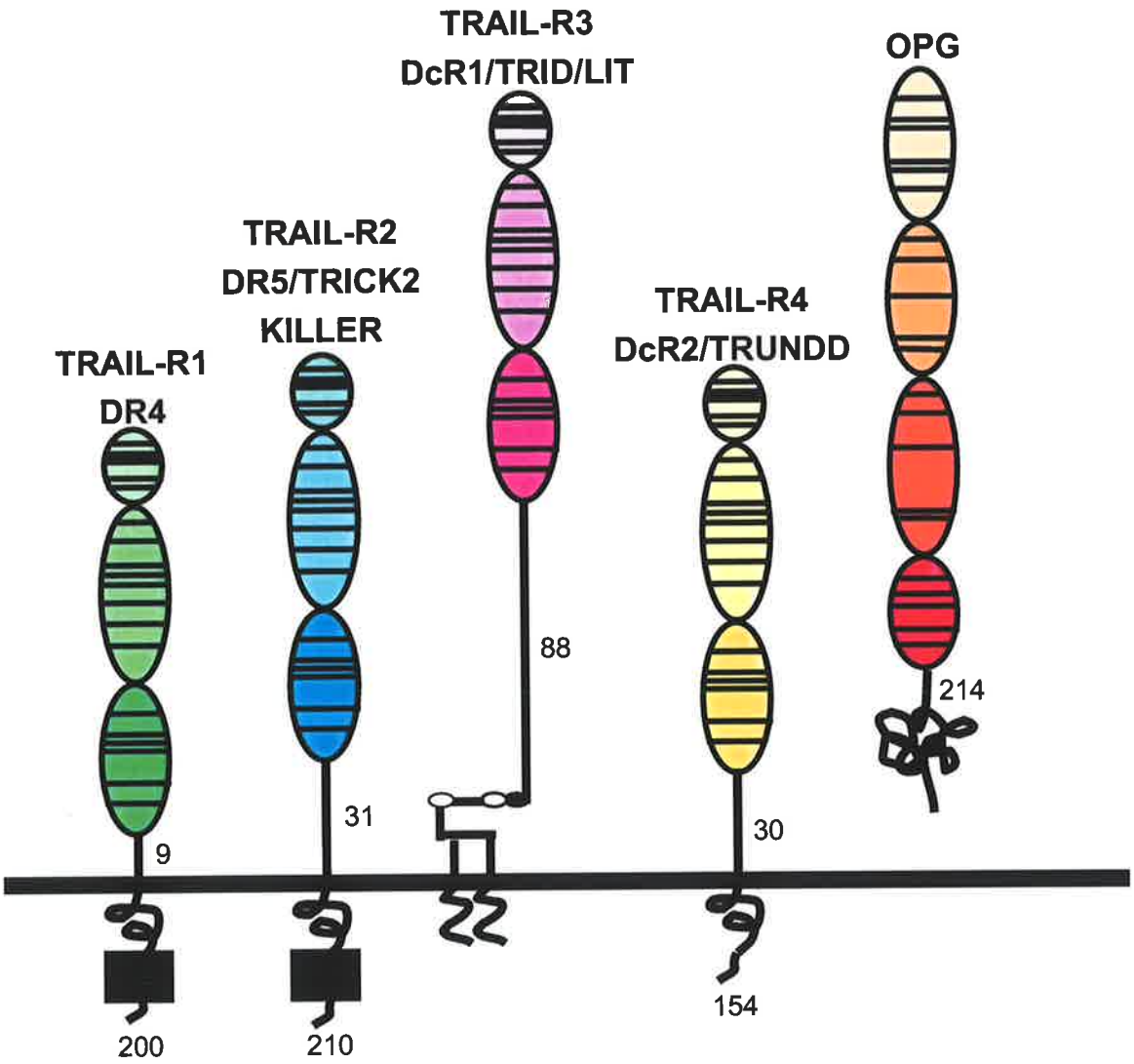


Figure 1.8 The five receptors for TRAIL. Adapted from Degli-Esposti *et al.*, 1999. Numbers indicate amino acid length.

to TRAIL-induced apoptosis. It appears that these receptors have the ability to act as 'decoys', capable of binding TRAIL and therefore competing with the death -domain containing receptors DR4 and DR5.

The four membrane-bound TRAIL receptors are tightly clustered on human chromosome 8p21-22, suggesting that recent evolution occurred *via* gene duplication [Walczak *et al.* 1997; Degli-Esposti *et al.* 1997]. OPG also maps to chromosome 8 at position 23-24 of the q arm [Simonet *et al.* 1997].

Pan and colleagues identified the first of the TRAIL death receptors, DR4, using the EST data base system [Pan *et al.* 1997]. DR4 is a 468 amino acid type I transmembrane protein that contains a 23 amino acid signal sequence, a 226 amino acid extracellular region, a 19 amino acid transmembrane region and a 220 amino acid cytoplasmic region. Within the extracellular domain there are two cysteine-rich motifs homologous to those within the ligand-binding domain of other TNF receptor family members [Pan *et al.* 1997]. DR4 binds TRAIL with a very high affinity and is able to induce an apoptotic signal due to a functional death domain within the cytoplasmic region [Degli-Esposti 1999; Pan *et al.* 1997; Pan *et al.* 1997; Truneh *et al.* 2000]. DR4 mRNA is expressed in most of the same tissues as TRAIL mRNA [Pan *et al.* 1997; Pan *et al.* 1997].

Soon after the discovery of the first TRAIL receptor, several groups reported a second receptor, DR5, also known as TRAIL-R2/TRICK2A/TRICKB/KILLER [Sheridan *et al.* 1997; Screaton and Xu 2000; Walczak *et al.* 1997; Wu *et al.* 1997]. DR5 is a 411 amino acid type I transmembrane protein that contains a 51 amino acid signal sequence, a 132 amino acid extracellular region, a 22 amino acid transmembrane region and a 206 amino acid cytoplasmic region [Pan *et al.* 1997]. There are also two cysteine-rich motifs in the extracellular region of DR5 also present in DR4. Northern

blot analysis revealed that most tissues expressing DR4 mRNA also expressed DR5 mRNA [Pan *et al.* 1997].

DcR1, also known as TRID, TRAIL-R3 and LIT, was identified using the sequence of the ligand binding domain of TNFR1 [Pan *et al.* 1997; Sheridan *et al.* 1997; Walczak *et al.* 1997]. The 299 amino acid DcR1, contains a 23 amino acid signal sequence, a 217 amino acid extracellular region and a 19 amino acid transmembrane region [Pan *et al.* 1997]. The two cysteine-rich motifs present in the extracellular region have 58% and 54% homology with DR4 and DR5 respectively. Due to the absence of a cytoplasmic region, the receptor is bound to the cell surface *via* a glycosyl-phosphatidylinositol (GPI) anchor and its ability to transmit an intracellular signal has yet to be determined. It is, however, able to bind TRAIL at the cell surface, possibly acting as a 'decoy' and inhibiting a response by the death domain-containing receptors DR4 and DR5. DcR1 mRNA is not as widely expressed as DR4 and DR5 mRNA [Pan *et al.* 1997; Sheridan *et al.* 1997; Degli-Esposti *et al.* 1997]. An absence of DcR1 expression has been identified in many tumour types, however, the presence or absence of DcR1 alone does not appear to correlate with sensitivity to TRAIL-induced apoptosis [Bretz *et al.* 1999; Degli-Esposti 1999].

The fourth receptor, DcR2, was identified from a cDNA library using the sequence of the DcR1 ligand-binding domain as a probe [Degli-Esposti *et al.* 1997; Marsters *et al.* 1997]. DcR2 is a classical type 1 transmembrane protein that shares 58% and 57% homology with DR4 and DR5 and 70% homology with DcR1. All four receptors possess two cysteine motifs within the extracellular region, necessary for ligand binding. DcR2 binds to TRAIL with an extremely high affinity and soluble DcR2 protein is able to block TRAIL-mediated apoptosis in TRAIL-sensitive cell lines [Marsters *et al.* 1997; Truneh *et al.* 2000]. Despite the similarities in ligand binding between TRAIL receptors, DcR2 contains a truncated death domain within the

cytoplasmic region and does not mediate apoptosis [Degli-Esposti *et al.* 1997; Marsters *et al.* 1997]. DcR2 gene expression in normal tissues is as widespread as DR4 and DR5 gene expression. As with DcR1, the presence or absence of DcR2 mRNA does not correlate with sensitivity or resistance of cells to TRAIL-mediated apoptosis [Degli-Esposti 1999; Bretz *et al.* 1999]. Moreover, Zhang and colleagues demonstrated that mRNA expression of DcR2 within the cell did not reflect receptor protein expression at the cell surface [Zhang *et al.* 1999]. Importantly, it was also demonstrated that mRNA transcripts located within the cytoplasm were redistributed to the cell surface upon activation.

The fifth TRAIL receptor, osteoprotegerin (OPG), exists as a secreted protein and has been identified as an antagonist of RANKL-mediated bone resorption [Truneh *et al.* 2000; Emery *et al.* 1998; Degli-Esposti 1999]. Therefore, unlike any of the other TRAIL receptors, OPG lacks a transmembrane domain [Simonet *et al.* 1997]. Reports have also identified that OPG has an affinity for TRAIL and is able to inhibit TRAIL-mediated apoptosis as seen with DcR1 and DcR2 [Truneh *et al.* 2000; Emery *et al.* 1998]. Not only is OPG able to bind to TRAIL but it also binds to an osteoblast differentiation factor RANKL also known as ODF, TRANCE and OPGL, which is involved in regulating bone resorption [Emery *et al.* 1998; Simonet *et al.* 1997]. It has been shown that TRAIL blocks the anti-osteoclastogenic activity of OPG suggesting a potential cross-regulatory mechanism [Emery *et al.* 1998]. The physiological role of OPG in TRAIL signalling is unclear although a recent report has suggested that cancer-derived OPG may be an important survival factor in hormone-resistant prostate cancer cells. This is supported by a reduction of TRAIL-induced apoptosis in cancer cells producing higher than normal levels of OPG [Holen *et al.* 2002].

1.6.2 Molecular mechanisms of TRAIL-induced apoptosis

Apoptosis induced by TRAIL occurs *via* the extrinsic pathway, as for all members of the TNF superfamily. Ligation of TRAIL to either DR4 or DR5 death receptors results in trimerisation of the receptor and clustering of the receptor's intracellular DD [Ozoren and El-Deiry 2003]. This leads to the recruitment of the adaptor protein Fas-associated death domain (FADD/Mort1), through its own death domain, to the receptor-ligand complex followed by binding of procaspase-8 to form the DISC [French and Tschopp 1999; Degli-Esposti 1999; Walczak and Krammer 2000; Bodmer *et al.* 2000; Wang and El-Deiry 2003; Wang *et al.* 2001; Kischkel *et al.* 2001]. The formation of the DISC leads to the autocleavage of procaspase-8 and release of active caspase into the cytosol [Medema *et al.* 1997; Kischkel *et al.* 2001; Boldin *et al.* 1996; Juin and Evan 2000; Zhang *et al.* 1999]. Procaspase–10 has been shown to be recruited to the DISC and become activated, however it remains controversial as to whether caspase-10 can initiate apoptosis in the absence of caspase-8 [Kischkel *et al.* 2001; Sprick *et al.* 2002]. Sufficient levels of caspase-8 are required to activate caspase-3 and initiate the caspase cascade [Kim *et al.* 2000]. Amplification of the apoptotic signal occurs when the extrinsic and intrinsic pathway are linked *via* caspase-8 cleavage of Bid, leading to its translocation to the mitochondria to promote cytochrome *c* and Smac/DIABLO release through interaction with Bax and Bak [Deng *et al.* 2002; Green 2000].

Two different cell types have been identified with respect to the degree of involvement of the intrinsic and/or extrinsic apoptotic pathway, following death receptor signalling. In Type I cells, death receptor ligation is able to recruit and activate sufficient levels of caspase-8 to induce apoptosis, by initiating the caspase cascade, without the need of the mitochondrial intrinsic pathway. On the contrary, type II cells produce insufficient levels of activated caspase-8 and additionally require Bcl-2 and the mitochondrial intrinsic pathway to amplify the apoptotic signal after ligand binding

[Ozoren and El-Deiry 2002]. The difference between the two cell types is based on the levels of caspase-8 recruited by FADD to the DISC [Scaffidi *et al.* 1999; Ozoren and El-Deiry 2002]. The two different cell types have been identified in response to FasL and TRAIL [Burns and El-Deiry 2001; El-Deiry 2001; Ozoren *et al.* 2000].

The DISC can be inhibited by FLICE inhibitory protein (also known as FLIP) [Wang *et al.* 2000; Irmler *et al.* 1997; Kataoka *et al.* 2000]. Inhibition of the DISC by FLIP leads to the activation of signalling pathways such as NF-κB and Erk [Kataoka *et al.* 2000].

1.6.3 TRAIL sensitivity and resistance

By comparison with normal cells, many cancer cells (because some are as resistant as normal cells) are more sensitive to TRAIL-induced apoptosis after TRAIL treatment. A proposed explanation for the observed difference in TRAIL sensitivity is a greater expression of TRAIL death receptors in cancer cells [Zhang *et al.* 1999]. This, however, is not supported by mRNA expression studies of the death receptors, which show them to be widely distributed in both normal and malignant tissues [Chaudhary *et al.* 1997]. The discovery of the decoy receptors offered a possible explanation, however, it was assumed that only normal cells would express surface decoy receptors [Gura 1997]. This assumption proved incorrect and the link between TRAIL receptor expression and TRAIL sensitivity remains unclear [Hersey and Zhang 2001; Ozoren and El-Deiry 2003].

The cellular FLICE-inhibitory proteins (c-FLIPS) have been identified, which are able to negatively regulate death receptor signalling. These proteins have sequence homology to caspase-8 and –10 and can be recruited to the DISC, preventing the interaction between FADD and the procaspases [French and Tschopp 1999; Tschopp *et al.* 1998; Jiang *et al.* 1999; Igney and Krammer 2002; Igney and Krammer 2002].

Multiple splice variants of c-FLIP have been reported, designated c-FLIP$_S$ and c-FLIP$_L$, representing the short and long forms respectively. The short form of c-FLIP has been identified as conferring TRAIL resistance in a sensitive colon carcinoma cell line [Burns and El-Deiry 2001]. A correlation of high FLIP expression and TRAIL resistance has been found in many cancer cells [French and Tschopp 1999]. In addition, high FLIP expression has also been implicated in enhanced tumour progression [French and Tschopp 1999]. Recent reports suggest that FLIP may promote caspase-8 activation, leaving FLIPs role in apoptosis unclear [Chang *et al.* 2002].

The role of caspases is central to the process of apoptosis and may be a factor in resistance. Individual caspase expression levels may have an impact on overall activity, with frameshift mutations and alternative splicing identified in cancer cell lines [Srinivasula *et al.* 1998]. Caspase mutations are a plausible cause for TRAIL resistance, however detection of such in human cancer is rare [Mandruzzato *et al.* 1997]. It is more probable that other factors hinder caspase function and confer resistance in cancer cells [Baylin and Bestor 2002]. Hypermethylation of regulatory sequences of the caspase-8 gene was found to inhibit caspase-8 expression in numerous tumour cell types both *in vitro* and *in vivo* [Teitz *et al.* 2000; Fulda *et al.* 2001; Baylin and Bestor 2002]. Demethylation treatment or gene transfer experiments to restore caspase-8 expression led to sensitisation of these cells to apoptotic signals.

Mutations in proteins of the Bcl-2 family can occur, such as Bax mutations in mismatch-repair (MMR)-deficient human tumours. Resistance to TRAIL-induced apoptosis can occur due to Bax inactivation, probably by preventing the release of Smac/DIABLO, needed to antagonise the IAP protein family [Deng *et al.* 2002; Burns and El-Deiry 2001]. Recent studies have shown that combination treatment of chemotherapy and TRAIL is able to reverse the resistance to TRAIL [Bouralexis *et al.* 2004; LeBlanc *et al.* 2002; Wu *et al.* 2004; Van Valen *et al.* 2003; Zhu *et al.* 2004].

Evidence suggests that chemotherapy sensitises the cells to the effects of TRAIL by mechanisms such as increased pro-apoptotic protein expression and increased death receptor expression.

Sensitivity to death receptor mediated apoptosis can be regulated by several factors and it is the sum of these factors, which decides between life and death of a cell.

1.6.4 Physiological role of the TRAIL system

The physiological role the TRAIL system is not well understood. Initially it was assumed that the functionally distinct receptors could explain the role of the TRAIL system and the apparent differences in sensitivity to TRAIL between normal and transformed cells. The competing 'decoy' and death receptors provided a simple explanation to the observed phenomenon. However the levels of TRAIL receptor expression do not always correlate with resistance or sensitivity to TRAIL-induced apoptosis suggesting the influence of other factors [Degli-Esposti 1999]. The cell surface expression of TRAIL has been discovered for cell types that had previously been known to induce apoptosis in target cells *via* an unknown mechanism. These include type II interferon (IFNγ)-stimulated monocytes [Griffith *et al.* 1999], type I interferon (INFα and IFNβ) [Walczak and Krammer 2000], TCR stimulated T cells [Kayagaki *et al.* 1999; Kayagaki *et al.* 1999], cytomegalovirus (CMV)-infected fibroblasts [Sedger *et al.* 1999], non-stimulated CD4+ T cells [Thomas and Hersey 1998; Mariani and Krammer 1998; Kayagaki *et al.* 1999], lipopolysaccharide activated monocytes and macrophages [Halaas *et al.* 2000], INFα and IFNγ- stimulated as well as measles virus-infected dendritic cells [Vidalain *et al.* 2000; Fanger *et al.* 1999] and natural killer (NK) cells [Kashii *et al.* 1999; Johnsen *et al.* 1999; Zamai *et al.* 1998; Kayagaki *et al.* 1999]. Interferon stimulation has been associated with surface

expression of TRAIL such as in human lymphoid cells, therefore, IFNs may play a role in mediating death in TRAIL-sensitive tumour cells [Kayagaki *et al.* 1999; Griffith *et al.* 1999]. The antiviral activity of IFN has been reported to induce TRAIL-mediated apoptosis in virally infected cells but not normal cells [Sedger *et al.* 1999]. Therefore the TRAIL apoptotic system may be important in controlling virally transformed cells. The expression of TRAIL, together with other apoptosis-related cytokines such as FasL, TNF and lymphotoxin-α (LT-α) by NK cells, has led to the speculation that TRAIL is one apoptotic component of many, aiding NK cells in their pursuit of defective cells for elimination [Kashii *et al.* 1999]. Other than its involvement in apoptosis, TRAIL has also been identified as an inhibitory protein in auto-immune inflammation by blocking cell cycle progression in rheumatoid arthritis [Song *et al.* 2000]. The absence of TRAIL led to hyper-proliferation of cells and increased expression of cytokines and auto-antibodies whereas treatment with TRAIL prevented cell cycle progression [Song *et al.* 2000]. A role for TRAIL in the immune privilege of uteri was also proposed, with the discovery of TRAIL expression in human placentas [Phillips *et al.* 1999]. The constitutive expression of TRAIL on murine NK cells in liver was recently reported and may play a role in prevention against tumour metastasis [Hayakawa *et al.* 2001]. These studies suggest that the TRAIL ligand-receptor system is critical in the destruction of virally infected cells, inhibition of auto-immune inflammation and surveillance of malignant transformations.

The differing activities of TRAIL further emphasise its complexity and continued studies will provide a better understanding of the TRAIL ligand-receptor system and hopefully identify the mechanisms that regulate tissue homeostasis.

1.6.5 Potential for cancer therapy

The preferential activity of TRAIL against cancer cells makes it potentially ideal as a cancer therapeutic alone or in combination with chemotherapy and radiation therapy. Normal cells appear to be resistant to TRAIL, which makes the use of TRAIL therapy more appealing [Ashkenazi 2002; Atkins *et al.* 2002; Evdokiou *et al.* 2002]. Most importantly, TRAIL-induced apoptosis is independent of p53 status, which makes it potentially effective against tumours that are resistant to conventional chemotherapy [El-Deiry 2001].

TRAIL, in its soluble recombinant form, induces apoptosis in a number of transformed cell lines derived from leukaemia, multiple myeloma, neuroblastoma, and cancers of the colon, lung, breast, prostate, pancreas, kidney, central nervous system and thyroid [Yu *et al.* 2000; Rieger *et al.* 1998; Keane *et al.* 1999; Mitsiades *et al.* 2001; Mizutani *et al.* 2001; Gazitt 1999; Walczak *et al.* 1999]. More importantly, the use of TRAIL *in vivo* has also been successful. Mice bearing solid tumours showed increased tumour cell apoptosis, suppressed tumour progression and improved survival, without any normal tissue toxicity, after the administration of soluble TRAIL [Ashkenazi *et al.* 1999; Walczak *et al.* 1999]. Significant anti-tumour activity without systemic toxicity has been reported for many human xenograft models in mice derived from multiple myeloma, malignant glioma, and cancers of the breast and colon [Pollack *et al.* 2001; Roth *et al.* 1999; Mitsiades *et al.* 2001; Kelley *et al.* 2001]. Additionally, TRAIL was also shown to inhibit growth of tumours immediately after implantation, as well as inducing apoptosis in established tumours.

The use of an alternative delivery system has been explored by means of adenoviral gene therapy. This method has been used in both *in vitro* and *in vivo* experiments with promising results [Kagawa *et al.* 2001; Griffith and Broghammer 2001; Griffith *et al.* 2000]. Anti-tumour activity of TRAIL gene therapy has been

verified in cancers such as colon, breast, prostate, glioblastoma and hepatocellular carcinoma [Yamashita *et al.* 2002; Lee *et al.* 2002; Norris *et al.* 2001].

To date, four recombinant versions of human TRAIL have been generated. One of the first versions generated contains TRAIL amino acids 114-281 fused to an amino-terminal polyhistidine tag [Pitti *et al.* 1996]. To promote and stabilise the formation of ligand trimers, the second version generated contains an amino-terminal modified yeast Gal4 leucine zipper fused to residues 95-281 [Walczak *et al.* 1999]. The third variant contains the TRAIL amino acids 95-281 fused to an amino-terminal 'Flag' epitope tag. In some cell lines the use of anti-flag antibodies cross-link with this version of TRAIL and improve its activity [Bodmer *et al.* 2000]. The fourth version generated has potential to be the most clinically favoured because it contains amino acids 114-281 of human TRAIL but lacks any exogenous sequence and therefore is the least probable to be immunogenic in humans [Ashkenazi and Dixit 1999]. Non-tagged, recombinant soluble TRAIL is produced by bacteria at a neutral pH, as a stable homotrimer, optimised by the addition of zinc and reducing agent to the culture media [Kelley *et al.* 2001; Lawrence *et al.* 2001].

The use of the non-tagged, zinc-bound version of TRAIL is reported not harmful to most normal human cells, with some cells including astrocytes, hepatocytes and keratinocytes sensitive to the tagged, non-optimised versions of TRAIL [Ashkenazi *et al.* 1999; Walczak *et al.* 1999; Lawrence *et al.* 2001; Jo *et al.* 2000; Qin *et al.* 2001]. The observed difference may be determined by the amount of multimerisation of the death receptors DR4 and DR5. The tagged versions of TRAIL, which lack a zinc atom, have low solubility and aggregate or precipitate at high concentrations. This is also seen with the antibody cross-linked version of TRAIL. This may result in over multimerisation of the death receptors resulting in an overwhelming death signal and eventual apoptosis. Conversely, the non tagged, zinc-bound version of TRAIL is highly

stable and soluble as a trimer, forming only trimeric death receptor multimerisation complexes, which are well below the apoptotic threshold in normal cells, but sufficient to induce apoptosis in cancer cells due to a lowered threshold [Evan and Littlewood 1998; Jo *et al.* 2000; Lawrence *et al.* 2001; Ashkenazi *et al.* 1999]. This hypothesis is also supported by the observation that unlike the poly-histidine tagged version of TRAIL, which binds irreversibly to the death receptors, the untagged, zinc-bound version of TRAIL has reversible binding [Jo *et al.* 2000; Lawrence *et al.* 2001].

Studies in mice show no obvious toxicity to various versions of recombinant TRAIL, however mice are more resistant to toxicity than primates [Walczak *et al.* 1999]. Non-human primates, were used to determine safety of the untagged zinc- bound TRAIL. Cynomolgus monkeys and chimpanzees were intravenously injected with TRAIL with varying doses over 7 days, with no toxicity observed [Ashkenazi *et al.* 1999; Lawrence *et al.* 2001; Kelley *et al.* 2001]. More importantly, there was no evidence of hepatotoxicity, suggesting that this version of the recombinant molecule may be the best candidate for clinical assessment.

1.6.6 Combined treatment with TRAIL and other agents

Response to cancer therapy is determined by the induction of apoptosis, which is limited by lack of selectivity in the currently available apoptosis-inducing agents. In addition, most cancers are resistant to growth arrest and apoptosis. Current standard cancer therapy is restricted by its toxicity to normal cells and the consequent side effects observed. It is therefore central to the application of therapeutics that the apoptosis-inducing agents given to patients are effective and selective at killing cancer cells, with normal cells spared this fate. The approaches for therapy can range from selective inhibition of anti-apoptotic pathways, anti-angiogenic therapy, tissue-specific therapy such as immunotherapy, and the exploitation of cancer specific features such as

unrestricted cell cycles and hypoxicity. To ensure both effective and selective treatment, combinations of drugs are proving useful by providing multiple apoptotic signals *via* different pathways. Examples of this include the use of anti-apoptotic kinases, such as inhibitors of Raf-1, MEK and Akt, to increase sensitivity to chemotherapy [Dancey and Sausville 2003; Solit *et al.* 2003; Yu *et al.* 2001; MacKeigan *et al.* 2000]. This combines activators of both the extrinsic and intrinsic pathway, leading to caspase activation [Milner *et al.* 2002] and combination therapy of anticancer agents that potentiate the apoptotic signal.

In an effort to overcome resistance of cancer cells to TRAIL, combination therapy is proving to be valuable experimentally. Combination therapy can result in either a synergistic, additive or antagonistic apoptotic effect. A combination of agents that is more effective than the sum of both agents is said to show synergy. Additivism is no more and no less effective than expected and antagonism is a combination that is less effective than expected [Berenbaum 1977].

Synergistic combinations usually consist of one agent that sensitises the cell to the cytotoxic effects of the second agent. This may be by blocking anti-apoptotic pathways, such as down regulating expression of FLIP [Sayers *et al.* 2003], or the use of DNA damaging drugs, which induce the mitochondrial release of inhibitors of IAP to reactivate caspase-3 [Leverkus *et al.* 2003]. The use of synergistic combinations may also cause unwanted synergy in normal cells, which may be translated into side effects in the clinic. It is therefore crucial to find combinations that are selective, to minimise the effects on normal cells.

The use of recombinant soluble TRAIL in combination with other agents is being actively investigated in the field of cancer therapy [Nagane *et al.* 2001]. Studies in OS, Ewing's sarcoma, leukaemia, colorectal, multiple myeloma, neuroblastoma, melanoma, breast, bladder, glioma, liver, lung, ovarian, pancreatic, renal and thyroid cancer have

shown that combination treatment between TRAIL and chemotherapeutic drugs or radiation, results in greater tumour cell death than standard chemotherapy alone [Ahmad and Shi 2000; Bouralexis *et al.* 2004; Cuello *et al.* 2001; Chinnaiyan *et al.* 2000; Dejosez *et al.* 2000; Eggert *et al.* 2001; Evdokiou *et al.* 2002; Gliniak and Le 1999; Gong and Almasan 2000; Griffith *et al.* 1998; Jazirehi *et al.* 2001; Keane *et al.* 1999; Lacour *et al.* 2001; Mizutani *et al.* 2001; Nagane *et al.* 2000; Ng *et al.* 2002; Rohn *et al.* 2001; Sun *et al.* 2000; Van Valen *et al.* 2000; Wu *et al.* 2002; Yamanaka *et al.* 2000]. Chemotherapeutic drugs or radiation may activate an apoptotic pathway, which is different from the apoptotic pathway initiated by TRAIL, therefore generating a more potent apoptotic signal.

It has been observed that DNA damage and radiation causes upregulation of DR5 transcription and therefore may enhance the cytotoxicity of TRAIL through upregulation of its death receptor DR5. [Chinnaiyan *et al.* 2000; Gong and Almasan 2000; Sheikh *et al.* 1998; Wu *et al.* 1997]. Cotreatment of TRAIL with chemotherapeutic agents cisplatin and etoposide in malignant gliomas, resulted in an augmented apoptotic effect due to increased expression of DR5 [Nagane *et al.* 2000]. In vitro experiments using sublethal doses, which are commonly used for the treatment of malignant glioma in humans, caused upregulation of DR5 transcripts but did not induce apoptosis in most gliomas tested. Treatment with both TRAIL in combination with DNA damaging drugs caused synergistic apoptosis *in vitro* as well as growth suppression and even regression in established human glioblastoma xenografts in nude mice [Nagane *et al.* 2000]. Conversely, single treatments were ineffective, supporting the usefulness of combination therapy for gliomas and even TRAIL resistant therapy. Upregulation of DR4 has also been reported in response to DNA damaging agents [Guan *et al.* 2001]. There has been a recent suggestion that the synergy between TRAIL

and chemotherapeutic agents may be independent of DR4 and DR5 upregulation and that other mechanisms may be involved [Rohn *et al.* 2001].

Although most studies have been performed with cell lines, the effectiveness of TRAIL and chemotherapeutic drug combination treatments *in vitro* has also been shown to reflect sensitivity *in vivo.* Chemotherapeutic drugs such as doxorubicin, 5-flouracil and camptothecin augment TRAIL cytotoxicity both *in vitro* and *in vivo* for colon, breast and bladder cancer [Keane *et al.* 1999; Gliniak and Le 1999; Bonavida *et al.* 1999].

Therefore although the mechanisms are not clearly defined, combination treatment of TRAIL with standard chemotherapeutic agents is able to synergistically augment cell death *in vivo,* providing an alternative strategy for the treatment of human cancers. Ideally, a combination of TRAIL and another less toxic agent, in lieu of chemotherapy, may prove far superior in cancer treatment by reducing the well-known side effects associated with current chemotherapeutic agents. Scientists are in pursuit of such a new agent with many possible candidates currently being explored for future management strategies.

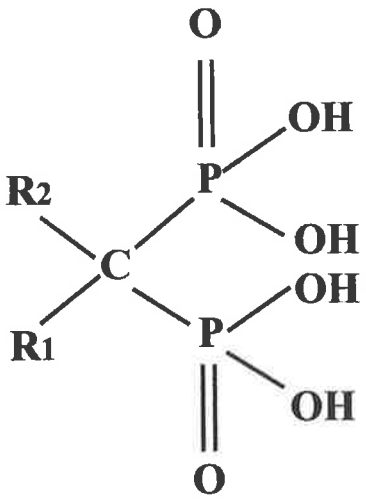
1.7 BISPHOSPHONATES

1.7.1 General properties

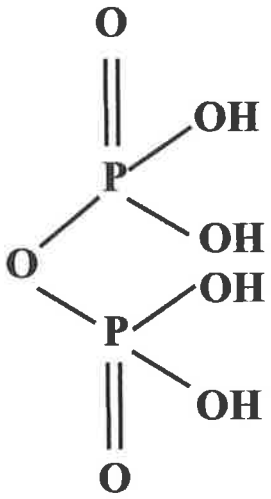
Bisphosphonates (BPs) are synthetic analogues of inorganic pyrophosphate, commonly used for the prevention and treatment of various bone diseases associated with enhanced bone resorption, such as Paget's disease and osteoporosis. First synthesised in 1865, BPs were found to inhibit precipitation of calcium carbonate and were mainly used as anti-corrosive and anti-scaling agents for industrial purposes. It was not until 1968 that the biological properties of BPs were first reported by Fleisch

and his group [Fleisch *et al.* 1968]. BPs are all characterised by a phosphorous-carbon-phosphorous group [Fleisch *et al.* 1968; Fleisch 2000]. They are similar to pyrophosphates but have a carbon atom replacing the central oxygen atom, thus allowing the binding of two extra side chains, R1 and R2 (see **Figure 1.9**). Structural changes of BPs occur at these side chains of the central carbon atom and even minor alterations can result in significant differences in the physicochemical and biological properties of the compound [Fleisch 2000; Shinoda *et al.* 1983]. The three-dimensional structure of BPs allows them to bind to divalent metal ions such as Ca^{2+}, Mg^{2+} and Fe^{2+} *via* the oxygen atom from each phosphate group, forming insoluble complexes [Ebetino *et al.* 1998]. If the R1 side chain is a hydroxyl (OH) or an amino group (NH_2) then the affinity for Ca^{2+} increases due to changes in structure and improved binding [Jung *et al.* 1973]. The highly selective localisation and retention of BPs in bone is the basis for its use in skeletal disorders. The degree of inhibition of bone resorption varies greatly depending on the structure of the BP.

BPs are divided into three main groups, as shown in **Table 1.2**: (i) The first generation group of compounds, such as etidronate and clodronate, is the non-nitrogen containing group of BPs and these are the weakest in their resorptive inhibitory function. They are structurally similar to ATP and are incorporated into metabolic pathways as non-hydrolyzable analogues of ATP, thereby inhibiting ATP-dependent function. An important example of this is the vacuolar H+-ATPase proton extrusion pump, which is essential for osteoclast function [Blair *et al.* 1989]; (ii) the second group, which includes pamidronate and alendronate, contain a nitrogen atom (N-BPs) on the side chain, which strongly increases BP activity [Rogers *et al.* 2000]; (iii) the third and most active group of N-BPs contain a cyclic substituent and either one nitrogen atom in the pyridyl ring, such as risedronate, or two nitrogen atoms in an imidazole ring, such as zoledronic acid (**Figure 1.10**) [Rogers *et al.* 2000]. This group



BISPHOSPHONATES



PYROPHOSPHATE

Figure 1.9 The general structures of bisphosphonates compared to pyrophosphate.

BISPHOSPHONATE CLASS	MECHANISM OF ACTION	STRUCTURE	POTENCY
Non-nitrogen-containing			
* First generation BPs -etidronate, clodronate	Cytotoxic, non-hydrolyzable analogues of ATP	Side chains lacking N atom Aliphatic side chain with single N atom	+ ++
Nitrogen-containing			
* Second generation BPs - pamidronate, alendronate - risedronate	Inhibit protein prenylation	Heterocyclic ring with single N atom	+++
* Third generation BPs -zoledronic acid		Imidazole ring with two N atoms	++++

Table 1.2 The evolution of Bisphosphonates. Bisphosphonates are divided into three groups based on their mechanism of action and their structure.

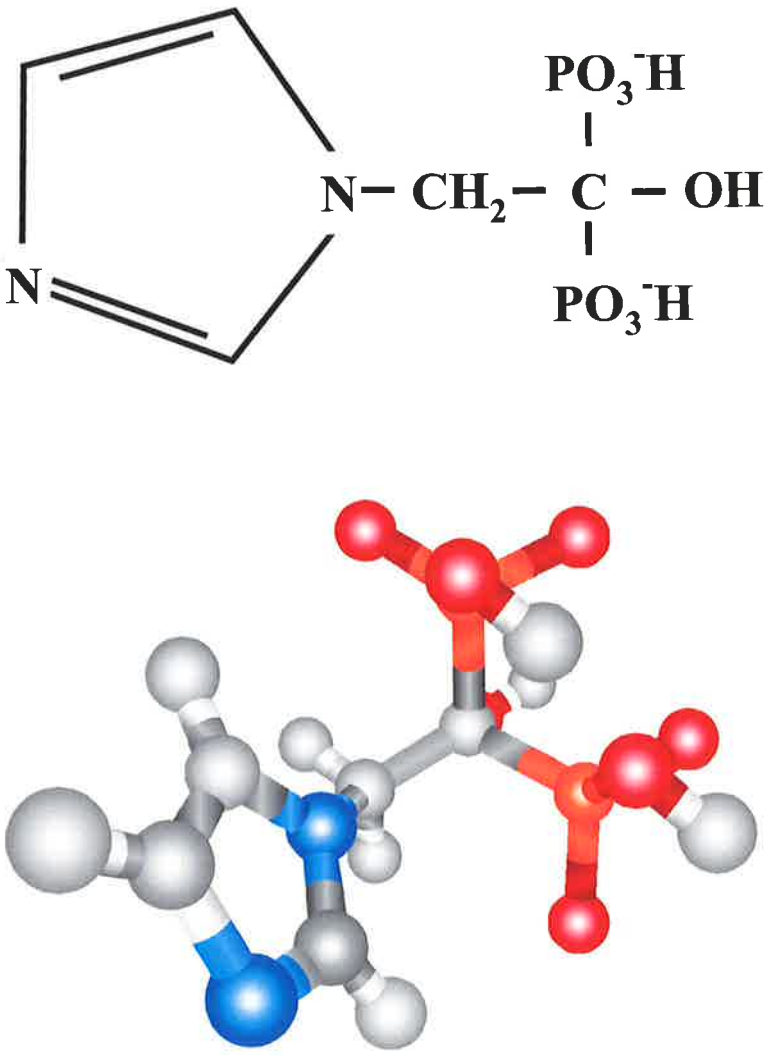


Figure 1.10 Chemical structure of zoledronic acid (2-[imidazol-1-yl]-1-hydroxyethylidene-1,1-bisphosphonic acid).

of N-BPs are up to 10,000 fold more potent than the non-nitrogen containing BPs, with respect to their ability to inhibit bone resorption *in vitro* [Green *et al.* 1994; Fleisch 2000]. Zoledronic is the most potent inhibitor of bone resorption of the currently available BPs [Green *et al.* 1994]. Studies have shown that ZOL preserves bone mass, structure and strength in eostrogen-deficient animals without affecting bone mineralisation [Binkley *et al.* 1998; Bare *et al.* 1997]. Already used in the treatment of bone disease such as Paget's disease, ZOL also shows exciting potential as an anticancer agent, as will be discussed throughout the thesis. Significantly, prolonged treatment with zoledronic acid seems to be safe and well tolerated [Ali *et al.* 2001; Rosen *et al.* 2003; Rosen *et al.* 2004].

1.7.2 The effect of bisphosphonates on bone cells

Bone remodelling by osteoblasts and osteoclasts is an ongoing process in adult bones. This allows the maintenance of the mechanical integrity of the skeleton and is also involved in calcium homeostasis [Rodan 1997]. The normal remodelling rate in a human adult is between 2 and 10 % of the skeletal mass per year. The process of remodelling starts with the recruitment of osteoclasts to a particular site, where they erode the bone to form a resorption cavity. This is tightly coupled with the formation of new bone by the osteoblasts, over the resorption cavity [Manolagas and Jilka 1995; Rodan 1996].

Bone destruction is a common manifestation associated with skeletal malignancy and is mediated primarily by the osteoclasts and their bone resorbing activity [Goltzman 2001; Taube *et al.* 1994]. The initial appearance of malignant cells within the bone microenvironment leads to the release of numerous agents produced directly by the tumour cells including hormones, growth factors and cytokines, which act on osteoblastic stromal cells to enhance the production of osteoclastic stimulating factors.

This leads to increased osteoclast number and increased bone resorption. Osteoclasts resorb bone by secreting proteases that dissolve the bone extracellular matrix and release bone mineral and growth factors [Chirgwin and Guise 2000]. Bone resorption occurs at the extracellular space/bone interface, under the ruffled border of the osteoclast's plasma membrane [Blair *et al.* 1989]. The factors released from the bone are able to stimulate tumour growth resulting in the establishment of a mutually beneficial relationship, often termed "the vicious cycle" due to its progressively destructive nature.

Due to their safety and efficacy, BPs have been used extensively over the years for the treatment of many diseases characterised by increased bone resorption [Body 2000]. BPs preferentially concentrate to bone surfaces that are actively remodelling, at the bone/osteoclast interface, where bone mineral is most exposed [Masarachia *et al.* 1996]. During acidification of bone mineral by osteoclasts, BP is released and is internalised by osteoclasts [Dunford *et al.* 2001]. Following internalisation, the primary action of BPs is to inhibit osteoclast activity.

The action of BPs on the osteoclasts involves various processes; reduction in osteoclast activity and a reduction in osteoclast number either by apoptosis or *via* decreased recruitment [Hughes *et al.* 1995]. Although several studies have shown that BPs to induce osteoclast apoptosis, it appears that the first effect is to reduce osteoclast activity, resulting in reduced bone resorption [Hughes *et al.* 1995; Coxon *et al.* 2000; Rogers *et al.* 2000]. The effect of BPs on osteoclast function is due to loss of ruffled border, altered actin cytoskeleton, decreased acid production, decreased enzyme activity, decreased protein prenylation, disruption of integrin-induced intracellular signalling and altered trafficking of membranes and intracellular molecules [Hughes *et al.* 1995; Coxon *et al.* 2000; Rogers *et al.* 2000]. Indirect effects *via* the osteoblasts also affect osteoclast activity.

Osteoblasts are key regulators in bone cell differentiation and function. BPs have been shown to directly influence osteoblast function [Giuliani *et al.* 1998; Garcia-Moreno *et al.* 1998; Khokher and Dandona 1989]. Recent investigations have reported BPs to cause a stimulatory effect on osteoblast proliferation [Giuliani *et al.* 1998; Fromigue and Body 2002; Viereck *et al.* 2002; Tsuchimoto *et al.* 1994]. The anabolic effects on osteoblasts are dose dependent with higher doses causing inhibition and even cell death. Maturation of osteoblasts has been reported after BP treatment, with an earlier commitment towards the osteoblastic phenotype [Im *et al.* 2004; Fromigue and Body 2002]. Evidence from *in vitro* and *in vivo* studies confirm that BPs inhibit osteoclastogenesis and the recruitment of osteoclast progenitors into bone [Sahni *et al.* 1993; Clohisy *et al.* 2001; Van Beek *et al.* 2002]. BPs can also influence changes in osteoblastic gene expression and the secretion of soluble factors involved in regulating osteoclast differentiation and activity [Giuliani *et al.* 1998; Reinholz *et al.* 2000; Tokuda *et al.* 1998; Vitte *et al.* 1996]. One example is increased osteoprotegerin (OPG) production in osteoblasts treated with BPs [Pan *et al.* 2004; Viereck *et al.* 2002]. Osteoclast development is regulated by the receptor activator NF-κB ligand (RANKL), produced by bone marrow stromal cells and osteoblasts [Lacey *et al.* 1998]. OPG is an antagonist soluble decoy receptor that binds RANKL [Grimaud *et al.* 2001]. The ratio between RANKL and OPG regulates osteoclastogenesis, therefore disruption of this balance by BPs may lead to reduced bone resorption. Overall, BPs act *via* multiple mechanisms to reduce osteoclast number and activity and thus inhibit bone resorption.

1.7.3 Molecular mechanism of nitrogen-containing bisphosphonates

The newer N-BPs inhibit farnesyl pyrophosphate synthase, a key enzyme of the mevalonate pathway, by blocking the pyrophosphate Mg^{2+} binding site of the enzyme [Luckman *et al.* 1998; Coxon *et al.* 2000; Dunford *et al.* 2001; Fisher *et al.* 2000; Rodan

1998; Rogers *et al.* 2000]. The mevalonate pathway produces various end products that are important for many different cellular functions. These include the production of isoprene units incorporated into sterols such as cholesterol, essential in membrane integrity; steroid production; ubiquinone, for electron transport and cell respiration; farnesyl and geranylgeranyl isoprenoids, involved in the post-translational modification of proteins; dolichol, for glycoprotein synthesis; and isopentenyladenine, required for tRNA function and protein synthesis [Goldstein and Brown 1990; Kabakoff *et al.* 1990; Edwards and Ericsson 1998; Wong *et al.* 2002].

The post-translational lipid modification process that occurs as part of the mevalonate pathway is called prenylation. More specifically, prenylation is divided into geranylgeranylation and farnesylation, which involves the covalent attachment of hydrophobic geranylgeranyl (20 carbon) and farnesyl (15 carbon) prenyl lipids, to a conserved C-terminal cysteine residue contained within prenylation motifs on the target proteins [Casey 1992; Zhang and Casey 1996]. These reactions are specifically catalysed by geranylgeranyl transferase and farnesyl transferase, respectively (shown in **Figure 1.11**). The GTPase family consists of over 100 proteins such as Ras, Rho, Rac and Rab, which are major substrates for prenylation and are involved in a large variety of cellular processes such as cytoskeletal organisation, nucleocytoplasmic transport, cell attachment, integrin signalling and microtubule organisation [Edwards and Ericsson 1998; Ridley *et al.* 1992; Ridley and Hall 1992; Takai *et al.* 2001; Zhang and Casey 1996]. N- BPs prevent prenylation by inhibiting the enzyme farnesyl pyrophosphate synthase, which results in depletion of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, utilised for prenylation [Dunford *et al.* 2001].

The role of GTPases in osteoclasts has not been widely investigated and although many GTPases are involved in general cell maintenance, cell type specific GTPases have also been identified [Lutcke *et al.* 1993]. It is possible that specific GTPases,

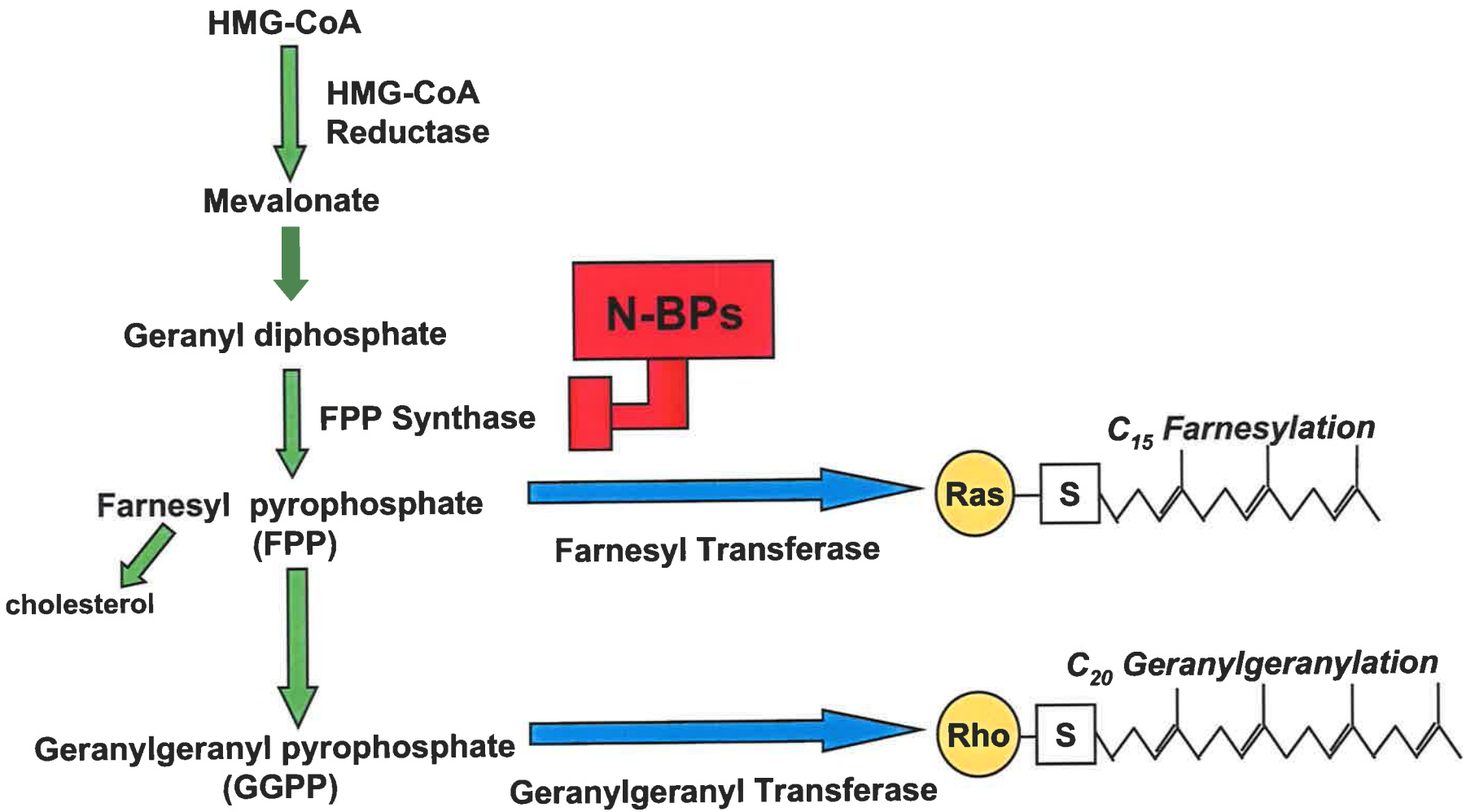


Figure 1.11 The mevalonate pathway. Nitrogen-containing bisphosphonates inhibit the enzyme as indicated above, preventing downstream processes including farnesylation and geranylgeranylation.

linked with the actin ring and ruffled border, unique to osteoclasts, may exist. Many GTPases have been identified in playing a role in the regulation of osteoclast activity, such as Rab7 involvement in bone resorption. It is therefore not surprising that inhibition of prenylation by N-BPs induces multiple negative effects on osteoclast activity [Fisher *et al.* 1999; Coxon and Rogers 2003; Zhao *et al.* 2001].

1.7.4 Anti-tumour effects of bisphosphonates

The use of BPs as inhibitors of bone resorption has been long known, however it was only until fairly recently that the anti-tumour potential of BPs was discovered. Shipman and his colleagues were the first to demonstrate the anti-cancer effects of N-BPs in myeloma cells [Shipman *et al.* 1997; Shipman *et al.* 1998; Shipman *et al.* 1998]. The mechanism of apoptosis in the malignant cells was confirmed to be the same as that seen in the osteoclasts, that is, inhibition of the mevalonate pathway leading to loss of important signalling proteins [Shipman *et al.* 1998; Shipman *et al.* 2000]. N-BPs clearly decreased myeloma cell proliferation and induced apoptosis, whereas the non-nitrogen containing BPs had little or no effect [Shipman *et al.* 1998]. Consistent with the proposed mechanism of N-BP action, the addition of geranylgeraniol and farnesol, both intermediates of the mevalonate pathway, prevented N-BP-induced apoptosis and partially reversed cytostasis. A wide variety of tumour cell lines have also been used to demonstrate the anti-tumour activity of N-BPs, including Ewings sarcoma [Sonnemann *et al.* 2003], leukaemia [Kimura *et al.* 2004], melanoma [Riebeling *et al.* 2002], breast cancer [Senaratne and Colston 2002], ovarian cancer [Sawada *et al.* 2002], prostate cancer [Lee *et al.* 2001], pancreatic cancer [Tassone *et al.* 2003] and OS [Evdokiou *et al.* 2003; Farese *et al.* 2004]. These *in vitro* studies have shown N-BPs to dose-dependently inhibit proliferation and induce apoptosis in tumour cell lines. The concentrations of N-BPs used to produce these cytostatic and apoptotic effects *in vitro*

were in the range of 5-2000 μM. This concentration range is within that achieved in bone at an active resorption site [Sato *et al.* 1991]. The potency of the N-BP and the cell line used, will determine the concentration of N-BP required to induce apoptosis [Fromigue *et al.* 2000].

Tumour cell adhesion and invasion are necessary for effective spreading of tumour cells. Van der Pluijm showed that N-BPs reduce tumour burden in bone by inhibiting breast cancer cell adhesion [van der Pluijm *et al.* 1996]. This suggested a preventative role for N-BPs in deterring tumour cells away from the bone environment, by preventing attachment. Reduced migration has been reported in other cancer cell types pretreated with low levels of N-BPs that did not induce apoptosis [Boissier *et al.* 2000]. BPs inhibit several enzymes required for tumour cell invasion *in vitro*, such as the matrix metalloproteinase enzymes (MMP-1, -2, -3, -7, -8, -9, -12, -13, -14) [Boissier *et al.* 2000; Heikkila *et al.* 2002; Teronen *et al.* 1999]. Investigation of MMP activity by several groups have produced conflicting results, which may be due to the varying N-BPs and cell lines used.

Angiogenesis is essential for the growth and survival of metastatic tumours, to ensure vascularisation and access to nutrient rich blood supply. *In vitro* experiments using zoledronic acid have shown clear inhibition of cell proliferation induced by growth factors bFGF and VEGF or serum [Wood *et al.* 2002]. Subsequent animal studies revealed that when zoledronic acid was administered systemically to mice, it potently inhibited angiogenesis induced by subcutaneous implants impregnated with bFGF [Wood *et al.* 2002]. The anti-angiogenic properties of N-BPs, specifically zoledronic acid, have also been reported in other *in vivo* models. 5T2 myeloma-bearing mice treated with zoledronic acid showed decreased tumour burden and reduced angiogenesis, evident by a marked decrease in micro-vessel density within the myeloma bone lesions [Croucher *et al.* 2003]. The additional ability of N-BPs to inhibit

angiogenesis makes them very appealing for the preventative treatment of metastatic cancers.

In vivo studies have clearly demonstrated a reduction in tumour-induced osteolysis, using BP treatment in numerous animal models of metastatic cancer including breast [Hall and Stoica 1994; Sasaki *et al.* 1995; Yoneda *et al.* 2000; Lipton 2000; Hiraga *et al.* 2001; Peyruchaud *et al.* 2001], prostate [Pollard and Luckert 1985; Pollard and Luckert 1986; Nemoto *et al.* 1988; Shevrin *et al.* 1991; Corey *et al.* 2003], bladder [Nemoto *et al.* 1991; Nemoto *et al.* 1992; Nemoto *et al.* 1993] and multiple myeloma [Croucher *et al.* 2003; Cruz *et al.* 2001; Dallas *et al.* 1999]. Within the bone microenvironment BPs work to inhibit bone resorption therefore decreasing the release of tumour-promoting growth factors from bone and thus hindering the progression of bone metastases. Not only is tumour-induced osteolysis decreased, but also the number and size of tumours within bone.

There is ongoing debate as to whether the inhibition of osteolysis seen with BP treatment is due to a direct anti-tumour effect *in vivo* or whether the BP induces a change in the bone environment that makes it less conducive to tumour cell growth. Supportive, but inconclusive evidence it plentiful. This includes evidence showing increased apoptosis of tumour cells within osteolytic lesions of mice [Yaccoby *et al.* 2002; Hiraga *et al.* 2001]; lowered serum levels of tumour markers in tumour-bearing animals treated with BPs [Yaccoby *et al.* 2002; Corey *et al.* 2003]; and modest increases in survival time [Sasaki *et al.* 1995; Cruz *et al.* 2001]. The effect of BPs on cancer cells in non-bone sites is unlikely, due to the chemical properties of the compound. BP concentrations can reach an adequate level in bone that may cause apoptosis of cancer cells, however such concentrations are not achievable in visceral organs *in vivo* [Fleisch 2000]. Although BPs cause few adverse effects in non-bone organs, changes in visceral metastases after BP treatment in tumour bearing animals have been reported. Results

have been contradictory, with trends towards either a decrease [Alvarez *et al.* 2003; Nobuyuki *et al.* 2001; Sasaki *et al.* 1998] or an increase in visceral metastases [Stearns and Wang 1996; Sasaki *et al.* 1998; Sasaki *et al.* 1995; Cruz *et al.* 2001]. This was despite a marked decrease in new bone metastases [Yoneda *et al.* 2000; Michigami *et al.* 2002]. One report noted an increase in bone metastases after BP treatment [Kostenuik *et al.* 1993]. These inconsistencies may be due to unusual characteristics of some animal tumour models and cannot be reproduced consistently. One study, using the MDA-MB-231 breast cancer animal model, resulted in marked inhibition of bone metastases with ibandronate treatment, but an increase in adrenal metastases [Michigami *et al.* 2002]. No increase in adrenal metastases was observed with co-administration of the chemotherapeutic agent doxorubicin with ibandronate [Michigami *et al.* 2002]. Cancer patients who receive BPs are also treated with anticancer agents, however it still remains possible that preventative BP therapy may have adverse effects on visceral metastases. It is imperative that the risk of visceral metastases due to preventative BP therapy be thoroughly investigated. An increased risk of non-osseous metastases may far outweigh the prophylactic benefit currently observed.

1.7.5 Clinical use of bisphosphonates as cancer therapy

The unique properties of the nitrogen containing BPs include the selective accumulation in bone, the suppression of osteoclastogenesis and the promotion of osteoclast apoptosis, which all lead to inhibition of bone resorption. For this reason, BPs are used for the treatment of cancer-related bone disease particularly for bone metastases, bone pain and hypercalcemia. Cancers frequently associated with metastatic bone disease include advanced, breast cancer [Rosen *et al.* 2003; Lipton 2000], prostate cancer [Saad *et al.* 2002; Adami 1997], lung cancer [Rosen *et al.* 2003] and multiple myeloma [Rosen *et al.* 2003; Sezer *et al.* 2003; Bruno *et al.* 2004; Berenson *et al.*

2002]. From the diagnosis of bone metastases, patients experience severe bone pain often associated with increased skeletal morbidity, rendering them very susceptible to fractures that often require surgery [Martin and Moseley 2000]. BP treatment is the current standard practice for palliative treatment of bone metastases. Clodronate and the more potent N-BPs, including pamidronate and zoledronic acid, are commonly used [Body 2003]. A large number of clinical trials have assessed the clinical benefit of BPs in patients with bone metastases. These studies have measured the occurrence of skeletal-related events (SREs), which are defined as pathologic fracture, spinal cord compression, bone pain that required palliative radiation therapy, bone surgery, or hypercalcemia of malignancy.

The N-BPs pamidronate and zoledronic acid have proven to be more effective in large non-randomised clinical trials than the non-N-BP, clodronate. However only zoledronic acid has demonstrated significant benefits in patients with prostate cancer [Rogers *et al.* 2000; Coleman and Seaman 2001]. Trials using breast cancer patients with at least one bone lesion indicate that treatment with 4 mg of zoledronic acid was more effective than 90 mg of pamidronate in reducing skeletal complications [Rosen *et al.* 2004]. The use of zoledronic acid in four randomised, controlled multicentre, phase III trials involving over 3,000 patients, demonstrated significant improvement in patients with bone lesions secondary to breast cancer, multiple myeloma, prostate cancer, lung cancer and other solid tumours [Rosen *et al.* 2003; Berenson *et al.* 2001; Rosen *et al.* 2004; Rosen *et al.* 2003]. Zoledronic acid is the first BP to show significant clinical benefit in patients with bone metastases from various primary tumours and therefore seems far superior to other BPs.

1.7.6 Combined treatment with bisphosphonates and other agents

Until recently, most *in vitro* and *in vivo* experiments were designed to assess to the anti-cancer activity of BPs as a single agent. This is unlike the clinical scenario, where BPs are given to patients with metastatic bone lesions as adjuvant therapy with conventional antineoplastic agents [Body *et al.* 1998; Diel *et al.* 1998]. In 1996, Sterns and Wang found that the combination of alendronate and paclitaxel showed enhanced anti-cancer activity in tumour bearing mice models of human prostate cancer. Alendronate alone was found to suppress metastases to the bone with no effect on visceral metastases. However, combination of alendronate with paclitaxel not only prevented metastases to the bone, but also prevented non-osseous metastases, resulting in increased survival in the combination treated mice [Stearns and Wang 1996].

In two murine models of bone metastases using breast cancer cells, mice treated with either ibandronate or zoledronic acid and UFT (a pro-drug of flourouracil) resulted in a reduction in bone metastases, and lung and liver metastases and increased survival in the combination treated mice [Yoneda *et al.* 1999]. A reduction in both bone and visceral metastases has also been observed with minodronate and etoposide dual treatment in mice bearing small-cell lung tumours [Yano *et al.* 2003]. More importantly, the combination treatment prolonged the survival of the cancer-bearing mice. Zoledronic acid and Glivec also showed a significant improvement in survival in a mouse model of leukaemia [Kuroda *et al.* 2003].

Enhanced anti-tumour behaviour *in vitro* was also demonstrated using a combination of ibandronate and taxoid. Invasion and migration assays of breast cancer cells treated with this combination resulted in a dose dependent additive effect on inhibition of cell adhesion and cell migration [Magnetto *et al.* 1999].

In addition, experiments have revealed that the combination of zoledronic acid, with the anti-cancer agents dexamethasone and paclitaxel, leads to clear synergistic

apoptotic effects in myeloma and breast cancer cell lines [Tassone *et al.* 2000; Jagdev *et al.* 2001].

Farnesyl transferase inhibitors (FTIs) act by inhibiting farnesyl transferase, the enzyme required for the covalent attachment of a farnesyl lipid to proteins. Some FTIs are also able to inhibit geranylgeranyl transferase but their potency varies depending on the FTI. The use of multiple inhibitors of the mevalonate pathway, specifically FTIs with N-BPs, have led to synergistic anti-tumour activity on *in vitro* murine carcinoma cell invasiveness and *in vivo* metastasis in a murine model of spontaneous lung metastases, supporting the use of this combination for the prevention of metastatic progression [Andela *et al.* 2002]. The limitations of N-BPs for the prevention of metastases, is that it is limited to the skeleton. Use of combination treatment including FTIs, potentially extends its usefulness in the clinical setting to include prevention of visceral metastases.

Specific resistance to N-BPs has been reported in myeloma cell lines after continuous exposure to alendronate for 6 weeks [Salomo *et al.* 2003]. This was due to increased activity of farnesyl pyrophosphate synthase, the target enzyme inhibited by N-BPs, without upregulation of gene transcription suggesting post-transcriptional regulation. The development of resistance is unlikely to occur *in vivo,* as the levels of N-BPs achievable would be the highest at the osteoclast/resorption site interface. More importantly, myeloma cells remained sensitive to conventional anti-neoplastic agents used for the treatment of myeloma thus not compromising N-BP treatment [Salomo *et al.* 2003].

It is apparent from the numerous independent reports that the synergistic and additive behaviour of BPs in combination with other agents is far more beneficial for the treatment of cancer than either agent alone. The interaction with other agents potentially reduces the dose of N-BPs required for the treatment of cancers that

previously were only susceptible to N-BP-induced apoptosis at unacceptably higher doses. These data provide solid evidence for future preclinical and clinical investigations, to identify suitable combinations of N-BPs with other anti-cancer and novel agents, for synergistic anti-tumour activity in the treatment of cancer patients.

1.7 OTHER INHIBITORS OF THE MEVALONATE PATHWAY

1.7.1 Statins

Developed in the late 1970's, the statins, also known as HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors, are a class of drugs that inhibit the rate-limiting step of the mevalonate pathway required for cholesterol biosynthesis (**Figure 1.12**) [Goldstein and Brown 1990]. Statins are characterised by an open-ring side chain, which is able to bind to the active site of HMG-CoA reductase, inhibiting the enzyme [Istvan *et al.* 2000]. Compared to the natural substrate, statins are able to bind approximately 1,000 times more effectively to HMG-CoA reductase [Istvan and Deisenhofer 2001]. Inhibition of cholesterol biosynthesis leads to increases in hepatic LDL receptor, which promotes hepatic LDL-cholesterol uptake and clearance of cholesterol from the bloodstream, therefore lowering endogenous cholesterol levels [Ness *et al.* 1996]. For this reason, statins are widely used for the treatment of hypercholesterolemia. Like the N-BPs, the statins also inhibit protein prenylation, preventing the post-translational lipid attachment modifications of many proteins essential for their function, including the GTPases. Other beneficial uses of statins include, anti-inflammatory, anti-thrombotic and immunosuppressive effects [Rosenson *et al.* 1999; Rosenson and Tangney 1998; Bellosta *et al.* 2000]. Recent reports of statin

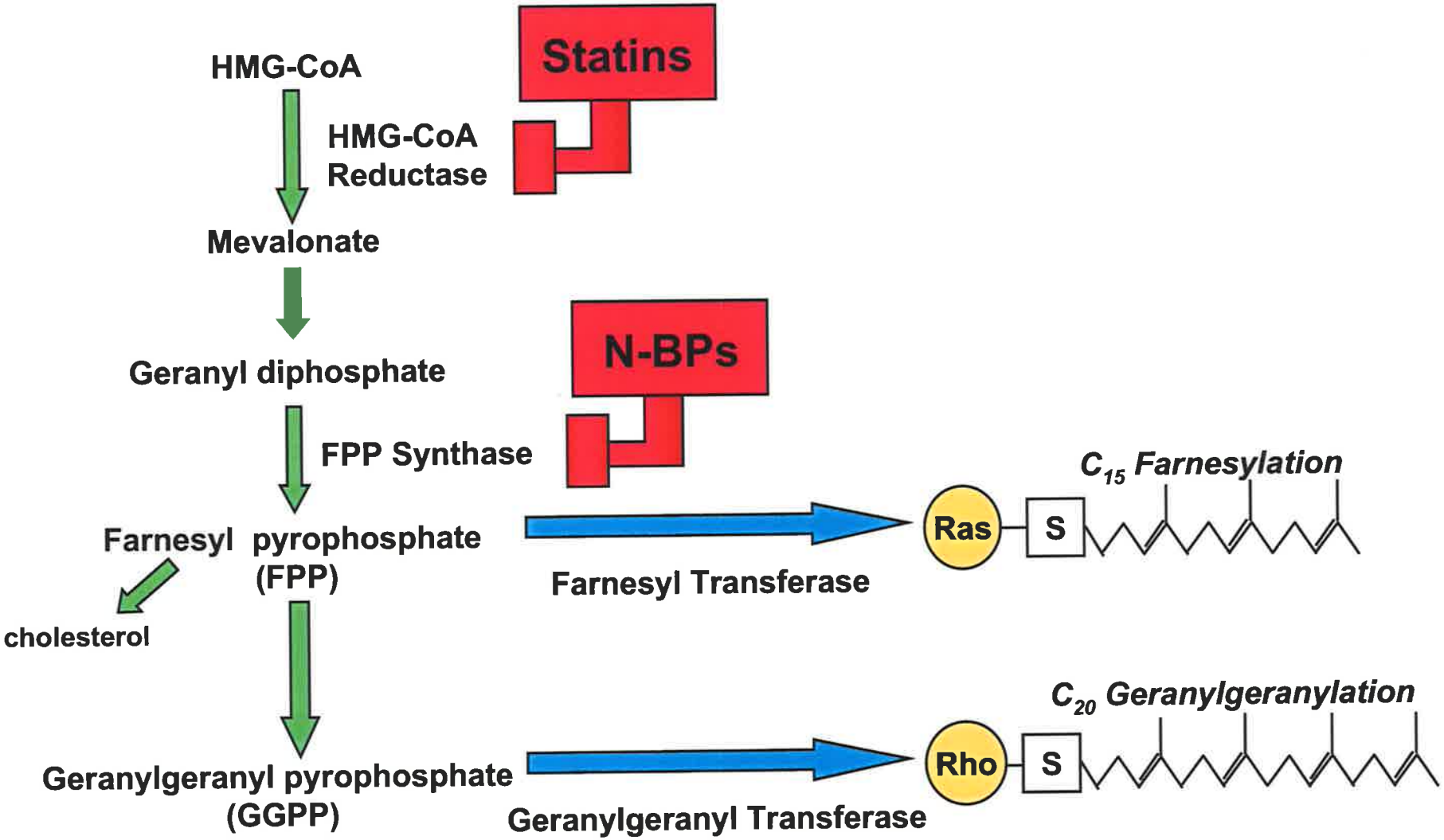


Figure 1.12 The mevalonate pathway. Statins inhibit the enzyme as indicated above, preventing downstream processes including cholesterol biosynthesis, farnesylation and geranylgeranylation.

activity have included the stimulation of bone formation and anti-cancer activity [Bellosta *et al.* 2000].

In 1999, Mundy reported that statins were potent stimulators of bone formation *in vitro* due to associated increases in BMP-2 gene expression in bone cells [Mundy *et al.* 1999]. Subsequent reports supported statin-induced BMP-2 expression in human OS cells, which was inhibited by the addition of downstream metabolites of the mevalonate pathway, indicating that gene activation was directly due to enzyme inhibition [Sugiyama *et al.* 2000]. It has been suggested that the degree of water solubility of statins, determines their ability to induce increases in BMP-2 expression. Lipophilic statins, such as lovastatin are far better at increasing BMP-2 expression than water-soluble statins, such as pravastatin [Sugiyama *et al.* 2000]. Lipophilic statins are osteogenic and promote osteoblastic calcification independently of BMP-2 [Izumo *et al.* 2001]. *In vivo* studies have confirmed increases in cancellous bone mass and bone strength [Oxlund *et al.* 2001]. In humans, several clinical studies have described the skeletal effects of statins. In a study of 6,110 subjects, a 71% reduction of hip fractures was reported among statin users [Wang *et al.* 2000]. These results have been confirmed by other population-based case-control studies, with significant reductions of fractures and associated increased BMD in statin users [Chan *et al.* 2000; Meier *et al.* 2000; Pasco *et al.* 2002]. This was compared with users of other lipid lowering agents to rule out that hyperlipidemia itself is not associated with fewer fractures [Bauer 2003].

It is evident from the escalating use of statin therapy in the past 15 years that long-term use is safe and well tolerated. Initial concerns regarding associated increases in cancer incidence were raised, however long term follow up trials have demonstrated no association between the use of statins and increased risk of cancer [Bjerre and LeLorier 2001; Hebert *et al.* 1997; Pedersen *et al.* 2000].

Analogous to the N-BPs, statins also exhibit anti-proliferative, anti-invasive and apoptotic effects in transformed cells [Wong *et al.* 2002; Chan *et al.* 2003]. The mechanism of the effects of statins has been shown to be the inhibition of protein prenylation [Keyomarsi, 1991; [Denoyelle *et al.* 2001; Xia *et al.* 2001]. All effects were reversed upon the addition of an intermediate of the mevalonate pathway, such as geranylgeraniol, confirming the importance of prenylation, particularly geranylgeranylation [Crick *et al.* 1998; Kusama *et al.* 2001; Agarwal *et al.* 1999]. Tumour cell types sensitive to statin-induced apoptosis include acute myeloid leukaemia (AML) [Dimitroulakos *et al.* 1999; Newman *et al.* 1994]; juvenile myelomonocytic leukaemia, squamous carcinoma of the head and neck and cervix, rhabdomyosarcoma, medulloblastoma, neuroblastoma [Dimitroulakos *et al.* 2001]; mesothelioma [Rubins *et al.* 1998]; astrocytoma [Jones *et al.* 1994]; and pancreatic cancer [Muller *et al.* 1998]. Statins, like BPs, trigger cell death in a tumour specific manner. In AML, statin induced apoptosis occurs in both primary tumours and tumour cell lines but not myeloid progenitor cells from normal bone marrow [Dimitroulakos *et al.* 1999; Newman *et al.* 1997].

In addition to their *in vitro* efficacy, statins have been used in many different animal models and shown to have anti-tumour effects. Anti-tumour effects were observed *in vivo* in leukaemia, glioma, neuroblastoma and liver, pancreatic and lung cancer [Kikuchi *et al.* 1997; Clutterbuck *et al.* 1998; Maltese *et al.* 1985; Kawata *et al.* 1992; Hawk *et al.* 1996]. A significant reduction in metastases was also observed for lymphoma, fibrosarcoma, melanoma, breast and colon cancer [Matar *et al.* 1998; Matar *et al.* 1999; Alonso *et al.* 1998; Broitman *et al.* 1996; Jani *et al.* 1993]. The use of statins in chemoprevention has been demonstrated in radiation-induced mammary tumourigenesis and chemical-induced colon tumourigenesis [Inano *et al.* 1997; Narisawa *et al.* 1996; Narisawa *et al.* 1996].

Clinical safety and efficacy of statins as anticancer agents has been evaluated in numerous clinical trials. Phase 1 clinical studies evaluated maximum tolerable dose and toxicity of lovastatin in advanced malignancies with doses reaching up to 30-45 mg/kg/day [Thibault *et al.* 1996; Larner *et al.* 1998]. Clinical efficacy of lovastatin has been reported with conflicting results. A phase II study in gastric adenocarcinoma generated disappointing results, however a randomised trial in patients with advanced liver cancer resulted in median survival of 18 months in the statin treated group compared with only 9 months in the control group [Kim *et al.* 2001; Kawata *et al.* 2001]. This suggests that statins may be of value in disease control when combined with standard chemotherapeutic therapy [Miller *et al.* 1993]. There is increased interest in the use of statin therapy for the treatment of cancer and clinical trials are ongoing. In advanced or recurrent cancers, statins are more likely to be effective when given in combination with other agents.

Combination therapy of statins with other agents has been investigated and found to act both additively and synergistically. These include the agents 5-flouracil, N,N'bis(2-chloroethyl)-N-nitrosurea, cisplatin, doxorubicin, cytosine arabinoside and non-steroidal anti-inflammatory drugs (NSAIDS) [Agarwal *et al.* 1999; Agarwal *et al.* 1999; Soma *et al.* 1992; Feleszko *et al.* 1998; Feleszko *et al.* 2000; Holstein and Hohl 2001; Wong *et al.* 2001]. Combination therapy has also been successful in animal models, with synergism observed in three tumour models in mice using lovastatin and doxorubicin [Feleszko *et al.* 2000]. Additionally, lovastatin has been used to potentiate anti-tumour activity of TNF-α and carmustine, in mouse tumour models of melanoma and astrocytoma [Feleszko *et al.* 1999; Feleszko *et al.* 1995; Soma *et al.* 1992]. One conflicting report by Bardeleben, suggests that the statin lovastatin, protects cells from doxorubicin and etoposide-induced cell death *in vitro* [Bardeleben *et al.* 2002]. These experiments have been repeated in an independent laboratory, where no evidence for

protection against doxorubicin and etoposide was found (unpublished data, Labrinidis *et al.*). Therefore, there is uncertainty regarding the validity of Bardeleben's data.

In a study of the relationship between cell cycle progression and TRAIL-induced apoptosis, it was found that a combination of simvastatin or lovastatin and TRAIL significantly augmented apoptosis in tumour cells but not normal cells [Jin *et al.* 2002]. This has been the first documented report of statin and TRAIL combination treatment *in vitro* and signifies the therapeutic potential for combining inhibitors of the mevalonate pathway with death receptor-mediated apoptosis.

The identification and improvement of new and known combinations of agents with statin therapy, needs to be investigated further. Additional optimisation and evaluation of statin therapy, alone and in combination, will improve efficacy in the clinical setting.

1.8.2 Prenyl transferase inhibitors (PTIs)

Post-translational modification or prenylation refers to the covalent addition of a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid onto acceptor proteins. Many small GTPase proteins require prenylation for proper biological function [Zhang and Casey 1996]. This requires the enzyme farnesyl transferase or geranylgeranyl transferase [Casey 1992]. The need to develop inhibitors of these enzymes was quickly recognised when it was discovered that the Ras GTPase protein was a major contributor to human cancer with expression of the Ras oncoprotein identified in over 30% of cancers [Sebti and Hamilton 1997]. Four different forms of the Ras protein, H-, N-, K_A-, and K_B-Ras require farnesylation to induce malignant transformation [Casey 1992; Zhang and Casey 1996]. Alternatively, R-Ras and TC-21 require geranylgeranylation to mediate Ras transformation and malignancy [Zohn *et al.* 1998]. Studies with the mutated K-Ras demonstrated that the protein is able to be cross-prenylated, that is either

farnesylated or geranylgeranylated [James *et al.* 1995; Rowell *et al.* 1997]. Other GTPases, such as RhoA, Rac1 and cdc42 also require geranylgeranylation to promote tumourigenesis [Zohn *et al.* 1998].

This led to the development of farnesyl transferase inhibitors (FTIs) and geranylgeranyl transferase inhibitors (GGTIs), (or in general terms referred to as PTIs), as novel anticancer agents, to specifically block the farnesylation and geranylgeranylation process, as shown in **Figure 1.13** [Sebti and Hamilton 2000]. This new approach to cancer therapy mimics the mechanism of action of N-BPs and statins. That is, inhibition of prenylation, the gradual depletion of prenylated proteins, an increase in unprenylated proteins leading to the inability of the cell to function and ultimately cell death. As is seen with the other inhibitors of the mevalonate pathway, variations in structure greatly affect the bioavailability and potency of the PTIs.

It is not surprising that PTIs induce much the same effect in tumour cells as other inhibitors of the mevalonate pathway, such as anti-proliferation and apoptosis in human cancer cells [Ashar *et al.* 2001; Bolick *et al.* 2003; Morgan *et al.* 2003; van de Donk *et al.* 2003; Vogt *et al.* 1997]. To elucidate the processing required for protein function, most *in vitro* studies to date have focused on the ability of FTIs and or GGTIs to potently and selectively inhibit prenylation of specific Ras mutants [Sebti and Hamilton 2000; Lobell *et al.* 2001]. It has been shown that the ability of FTIs to inhibit growth of human cancer cell lines does not correlate with Ras mutation status [Sepp-Lorenzino *et al.* 1995; Nagasu *et al.* 1995].

The efficacy of PTIs in animal models has been investigated with encouraging results. Although K-Ras requires both FTIs and GGTIs to inhibit prenylation *in vitro*, each alone is able to inhibit tumour growth in nude mouse xenograft models [Sun *et al.* 1998]. This verifies the concept that additional proteins requiring farnesylation, other than K-Ras, are critical for tumour growth and survival. Similarly, geranylgeranylated

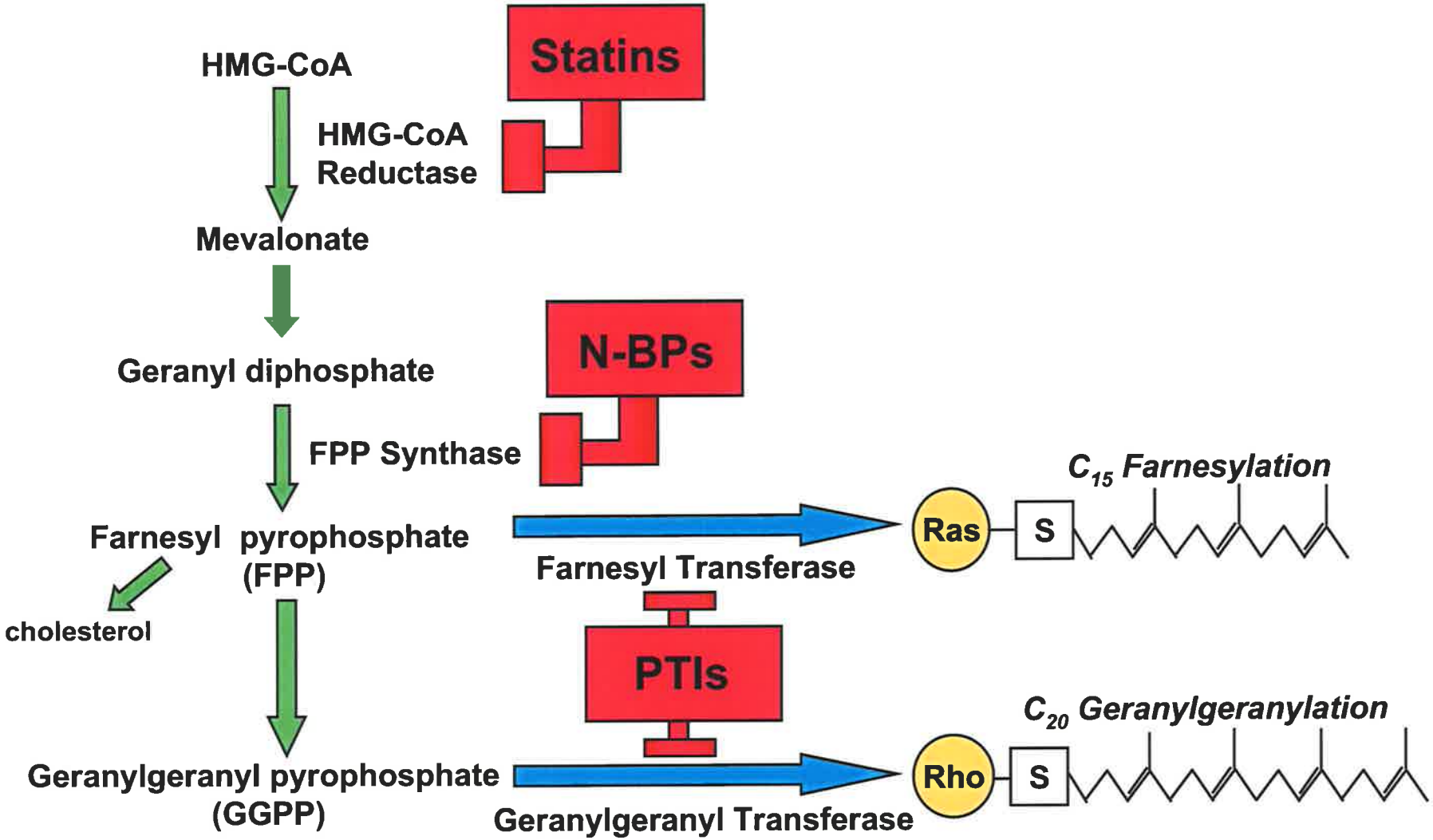


Figure 1.13 The mevalonate pathway. PTIs inhibit the enzymes as indicated above, preventing farnesylation and geranylgeranylation.

proteins are also vital for tumour progression, as GGTI treatment led to tumour growth inhibition, supporting previous *in vitro* studies that RhoA and Rac1 are involved in tumourigenesis [Zohn *et al.* 1998]. Numerous laboratories have shown human tumour growth inhibition in nude mice xenografts treated with FTIs [Sebti and Hamilton 2000; Cox and Der 1997; Gibbs and Oliff 1997; Sun *et al.* 1999]. Lantry demonstrated the ability of FTIs to inhibit the growth of chemically induced lung tumours in nude mice [Lantry *et al.* 2000]. These results illustrate the anti-tumour potential of FTIs, by not only inhibiting tumour growth in xenografts but also in the tumours own microenvironment. FTIs do not simply inhibit tumour growth, but actually cause tumour regression. In a transgenic mouse model of mammary cancer, Ha-*ras* oncogenes expressed under the control of mouse mammary tumour virus (MMTV) promoter, cause the development of aggressive mammary and salivary carcinomas [Gibbs and Oliff 1997]. Upon FTI treatment of animals bearing tumours, regression was observed in 100% of the mice. Variants of the Ras oncogene produced varying results *in vivo,* suggesting that genetic differences may influence prognosis.

Combination therapy using both FTIs and GGTIs was found to significantly enhance the chemotherapeutic effect of human colon cancer cells both *in vitro* and *in vivo* [Di Paolo *et al.* 2001]. It seems that inhibition of both geranylgeranylation and farnesylation triggers a magnified apoptotic signal that enhances cell death.

The use of standard cytotoxic agents in combination with PTIs, has been reported by several independent groups [Adjei *et al.* 2001; Shi *et al.* 2000; Sun *et al.* 1999]. Sun demonstrated that the co-addition of cisplatin, taxol or gemcitabine with either FTI or GGTI, resulted in greater anti-tumour efficacy than monotherapy [Sun *et al.* 1999]. Similarly, taxol and desoxypothilone in combination with FTI produced synergistic killing effects in breast cancer cell lines [Moasser *et al.* 1998]. Theoretically, cytotoxic agents that do not induce cell cycle arrest should be more effective agents in

combination with FTIs. This holds true for cisplatin, which is not cell cycle specific and has a synergistic apoptotic effect with FTIs [Sun *et al.* 1999].

Combining FTIs and radiation therapy has been one approach to overcoming radiation resistance. Ras activation has been identified as the link to increased radiation resistance in transformed cells [FitzGerald *et al.* 1985]. Inhibition of farnesylation by the addition of FTIs to H-Ras transformed fibroblasts, led to the sensitisation of the cells to radiation-induced apoptosis [Bernhard *et al.* 1996]. K-Ras transformed cells however required both FTI and GTI to become sensitised to radiation [Bernhard *et al.* 1998]. Other tumour cell lines tested using a variety of FTIs *in vitro* and *in vivo* include bladder, breast, colon and lung [Sebti and Hamilton 2000; Kohl *et al.* 1995; Britten *et al.* 2001; End *et al.* 2001].

The most important feature of FTIs is their very low toxicity to normal cells at concentrations that affect transformed cells [Sebti and Hamilton 2000]. Long term use of FTIs in mice, with over two months worth of daily injections, showed no toxicity or weight loss in the animals treated [Sun *et al.* 1999; Sun *et al.* 1998]. Many reports have also observed the lack of toxicity with FTI use [Cox and Der 1997; Gibbs and Oliff 1997; Sebti and Hamilton 1997; Sebti and Hamilton 2000]. The reason for lack of toxicity is unknown. Lower FTI concentrations may be adequate to inhibit Ras processing in transformed cells compared to other proteins, such as nuclear lamins, in normal cells [Garcia *et al.* 1993]. Another explanation may be that Ras signalling occurs through some but not all pathways so that FTI inhibition of oncogenic Ras does not affect the function of normal R-Ras2/TC21, which is expressed ubiquitously in many cells [Carboni *et al.* 1995].

On the other hand, GGTIs have been reported as showing a higher level of toxicity *in vivo* than FTIs [Lobell *et al.* 2001]. This has not been consistent, with encouraging *in vivo* results reported by Di Paolo using the GGTI, BAL9611 [Di Paolo

et al. 2001]. GGTIs have biological activities such as inhibition of human tumour growth *in vitro* and *in vivo* that are highly desirable for novel anticancer agents. There is therefore a pressing need to evaluate current forms of GGTIs and to identify new, structurally different forms, that exhibit enhanced anti-cancer activity with low toxicity. The recent development of inhibitors capable of inhibiting both farnesylation and geranylgeranylation, known as dual inhibitors, looks very promising, with increased efficacy and reduced toxicity in both *in vitro* and xenograft models of cancer [Vigushin *et al.* 2004; Dinsmore *et al.* 1999; Dinsmore *et al.* 2004].

FTIs have displayed impressive efficacy in preclinical models of cancer. Phase I trials have evaluated the maximum tolerable dose and have demonstrated that they are well tolerated and suitable for prolonged administration [Brunner *et al.* 2003]. Karp et al. reported encouraging results in patients with leukaemia, with a 30% response rate [Karp *et al.* 2001]. Phase II trials in haematological malignancies have confirmed improved response rates of 60% in chronic myelogenous leukaemia [Keating 2002], 30% in myelodysplastic syndromes [Kurzrock *et al.* 2001; Kurzrock *et al.* 2001], 29% in myeloproliferative disorders [Gotlib *et al.* 2002] and 75% in multiple myeloma [Alsina *et al.* 2002]. Solid tumour malignancies on the other hand have been disappointing in clinical trials using single-agent FTIs in bladder cancer [Winquist *et al.* 2001], prostate cancer [Haas *et al.* 2002], colorectal cancer [Sharma *et al.* 2002], and pancreatic cancer [Cohen *et al.* 2002]. Breast cancer, small cell lung cancer and glioma cancers however have provided positive results in clinical trials suggesting that tumours with specific molecular characteristics are more susceptible to FTI therapy [Johnston *et al.* 2002; Heymach *et al.* 2004; Cloughesy *et al.* 2002]. Preliminary results of phase III trials have been reported for colorectal cancer and pancreatic cancer. Both trials showed no statistical difference between the placebo group and the group for median overall survival [Cunningham *et al.* 2002; Van Cutsem *et al.* 2002]. Other phase III trials are

still under way. Although this has dampened enthusiasm for the use of FTIs, many of the initial studies were based on the assumption that K-Ras mutations are sensitive to FTIs. Subsequent studies suggest that sensitivity to inhibition of tumours by FTIs is independent of Ras mutation status and tumours with these mutations may actually be more resistant to FTIs. Clinical trials are often carried out on patients in the late stages of their disease with often highly metastatic cancers that are not responsive to FTI monotherapy. It may be more beneficial to treat patients in the early stages of their disease where the anti-angiogenic and pro-apoptotic properties of FTIs may prove more favourable. Preclinical studies investigating synergistic interactions using combinations of FTIs with other classes of anticancer agents, proved to be highly effective in inducing cancer cell death and may well be the therapy of choice in treating patients in the clinic.

1.9 AIMS AND SIGNIFICANCE OF PROJECT

Osteosarcoma is the most common primary malignancy of the bone in children and adolescents. The treatment of OS has undergone considerable changes over the past 20 years with the efficacy of chemotherapy significantly improving long-term survival compared to surgery alone. Despite this, patients continue to have a poor prognosis with the frequent acquisition of drug-resistant phenotypes and the occurrence of "second malignancies" associated with chemotherapy. It is therefore vital to find new alternative approaches for the treatment of OS.

Bisphosphonates (BPs), statins and prenyl transferase inhibitors (PTIs) all act by inhibiting the mevalonate pathway, a biosynthetic pathway responsible for the production of many intracellular products. Preclinical data clearly demonstrates the cytostatic, anti-proliferative and pro-apoptotic properties of these agents. More

importantly, N-BPs, statins and PTIs exhibit anti-cancer properties that are amplified in combination with chemotherapeutic agents. The identification of novel combinations that induce significant cell death but lack the associated toxicity observed with current therapy, is highly desirable. BPs high affinity for bone, make it ideal for the treatment of OS as bioavailability is optimal, whereas the use of statins and PTIs are useful in OS metastases and other soft tissue sarcomas.

TRAIL, a member of the TNF family, is capable of inducing apoptotic cell death in a wide variety of tumour cell lines, but does not seem to be cytotoxic to many normal cell types *in vitro,* or *in vivo*. It was found that TRAIL-resistant tumour cells can frequently be sensitised to TRAIL by treatment with cytostatic or cytotoxic agents. In contrast normal human osteoblasts are not sensitive to TRAIL alone and are not sensitised by conventional chemotherapeutic agents. These observations suggest that TRAIL may be a useful anti-tumour agent for OS and that it compares favourably with its TNF-ligand superfamily counterparts, Fas and TNF-α, which are cytotoxic to normal cells.

The aim of the current study was to investigate the effect of N-BPs, statins and PTIs in combination with TRAIL, in delivering an apoptotic stimulus to OS cells. Such an outcome would bring the hope of achieving substantial apoptotic response in this tumour type without the toxic side effects of current therapies.

The specific aims of this study are:

1. To investigate the apoptotic effect induced by the bisphosphonate, zoledronic acid, on OS cell lines.

2. To investigate the additive/synergistic interactions observed between zoledronic acid and TRAIL and to elucidate the molecular mechanisms involved.

3. To assess whether other inhibitors of the mevalonate pathway, specifically statins and prenyl transferase inhibitors, exhibit additive/synergistic activity in combination with TRAIL.

4. To establish an *in vivo* OS model and to assess the effects of zoledronic acid and TRAIL therapy as single agents and in combination.

CHAPTER TWO

MATERIALS AND METHODS

2.1 CELL LINES AND TISSUE CULTURE

2.1.1 Osteosarcoma cell lines

The cell lines listed below were purchased from the American Type Culture Collection (ATCC), Manassus, VA, USA.

HOS

BTK-143

G-292

MG-63

SJSA-1

Saos2

K-HOS

The cell line listed below was provided by Dr Berlin, Department of Orthopaedics, Sahlgrenska Hospital, Goteborg, Sweden.

KRIB

2.1.2 Normal human bone donor cells

Human osteoblastic cells were obtained from trabecular bone of osteoarthritic patients at joint replacement surgery, as described previously [Gronthos *et al.* 1999] or were grown from needle aspirates from the iliac crest of normal healthy young (25-35 years) donors. Briefly, specimens of bone were dissected into 0.3-0.5 mm pieces and washed extensively in phosphate buffered saline (PBS), pH 7.4. The fragments were seeded as explants into 75 cm^2 culture dishes (Corning, Costar Corp. Cambridge, MA, USA) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in αMEM containing 10% heat-inactivated fetal calf serum and L-ascorbic acid 2-phosphate. The medium

was replaced at 4-day intervals. Cells typically reached confluence after 4-6 weeks. Cells were then subcultured by treatment with a 0.1% (w/v) mixture of collagenase and dispase. Cells from first passage were used for all experiments.

2.1.3 Cell culture

All tissue culture was performed under sterile conditions within laminar flow hoods. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. A haemocytometer and trypan blue dye exclusion was used to determined cell density. Cells were incubated in 0.4%(w/v) trypan blue in phosphate buffered saline (PBS) exclusion and counted using a light microscope. The osteosarcoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), (supplied by the Media Production Unit, IMVS, SA). This was supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), gentamicin (160 µg/ml) and 10% fetal bovine serum. The medium was filter sterilised and stored at 4°C.

Cell lines were maintained by subculturing every 3 to 4 days using 0.05% (w/v) Trypsin digestion (supplied by the Media Production Unit, IMVS, SA).

2.2 CHEMICALS AND REAGENTS USED

2.2.1 General chemicals and reagents

The following chemicals and reagents were purchased from:

Sigma Chemical Co. Ltd., St Louis, MO, USA.

Ampicillin

β-mercaptoethanol

Bovine Serum Albumin

Bromophenol Blue

Dithiothreitol (DTT)

Ethylenediaminetetraacetic acid (EDTA)

Ethidium Bromide

Formamide

Hepes

Puromycin

Coomasie Stain

MOPS

Polyxyethylene Sorbitan Monolaerate (Tween 20)

Phenylmethylsulphanyl Fluoride (PMSF)

Saponin

Sephadex G_{50}

Sodium dodecyl sulphate (SDS)

Sodium Bicarbonate

Sodium Chloride

Sodium Vanadate

Sucrose

Tris-HCl

TRIS[hydroxymethyl]aminomethane

Triton-X-100

BDH Laboratory Supplies, Poole ,U.K.

Acetic Acid

Chloroform

Crystal violet

Dimethyl Sulphoxide (DMSO)

Ethanol

Formaldehyde

Formalin

Glycerol

Methanol

Sodium Chloride

Sodium Hydroxide

Roche Molecular Biochemicals, Castle Hill, NSW, Australia.

Cell Proliferation Reagent (WST-1)

4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI)

Glycogen (molecular grade)

Magnesium chloride

Invitrogen, BV CH, Groningen, Netherlands.

4-12% Bis-Tris Pre-Cast Gels

NuPage Antioxidant

NuPage LDS Sample buffer 4X PVDF membrane

NuPage MES SDS Running Buffer 20X

NuPage Sample reducing agent NuPage Bis-Tris Gels

NuPage Transfer Buffer 20X Superscript II

SeeBlue plus 2 protein markers

Sources of other routinely used reagents:

DNA grade Agarose	Progen Industries, QLD, Australia
Low Melting point Agarose	Progen Industries, QLD, Australia
CHAPS	Pierce, Rockford, IL, USA
Dulbeccos Mod. Eagles. Media	ICN Biomedicals, Aurora, OH, USA
α-Minimum Essential Media	ICN Biomedicals, Aurora, OH, USA
Diploma instant skim milk	Bonlac Foods Ltd, VIC, Australia
Dextran	Progen Industries, QLD, Australia
Foetal Calf Serum	TRACE Laboratories, VIC, Australia
Reagent A	Pierce, Rockford, IL, USA
Reagent B	Pierce, Rockford, IL, USA
DNA molecular weight markers	GeneWorks Pty Ltd, SA, Australia
SYBR-Gold Nucleic acid stain	Molecular Probes, Eugene, OR, USA
Recombinant human TRAIL	Peprotech, Rocky Hill, NJ, USA
Trypsin-EDTA	Media Production Unit, IMVS, SA, Australia
L-Glutamine	Media Production Unit, IMVS, SA, Australia
Hybond H^+ nylon membrane	Amersham Pharmacia Biotech, UK
Univert mountant	Merck, Kilsyth, VIC, Australia

2.2.2 Novel anticancer agents

Recombinant human TRAIL	Genentech Inc. San Francisco, CA, USA
Zoledronic Acid (Zometa)	Novartis Pharmaceuticals, Australia Pty Ltd. (Sydney, NSW, Australia)
Lovostatin	Sigma-Aldrich Co.
Mevastatin	Sigma-Aldrich Co.
FTI-277	Calbiochem, Alexandria, NSW, Australia

GGTI-298	Calbiochem, Alexandria, NSW, Australia

2.2.3 Enzymes and inhibitors of caspase activity

The following enzymes and inhibitors were used to detect or inhibit caspase activation.

Caspase-3 inhibitor II (Z-DEVD-fmk)	Calbiochem, Alexandria, NSW, AUS
Caspase-3/CPP32 Fluorogenic-Substrate (Ac-DEVD-AFC)	Calbiochem, Alexandria, NSW, AUS
Caspase Inhibitor III (Boc-D-fmk)	Calbiochem, Alexandria, NSW, AUS
Caspase Inhibitor I (Z-VAD-fmk)	Calbiochem, Alexandria, NSW, AUS

2.2.4 Antibodies and recombinant proteins

A list of Antibodies used in this thesis is listed below.

Table 2.1

Specificity	Antibody	Isotype	Source
TRAIL-R1	M271	Mouse IgG2a	Immunex, Seattle, WA, USA
TRAIL-R2	M413	Mouse IgG1	Immunex, Seattle, WA, USA
TRAIL-R3	M430	Mouse IgG1	Immunex, Seattle, WA, USA
TRAIL-R4	M444	Mouse IgG1	Immunex, Seattle, WA, USA
OPG	805	Mouse IgG1	R&D systems, Minneapolis, MN, USA
OPG	8051	Mouse IgG2A	R&D systems, Minneapolis, MN, USA
Poly-(ADP-Ribose)-polymerase		Rabbit IgG	Roche Molecular Biochemicals, Castle Hill, NSW, Australia

Unprenylated RAP1A (C-17)	sc-1482	Rabbit IgG	Santa Cruz Biotech. California, USA
Total RAP 1A	sc-65	Goat IgG	Santa Cruz Biotech. California, USA
BAX (N-20)	sc-493	Rabbit IgG	Santa Cruz Biotech. California, USA
Bcl-2 (N-20)	sc-492	Rabbit IgG	Santa Cruz Biotech. California, USA
Actin (I-19)	sc-1616	Mouse IgG1	Santa Cruz Biotech. California, USA
1D4.5		Mouse IgG2a	Dr Leonie Ashman, University of Newcastle, NSW, AUS
1B5		Mouse IgG1	Dr Leonie Ashman, University of Newcastle, NSW, AUS
Sheep IgG -FITC		Mouse Ig	Southern Biotechnology, Birmingham, AL, USA
Goat IgG-PE		Mouse IgG	Southern Biotechnology, Birmingham, AL, USA

2.2.5 Solutions, buffers and kits

DNA loading buffer (6X): 0.25% Bromophenol Blue, 40% (w/v) sucrose in water.

RNA loading buffer (2X): 50% Formamide, 6.2% Formaldehyde, 10% glycerol, 30μg/ml Ethidium bromide.

MOPS buffer: 0.2M MOPS, 50mM Na acetate, 10mM EDTA (pH 8.0).

Protein Lysis Solution: 25mM MOPS (pH 7.2), 60mM β-glycerolphosphate, 15mM $MgCl_2$, 5mM EGTA, 1mM DTT, 0.1mM Na Vanadate, 1% Triton X-100, 1mM PMSF.

Caspase-3 Assay Buffer: 50mM HEPES, 0.1% CHAPS, 10% Sucrose, 10mM DTT, pH 7.4.

Caspase-3 Lysis Solution:	5mM EDTA, 5mM TRIS-HCl, 0.5% NP40 solution, pH 7.4.
Crystal Violet Solution:	1% Crystal Violet, in a 2% ethanol solution (total volume of 100mls).
TAE 50X:	40mM TRIS-acetate, 0.001M EDTA (pH 8.0).
EDTA (0.5M):	93.06gms EDTA in 400mls of water, pH to 8.0, make up to 500mls with water.
Anti-Fade (propylgallate):	2gms n-propylgallate, 80% glycerol, 20% 1x PBS, rotate at room temperature overnight until dissolved and store at 4° C.
Propidium Iodide(PI)/ Triton X-100	0.1% w/v Triton X-100 in 10mls PBS, 2 mg Dnase-free Rnase A, 30µl of 0.1% w/v Triton X-100 in 10mls PBS, 2 mg Dnase-free Rnase A, 30µl of 10mg/ml PI (PI stock-dissolve 1mg PI in 1 ml H_20 and store at 4oC)
WST-1 cell proliferation reagent assay kit	Roche Molecular Biochemicals, Manheim, Germany
BCA Protein Assay kit	Pierce Biotechnology Inc, Rockford, IL, USA
NuPAGE® System	Invitrogen Corporation, Carlsbad, CA, USA

2.2.6 Antibiotics

GENETICIN (G-418)	Invitrogen, BV CH, Groningen, Netherlands
Penicillin (100 IU/ml)	Media Production Unit IMVS, S.A., Australia
Streptomycin (100 µg/ml)	Media Production Unit IMVS, S.A., Australia

Gentamicin (160 μg/ml)	Media Production Unit IMVS, S.A., Australia
Puromycin (2 μg/ml)	Sigma Chemical Co. Ltd., St Louis, MO, USA.

2.3 SPECIALISED EQUIPMENT USED

2.3.1 Flow cytometer

Analysis was carried out using the Becton and Dickinson "FACscan" flow cytometer coupled with LYSIS II or CellQuest software. Graphs were aligned using the WinMDI 2.8 software package. See section 2.4.8 and 2.4.9 for a detailed description of receptor analysis and cell cycle analysis methods using flow cytometry.

2.3.2 Phospho-imager

Imaging for western blot analysis (refer to 2.4.9) and DNA electrophoresis imaging (2.4.7) was performed using the Typhoon 9410 Variable Mode Imager (Amersham Biosciences, Buckinghamshire, UK).

2.3.3 Plate reader

The Perkin Elmer LS50 spectrofluorometer was used to measure optical density of crystal violet assays, WST-1 assays and protein estimation assays as described in sections 2.4.2, 2.4.3 and 2.4.6, respectively.

2.3.4 Radiography

Radiographs were taken using the HP cabinet X-Ray System-Faxitron Series, model no: 43855A. Exposure time and intensity was optimised with final settings set for 18 sec at 60 kVp. Kodak min R-2000 film was used supplied by Kodak (Australasia) Pty Ltd, Melbourne, Australia.

2.3.5 Micro-computer tomography analysis

High-resolution computer micro-tomography analysis was performed using SkyScan-1072 micro-CT (Skyscan, Aartselaar, Begium). The SkyScan software CONE-REC, 3D creator and CTan were used for file conversion, 3D reconstruction of images and bone volume analysis, respectively.

2.4 METHODS

2.4.1 Measuring cell viability using crystal violet

For determination of cell viability, $1x10^4$ cells per well were seeded in 96 well microtiter plates and allowed to adhere to the plate overnight. The cell density varied between different experiments, based upon the size of well and experimental design; however, the level of confluency for experiments did not exceed 70%. Cells were then treated for 72 hours with various combinations of novel anti-cancer agents. Cell viability was determined by staining with crystal violet and measuring OD_{570} of cell lysates.

2.4.2 Measuring cell viability using WST-1 assay

For determination of cell viability, $1x10^4$ cells per well were seeded in 96 well microtiter plates and allowed to adhere to the plate overnight. Cells were then treated for 72 hours with various combinations of novel anti-cancer agents. Cell viability was determined using the WST-1 cell proliferation reagent assay kit, a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cells. Media was removed from the wells at termination of the experiment and 200µl of WST-1 solution made up in DMEM media (100µl of WST-1 for every 1000µl

of medium) was added and left to incubate between 30 mins to 3 hours at 37° C. The absorbance was measured using an ELISA reader at 420-480 nm at 30 min intervals. The reference wavelength used was 600 nm.

2.4.3 Measuring caspase activity

DEVD-caspase was assayed by cleavage of zDEVD-AFC (z-asp-glu-val-asp-7-amino-4-trifluoro-methyl-coumarin), a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly (ADP-ribose) polymerase [Medina *et al.* 1997]. Cells were seeded at a density of $5x10^5$ in 24 well plates were treated as indicated, washed once with HBSS and resuspended in 200 µl of NP-40 lysis buffer containing 5 mM Tris-HCl, 5 mM EDTA and 0.5% NP40, pH 7.5. After 15 min in lysis buffer at 4°C, insoluble material was pelleted at 15,000g and an aliquot of the lysate was tested for protease activity. To each assay tube containing 8 µM of substrate in 1 ml of protease buffer (50 mM Hepes, 10% sucrose, 10 mM DTT, 0.1% CHAPS, pH 7.4) was added to 20 µl of cell lysate. Reactions were allowed to proceed for 4 hour at room temperature in total darkness, whereupon fluorescence was quantified (Exc 400 nm, Emis 505 nm). Optimal amounts of added lysate and duration of assay were determined in preliminary experiments. One unit of caspase activity was taken as one fluorescence unit (at slit widths of 12.5 nm) per 4 hour incubation with the fluorogenic substrate.

In experiments where caspase inhibition was used, the tetrapeptide pan-caspase inhibitors Z-VAD-fmk, BOC-D-fmk and Z-DEVD-fmk were resuspended in DMSO, and were added to cells at the indicated concentrations as the same time as all other agents. Control cells were incubated with the equivalent concentration of DMSO.

2.4.4 DAPI staining

Cells were seeded on plastic chamber slides (Nunc, Inc, Naperville, IL, USA) allowed to attach overnight and treated as indicated. After two washes with PBS, cells were fixed in 200 µl of an ethanol/acetic acid (ratio of 6:1) for 10 min at room temperature. The cells were washed in Milli Q water and allowed to air dry overnight. The fixed cells were then incubated with 0.8 mg/ml of 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI) for 15 min at room temperature. The cells were then rinsed once with 1X PBS and once with MilliQ water, and allowed to dry in total darkness. The coverslips were mounted on propylgallate (anti-fade) and kept in the dark until ready to visualise. DAPI staining was visualised by fluorescence microscopy using an Olympus BH2-RFCA fluorescence microscope and imaged using a Photometrics Coolsnap Fx digital camera (Roper Scientific, NJ, USA).

2.4.5 Protein estimation

The amount of protein per sample was estimated using the BCA Protein Assay kit (Pierce Biotechnology Inc). A series of 11 standards from 0 to 40µg were added to a 96 well plate using BSA 0.0004g/ml in caspase lysis buffer or protein lysis buffer. Frozen samples obtained for either caspase assay or western blot analysis were thawed and 5µl were aliquoted into the plate. Fifty parts of Reagent A was added to 1 part Reagent B and 200µl was added to each sample. The plate was incubated at 37°C for 30 mins and scanned at 570 nm in a plate reader. The values obtained were compared against the standards and the amount of protein in µg was calculated.

2.4.6 DNA fragmentation

Cells were harvested by washing twice with PBS, after treatments as indicated, and incubated overnight at 37°C in lysis buffer. DNA was extracted twice with equal volumes of phenol-chloroform-isoamylalcohol (25:24:1) and then precipitated in ethanol. Samples were electrophoresed in a 1.2% agarose gel, stained with ethidium bromide and visualised under UV light.

2.4.7 Anoikis assay

Tissue culture plates were coated with polyHEMA (poly(2-hydroxyethylmethacrylate); (Sigma-Aldrich, Castle Hill, Australia) as follows: 1 ml of 10 mg/ml of polyHEMA in 95% ethanol was applied to each well and allowed to dry. After drying, the wells were washed in PBS and stored dry until use. Cells were plated onto polyHEMA-coated or uncoated control wells at 1x 10^5 cells per well in a six well plate. At the indicated times, cells were harvested from polyHEMA-coated wells by centrifugation, followed by trypsin/EDTA to yield single cell suspension. The number of viable cells was determined by trypan blue exclusion and counting on a haemocytometer.

2.4.8 Western blot analysis

Cells were treated as indicated and lysed in buffer containing 10 mM Tris HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 2 mM sodium vanadate and a cocktail of protease inhibitors. Lysates were centrifuged for 5 min at 12,000 rpm, the supernatant fraction of the lysate was then removed and stored at –70°C until ready to use. Cell extracts containing between 35-50μg of protein were mixed with an equal volume of sample buffer containing 12 mM Tris HCl pH 6.8, 6%

SDS, 10% β- mercaptoethanol, 20% glycerol, and 0.03% bromophenol blue. Protein samples were boiled for 5 min and electrophoresed under reducing conditions in 4-12% gradient polyacrylamide gels. Separated proteins were electrophoretically transferred to PVDF transfer membrane and blocked in PBS containing 5% blocking reagent, for 1 h at room temperature. Immunodetection was performed overnight at 4°C in PBS/blocking reagent, using antibodies described in Table 2.1, and diluted according to manufacturers instructions. Filters were rinsed several times with PBS containing 0.1% Tween 20 and incubated with 1:5000 dilution of anti-mouse or anti-rabbit alkaline phosphatase-conjugate for 1 h. Bound proteins were detected and quantitated using the Vistra ECF substrate reagent kit using a Fluorimager (Molecular Dynamics Inc).

2.4.9 Flow cytometric analysis of receptor expression

Analysis was carried out using the Becton and Dickinson "FACscan" flow cytometer coupled with LYSIS II or CellQuest software. Graphs were aligned using the WinMDI 2.8 software package. Cells were reseeded into fresh culture flasks one day prior to the assay, rinsed twice with PBS and detached using 2 mM EDTA in PBS at 37°C for 5 min. For flow cytometric analysis, all subsequent incubation steps were performed on ice and centrifugation steps were performed at 4°C. Cells were washed twice in PBS by centrifugation at 200g for 5min. For analysis of internal antigen expression, cells were pelleted by centrifugation and then resuspended at 10^7 cells/ml in 2% paraformaldehyde in PBS and incubated for a further 5 min. The volume was increased to 10 ml with PBS, and the cells were then pelleted and washed twice by centrifugation at 200g for 5 min in wash buffer (PBS + 0.1% Azide). The cells were then resuspended in permeabilisation buffer, incubated for 10 min, washed twice in wash buffer by centrifugation at 200g. Both intact and permeabilised cells were

resuspended at 2 x 10^6 cells/ml in blocking buffer (10% BSA/PBS + 0.1% Azide). 50µl aliquots of the cell suspensions were added to polypropylene FACS tubes. To each of these was added 50µl of monoclonal antibody solution (Mabs), as indicated, or isotype-matched non-binding control Mabs, each diluted to 10µg/ml in blocking buffer and incubated for 45 min. The cells were washed twice in 2ml/tube of wash buffer by centrifugation at 300g. To the resuspended cell pellets was added 50µl of FITC-labelled $F(ab')_2$ sheep anti-mouse Ig or goat anti-mouse IgG-PE, both diluted 1/50 in blocking buffer. The cells were incubated for a further 45 min in the dark, washed twice as above, then resuspended and fixed in 0.5ml of cold 1% w/v paraformaldehyde for analysis by flow cytometry.

2.4.10 Flow cytometric analysis of the cell cycle

Analysis was carried out using the Becton and Dickinson "FACscan" flow cytometer coupled with LYSIS II or CellQuest software. Graphs were aligned using the WinMDI 2.8 software package. Cells were detached using 2mM EDTA in PBS, transferred to centrifuge tube and centrifuged for 5 min at 1100 rpm. Supernatant was removed, cells were resuspended well into 0.5 ml PBS and transferred into tubes containing 70% ethanol for storage at –20 °C until required. Preparation of the cells for cell cycle analysis required centrifugation of the ethanol suspended cells for 5 mins at 1100 rpm. The ethanol was decanted and the pellet was resuspended in 5ml PBS and centrifuged again for 5 min at 1100 rpm. The pellet was resuspended in 1ml PI/Triton X-100 staining solution with Rnase A and incubated at 37°C for 15 min.

2.4.11 Immunofluorescence assay

Cells were seeded into 8-chamber slides (Nunc, Inc, Naperville, IL, USA) at 2 x 10^4 cells per well, and cultured for 48 hours. *In situ* immunofluorescence was performed on ice. Cells were rinsed once in PBS, fixed in 2% paraformaldehyde in PBS for 5 min, and permeabilised with 0.1% saponin in PBS containing 10% heat inactivated pooled normal human serum (permeabilization buffer), for 10 min. The cells were then washed thrice with PBS containing 0.1% BSA and 0.1% NaN_3 (wash buffer), and blocked in 5% normal goat serum containing 0.1% w/v NaN_3 (blocking buffer) for 30 min. The blocking buffer was removed and monoclonal antibodies (Mab) specific for human TRAIL receptors TR1, TR2, TR3, TR4, TRAIL, OPG (Mab 8051) or isotype-matched non-binding control Mabs, each diluted to 10 μg/ml in blocking buffer as above, were added for 45 min. The slides were washed thrice in PBS containing 0.05% (v/v) TritonX-100. To reveal primary antibody reactivity, cells were incubated with a 1/50 dilution in PBS of FITC-conjugated goat anti-mouse $F(ab')_2$ antisera for 30 min. The cells were then washed as above and stained for 1 min with 1 μg/ml DAPI solution, rinsed in PBS and mounted in Univert mountant. The labelled specimens were examined using an Olympus BH2-RFCA fluorescence microscope or an Olympus Bx 51 fluorescence microscope and imaged using a Photometrics Coolsnap Fx digital camera (Roper Scientific, NJ, USA).

2.4.12 Statistical analysis

All graphs were generated using Microsoft Excel 2000. This program was used to determine standard deviation (SD) based on experiments that were performed in triplicate and repeated at least three times.

2.5 *IN VIVO* ANALYSIS OF ANTI-TUMOUR ACTIVITY

2.5.1 Cell culture

See section 2.1.3 for a detailed description of cell culture protocol. For the purpose of *in vivo* transplantation, cells were removed from flasks by gentle 2mM EDTA digestion, followed by dilution of cells in sterile PBS.

2.5.2 Cell lines

The human osteosarcoma cell line K-HOS was used in this study because it has been demonstrated to produce an osteolytic lesion when implanted into bone. *In vitro* studies have also demonstrated the highly synergistic apoptotic effect of combination ZOL and TRAIL treatment in K-HOS cells.

2.5.3 Animals

Four to six week old Balb/c Nu/Nu female mice were housed under pathogen free conditions, at a constant temperature of 23°C, under controlled 12 hour light-dark cycles, in accordance with the protocol approved by the IMVS Animal Research Committee. Animals were acclimatised for one week prior to commencement of procedures. IMVS Animal Ethics approval was obtained for all procedures and animal care was in accordance with guidelines. During the experiment all animals were monitored closely and weighed at regular intervals to assess effect of implantation.

2.5.4 Tibial implantation

Cell suspensions of K-HOS and KRIB in sterile PBS at either (2×10^3, 5×10^3 or 1×10^4 cells/20µl were used for the pilot study. All other *in vivo* experiments used 6×10^3/ 10µl of K-HOS cells for intra-tibial implantation. The mice were anaesthetised by subcutaneous (s.c.) injection with 80 mg ketamine/kg body weight and 10 mg

xylazine/kg body weight. The left tibia was wiped with 70% ethanol and using a 2 gas-sterilised Hamilton syringe, a 26 gauge needle was inserted through the tibial plateau with the knee flexed and the cells were injected (**Figure 2.1**). All animals were administered with PBS in the contralateral limb, as a control. After tibial implant mice were randomly assigned into groups. Mice were weighed regularly and radiographs were taken every two weeks to determine the extent of osteolysis. At sacrifice, all the major organs and both hind limbs were harvested in 10% buffered formalin for histological analysis.

2.5.5 Treatment

Recombinant human TRAIL, supplied by Immunex (Seattle, WA, USA) was used at 30mg/kg body weight, administered by intraperitoneal (i.p.) injection. Zoledronic acid (ZOL), supplied by Novartis Pharmaceuticals (Sydney, NSW, Australia), was dissolved in sterile water and prepared in sterile 1x PBS. Zoledronic acid at 100μg/kg body weight was administered by s.c. injection.

Protocol 1: *In Vivo* analysis of ZOL treatment.

Zoledronic acid was administered s.c. into female nude mice at weekly intervals starting one week after tibial implantation (Day 8, 15, 22, 29).

Protocol 2: *In Vivo* analysis of ZOL and TRAIL combination treatment.

Zoledronic acid was administered to mice at weekly intervals starting one week after tibial implantation (Day 8, 15, 22, 29). TRAIL was administered daily for 5 days, starting one week after tibial implantation and then once weekly (Day 8-12, 15, 22, 29).

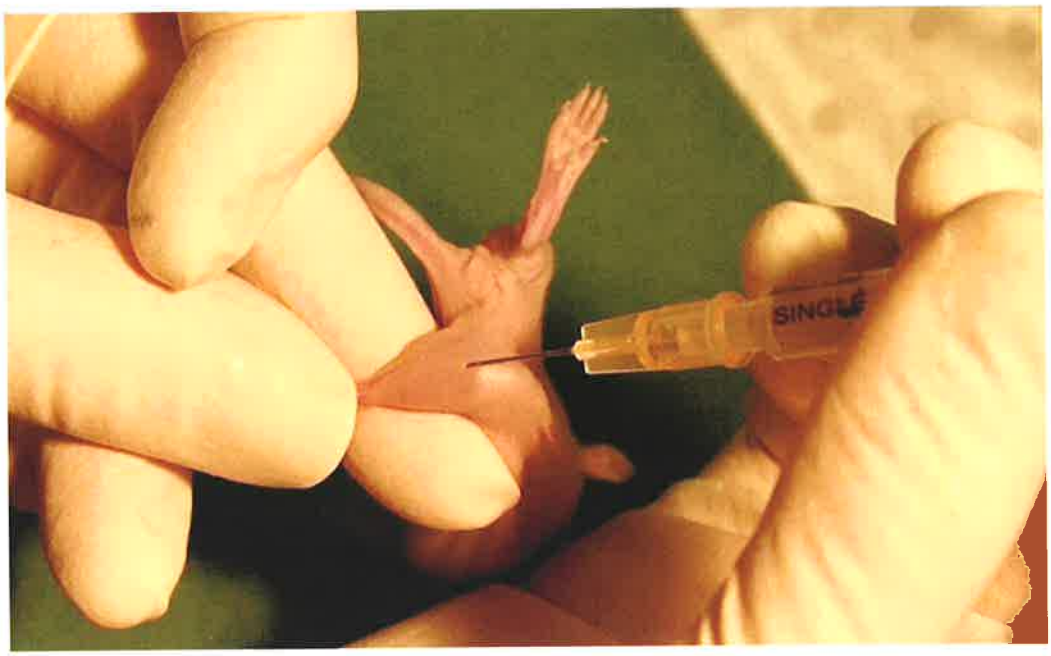


Figure 2.1 Intra-tibial injection of human OS cells into a nude mouse. The left leg was held with knee flexed to expose the proximal tibia for needle insertion just below the anterior tubercle.

2.5.6 Histology

The tibias were fixed in 10% buffered formalin, followed by acid decalcification in 10% EDTA solution and 7% nitric acid at room temperature. Decalcification was confirmed by radiography before sectioning. Sections were paraffin embedded, sectioned longitudinally at 6 μM and stained with haemotoxylin and eosin. Analysis was performed on a Nikon Eclipse TE300 inverted microscope (Nikon Corporation, Tokyo, Japan).

Histological sections were used to determine area of tumour burden using the *Scion* image analysis software. Sections were photographed and digital images were imported into the program. Using the program, the area of tumour burden was outlined and calculated as an area in mm^2 (*Scion* Image, Maryland, USA).

2.5.7 Radiography

Animals were anaesthetised by s.c. injection with 80 mg ketamine/kg body weight and 10 mg xylazine/kg body weight and were laid onto film with limbs spread for clear imaging. Refer to 2.3.4 for further details regarding equipment.

2.5.8 Micro-computer tomography analysis

Hind limbs required for micro-CT analysis were harvested in 100% ethanol. Both the right and left leg of each animal were mounted into the lid of the specimen tube containing 100% ethanol (to reduce dehydration). The specimen tube was securely placed into the SkyScan-1072 X-ray micro-CT Scanner and the specimen rotated 360^{o} to ensure a clear field of view. The specimen was also elevated or lowered to further improve imaging results. The program was commenced with voltage set at 100 kV & 98 μAmps, exposure time set at 1.7 sec, magnification set at 18 and beam hardening set to 100%. The TIFF files produced were converted into bmp files using the CONE-REC

software with Beam hardening set at 20%. Three dimensional (3D) reconstruction of images required the program 3D-creator with Step, Locality and Tolerance settings adjusted to 1 to give maximum image detail. Rotation of the images in any direction allowed the images obtained to be edited with the use of 3D-creator. CTan software was used for bone volume analysis of the scanned tibias. Using the two dimensional (2D) images obtained from the micro-CT scan the growth plate was identified and 150 sections starting from the growth plate/tibial interface and moving down the tibia, were selected. Histograms representing bone volume were generated and compared to the control tibia.

2.4.9 Statistical analysis

Fisher's exact test was used to assess the associations between treatment group and presence of osteolytic lesions and lung metastases. In each of these cases, the interaction between ZOL and TRAIL was assessed using the Breslow-Day test for homogeneity of odds ratios.

The 2-way Analysis of Covariance (ANCOVA) was used to assess the impact of ZOL and TRAIL and an interaction between the two on bone volume in the left leg where bone volume in the right leg was the covariate. Impact of ZOL and TRAIL, and an interaction between the two, on tumour area (where tumours were detected) was assessed using the 2-way Analysis of Variance (ANOVA).

CHAPTER THREE

ZOLEDRONIC ACID INDUCES APOPTOSIS IN HUMAN OSTEOSARCOMA CELLS.

3.1 INTRODUCTION

Although known for their ability to inhibit osteoclast-mediated resorption, BPs, have also been identified as having direct anti-tumour cell activity. *In vitro* experiments have shown the potential for N-BPs to dose-dependently inhibit the proliferation and survival of tumour cells, including the induction of apoptosis in human myeloma [Senaratne and Colston 2002; Stearns and Wang 1996] breast [Fromigue *et al.* 2000; Sato *et al.* 1991] and prostate [Lee *et al.* 2001] cancer cells. The concentrations of N-BPs used to produce these cytostatic and apoptotic effects *in vitro* vary, depending on the potency of the N-BP and the cell line used [Fromigue *et al.* 2000].

Other reported effects of N-BPs include a reduction in tumour cell adhesion and invasion, necessary for effective metastases of tumour cells, as well as decreased angiogenesis, vital for the growth and survival of metastases [van der Pluijm *et al.* 1996; Wood *et al.* 2002]. These additional effects of N-BPs on tumour cells, encourages investigation of these agents for the preventative treatment of metastatic cancers.

ZOL is a new generation N-BP and is the most potent of the currently available BPs in terms of inhibition of bone resorption [Fleisch 2000]. It has been demonstrated in phase I trials of patients with bone metastases or multiple myeloma, that ZOL is safe and well tolerated at all dose levels tested [Berenson *et al.* 2002; Berenson *et al.* 2001]. Zoledronic acid is the first N-BP to show a reduction in skeletal complications including osteolytic lesions and thus significant clinical benefit in patients with bone metastases from various primary tumours [Berenson *et al.* 2001; Coleman and Seaman 2001; Rosen *et al.* 2004; Rosen *et al.* 2003; Rosen *et al.* 2004; Rogers *et al.* 2000].

The effect of N-BPs on OS cells has not been extensively studied, although recently the anti-proliferative effect of pamidronate in human and rat OS cells and alendronate in canine OS cells, *in vitro,* has been investigated [Sato *et al.* 1991; Stearns

and Wang 1996; Farese *et al.* 2004]. To date, there have been no reports of the effects of the potent bisphosphonate, ZOL on human OS cells, other than a paper based on results obtained as part of this thesis [Evdokiou *et al.* 2003]. This chapter describes investigation of the cytotoxic effects of ZOL on human OS cell lines and the mechanisms involved.

3.2 MATERIALS AND METHODS

3.2.1 Cells and cell culture

The osteosarcoma cell lines and the normal human osteoblast-like cells used in this chapter are listed in section 2.1.1. The methods for cell preparation and propagation are described in section 2.1.2 and 2.1.3.

3.2.2 Measuring cell viability

The method used to assess cell viability after zoledronic acid treatment is described in section 2.4.2. and 2.4.3.

3.2.3 Measuring caspase activity

The caspase inhibitor used is listed in section 2.2.4. Caspase activity protocols used in this chapter are described in section 2.4.4.

3.2.4 DAPI staining of nuclei

The method used to prepare and stain cells with DAPI is described in section 2.4.5.

3.2.5 Cell surface receptor analysis after ZOL treatment

The antibodies used in cell surface receptor analysis are listed in section 2.2.5. The method used is described in section 2.4.10.

3.2.6 Cell cycle analysis after ZOL treatment

The method used is described in section 2.4.11.

3.2.7 Statistical analysis

Microsoft excel was used to obtain mean standard deviations of experimental data. Data shown in each case are from a representative experiment, which was repeated at least three times.

3.3 RESULTS

3.3.1 ZOL induces dose-dependent apoptosis

The effects of ZOL on cell proliferation were tested against a panel of eight human OS cell lines (HOS, BTK-143, G-292, Saos2, SJSA-1, MG-63, K-HOS and KRIB). Cells were treated with increasing concentrations of ZOL at 0, 1, 10, 25, 50, 100μM. Treatment with ZOL for 72 hour dose-dependently resulted in fewer cells, compared with untreated control cultures, in all eight OS cell lines, as shown in **Figure 3.1**. ZOL was most potent in the HOS, BTK-143, K-HOS, G-292 and KRIB cells, with IC_{50}'s of 8 μM, 10 μM, 12 μM, 15 μM and 25 μM, respectively. In contrast, there was a weaker response to ZOL in the SJSA-1, Saos2 and MG-63 cell lines, with IC_{50}'s of 35 μM, 50 μM and greater than 100 μM, respectively. The efficacy of ZOL to induce apoptosis and thus reduce cell number, varied considerably between cell lines. The differential sensitivity to ZOL can be seen more clearly in **Figure 3.2**.

It is clearly evident that the decrease in cell number is due to apoptosis and not an increase in control cell number, due to the morphology of the cells after ZOL treatment, as shown in **Figure 3.3**. This will be discussed later.

It was previously suggested that a possible mechanism for the bisphosphonate effects on cell growth was the ability of these compounds to act as calcium chelating agents [Rodan 1997]. The possibility was investigated by the addition of equimolar concentrations of EDTA, with respect to the concentration of ZOL that reduced cell viability by 50%. We found no significant effect of EDTA treatment on cell viability in any of the cell lines tested, suggesting that calcium chelation was not responsible for the observed effects (data not shown).

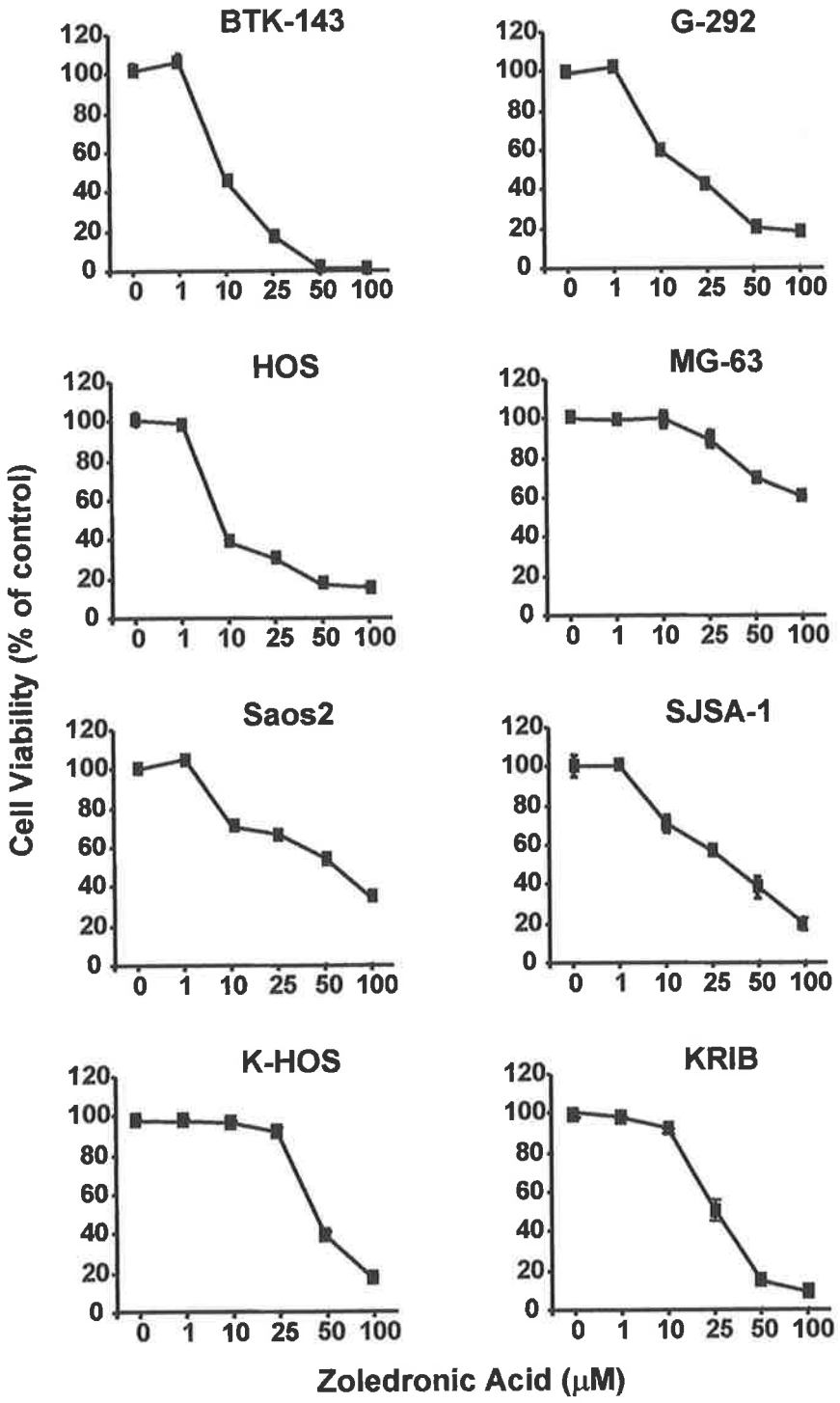


Figure 3.1 The effect of ZOL on OS cell number. OS cells were seeded 1 x 10^4 cells per well in 96 well microtiter plates and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of ZOL (1-100 μM). Cell viability was determined using the crystal violet assay and is expressed as % of control, as described in the chapter 2. Data shown in each case are from a representative experiment repeated at least three times: points are means ±SD.

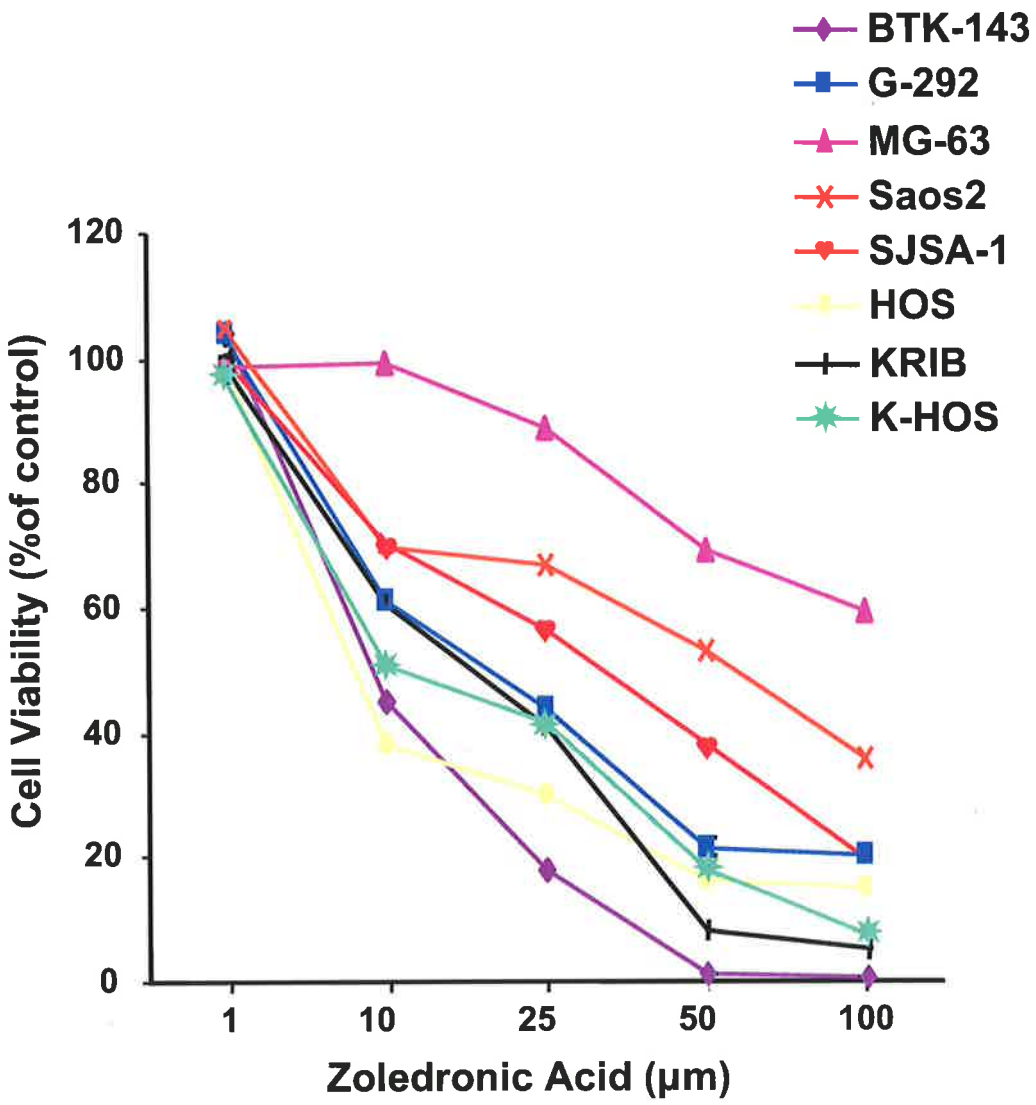


Figure 3.2 Differential sensitivity of OS cell lines. 1 x 10^4 cells per well were seeded in 96 well microtiter plates and allowed to adhere to the plate. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of ZOL (1-100 µM). Cell viability was determined using the WST-1 cell proliferation reagent assay kit, as described in chapter 2. Data is shown as a percentage compared to control untreated cells and are from a representative experiment repeated at least three times: points are means ±SD.

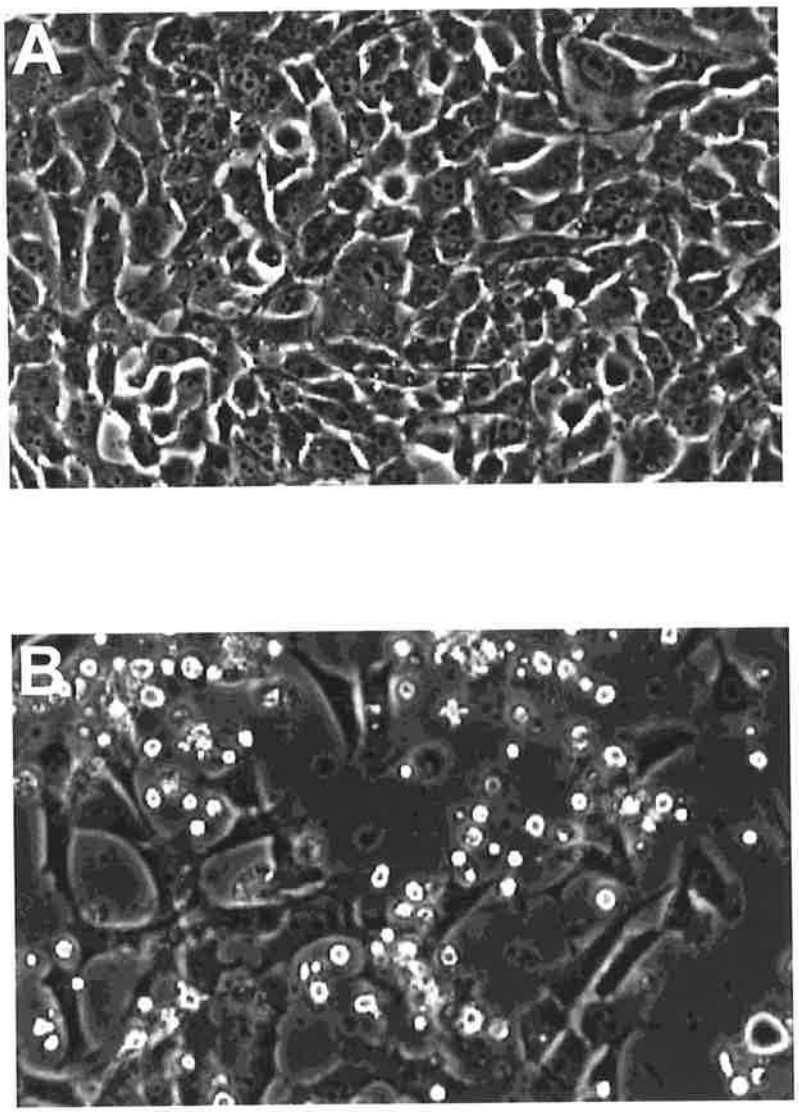


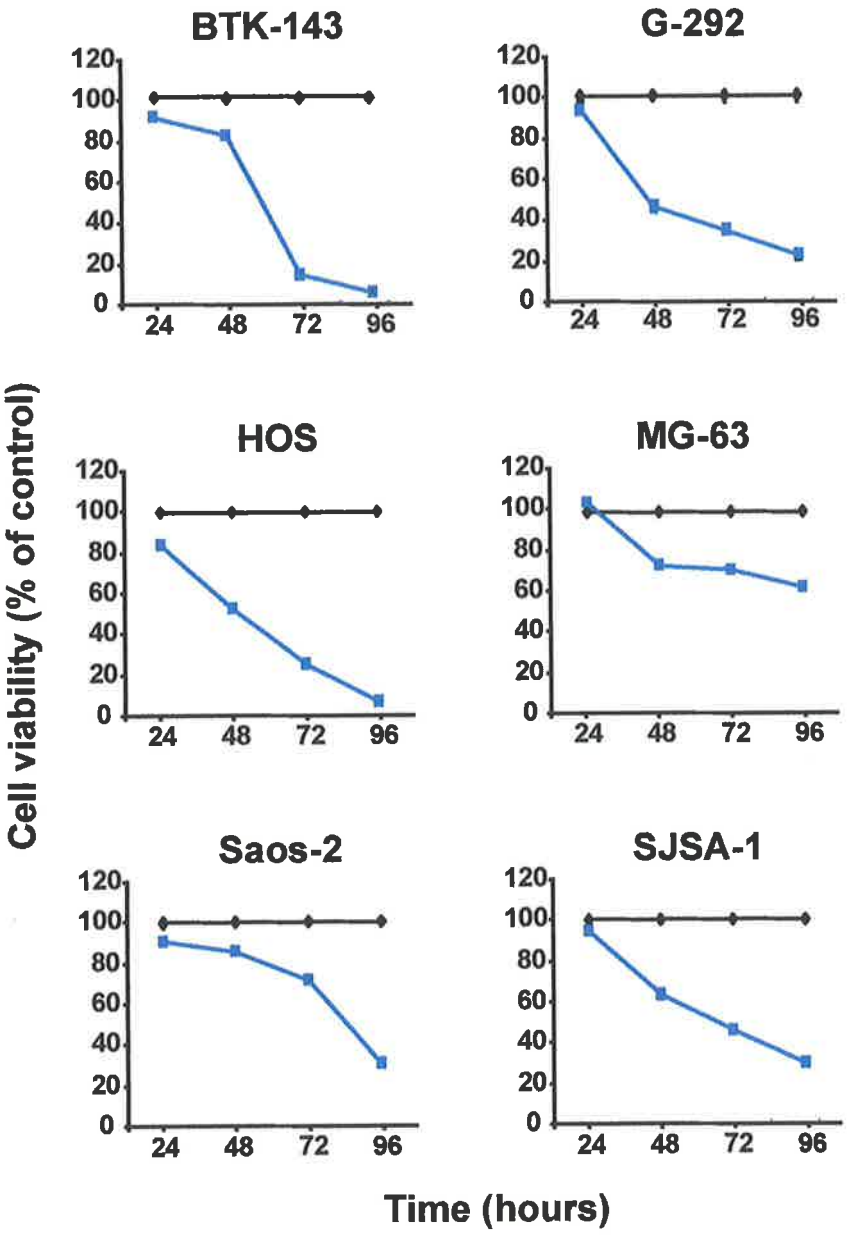
Figure 3.3 Zoledronic acid treatment leads to apoptosis. Photographic image of HOS cells treated with (**A**) fresh media or (**B**) media containing 25μM ZOL for 48 hours. Prior to apoptosis the cells begin to detach, seen by the characteristic 'stretching' of the cells (**B**) instead of the common round compact form they take in normal control media (**A**).

3.3.2 ZOL induces time-dependent apoptosis

The effects of ZOL on cell proliferation were assessed in six OS cell lines by counting viable cells, as assessed by trypan blue exclusion, over a 96 hour period in control cultures or in cultures treated with 25 μM ZOL as shown in **Figure 3.4**. The results demonstrate that, in the case of HOS, BTK-143 and G-292 cells, ZOL treatment resulted in a reduction in the number of cells, compared with the number of cells plated initially, indicating a time-dependent decrease in cell proliferation and survival. The effects of ZOL on cell viability were not immediate, in that there was a lag period of 48 to 72 hours before the onset of cell death. In the case of Saos2, MG-63 and SJSA-1 cell lines, the results suggest that ZOL at 25 μM had no effect on cell viability but the rate of proliferation was significantly decreased.

3.3.3 ZOL treatment leads to increased caspase activity

It has been demonstrated in other cell types that bisphosphonate-induced apoptosis leads to activation of the caspase cascade [Fromigue *et al.* 2000; Coxon *et al.* 2000; Reszka *et al.* 1999; Sato *et al.* 1991]. To assess whether the observed ZOL-induced apoptosis in OS cells might also be mediated by caspase activation, caspase-3 activity in HOS, BTK-143, G-292, Saos2, SJSA-1 and MG-63 OS cells after ZOL treatment was measured, in relation to the effect of ZOL on cell number. Caspase activity increased in all cell lines, in a dose- and time-dependent manner, following treatment with ZOL (shown in **Figure 3.5** and **3.6**, respectively). HOS, BTK-143, and G-292 cells were significantly more sensitive to ZOL than MG-63, SJSA-1 and SaOS-2 cells, in terms of reduced cell viability, and this was reflected by the relative concentrations of ZOL required for caspase-3 induction in the cell lines (**Figure 3.5**).



* Refer to amendments

Figure 3.4 The effect of ZOL on OS cell number. Cells were incubated for the times indicated in the absence (♦) or presence (▮) of 25 μM ZOL. The number of viable cells was quantitated using a heamocytometer and expressed as % of control. Data shown in each case are from representative experiments repeated at least three times: bars, ±SD.

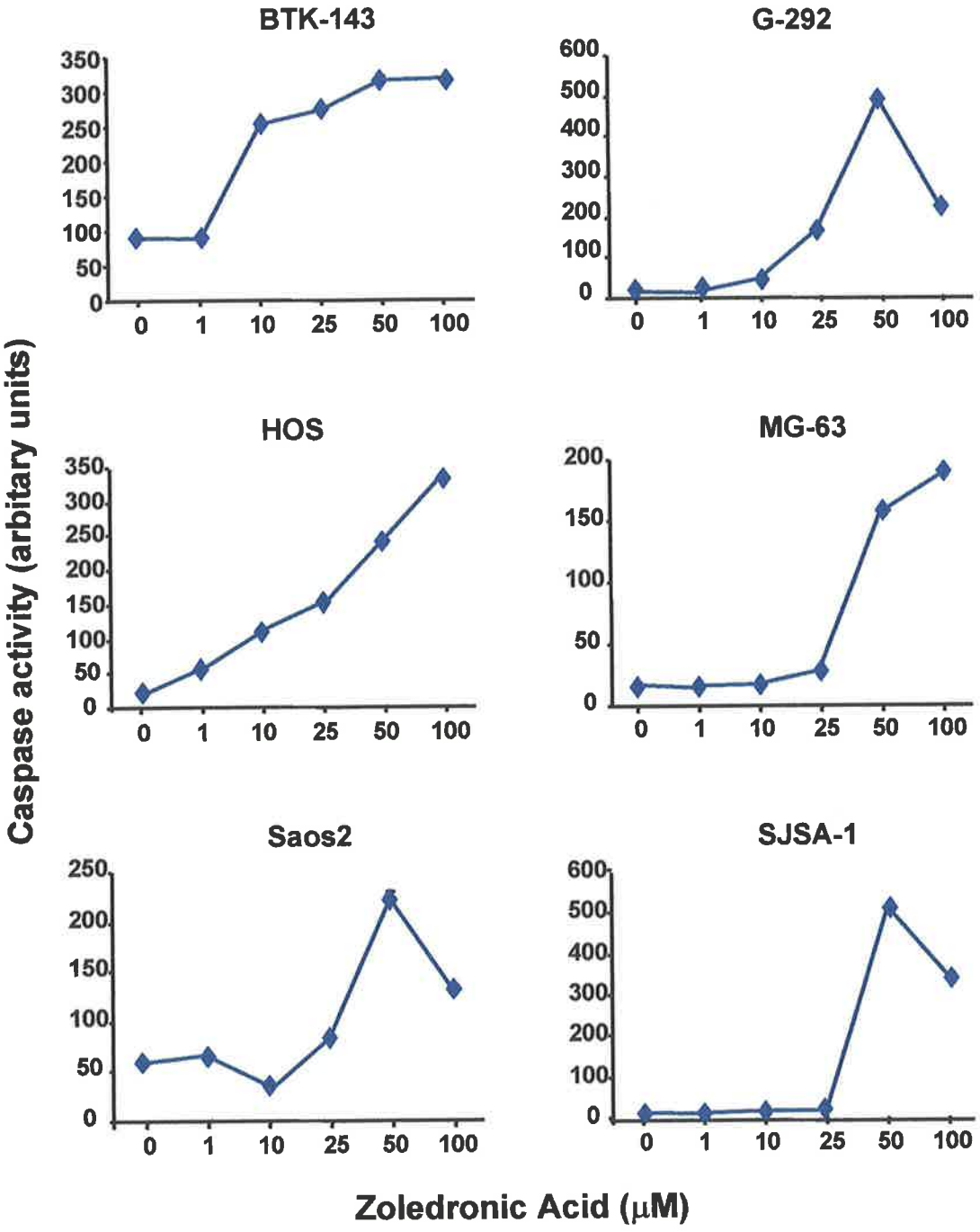


Figure 3.5 Increasing doses of Zoledronic acid leads to increased caspase activity in OS cells. BTK-143, G-292, HOS, MG-63, SJSA-1 and SaOS-2 OS cells were treated with a fixed concentration of ZOL (25 μM) for 72 hours. Cell lysates were used to determine caspase-3-like activity, using the caspase-3 specific fluorogenic substrate, zDEVD-AFC, as described in the chapter 2. The data show that the increase in caspase 3-like activity is well correlated with the ZOL-dose-dependent decrease in cell number (Figure 3.1).

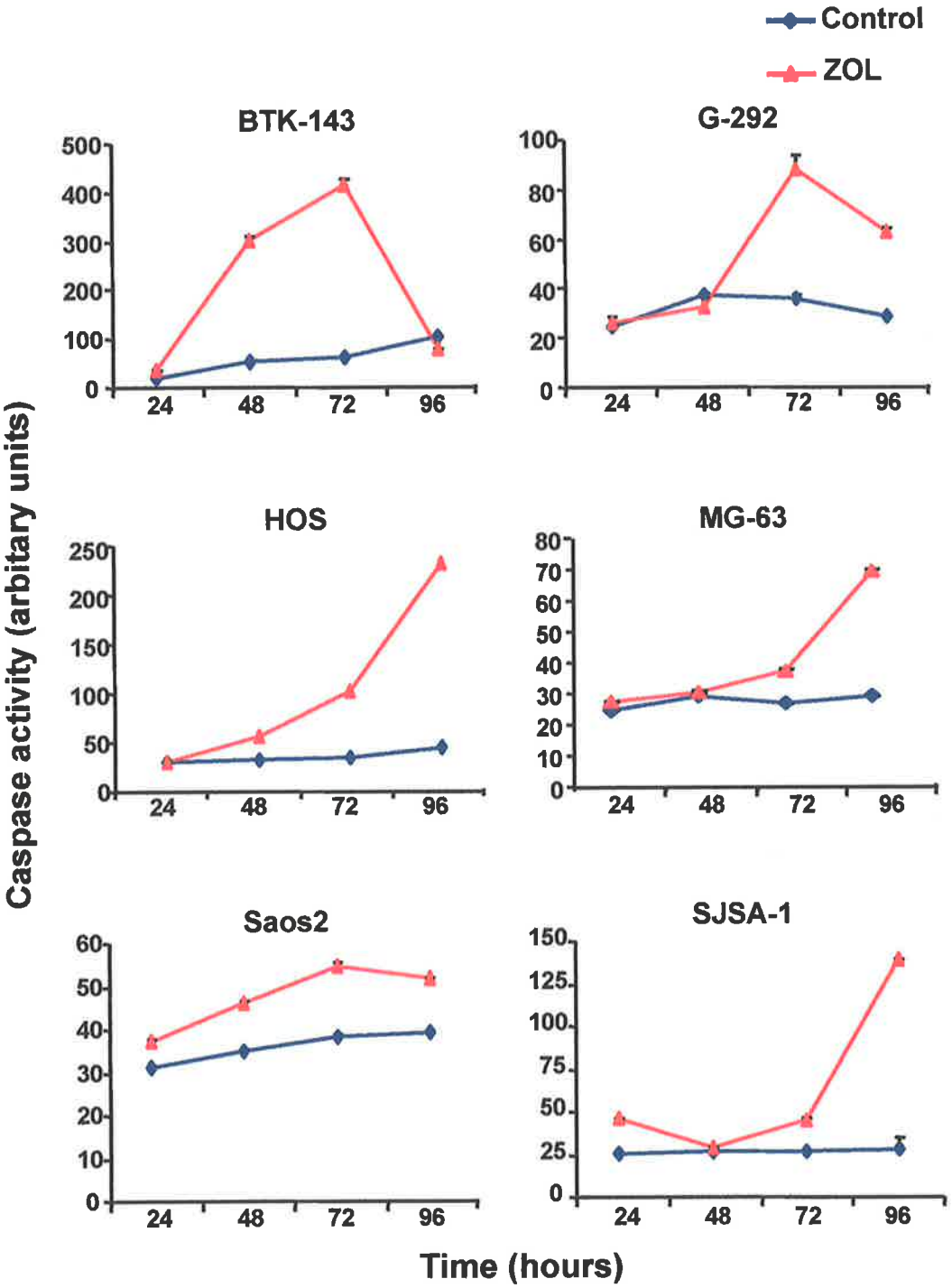


Figure 3.6 Zoledronic acid treatment leads to increased caspase activity over time in OS cells. BTK-143, G-292, HOS, MG-63, SJSA-1 and Saos2 OS cells were treated with a fixed concentration of ZOL (25 μM) for 24, 48, 72 and 96 hours. Cell lysates were prepared and caspase-3-like activity was determined, using the caspase-3 specific fluorogenic substrate, zDEVD-AFC, as described in the chapter 2. The data show that the increase in caspase 3-like activity is well correlated with the ZOL-time-dependent decrease in cell number (Figure 3.4).

Moreover, caspase activity in response to 25 μM ZOL was significantly increased above control levels by 72 hours in HOS, BTK-143 and G-292 cells. In contrast, there was only a minimal increase in caspase-3 activity in the MG-63, SJSA-1 and SaOS-2 cells after 72 hours of treatment (**Figure 3.6**).

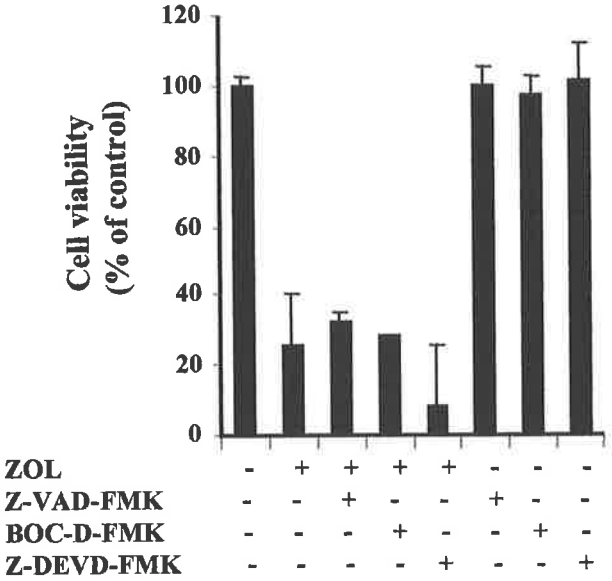
3.3.4 Caspase inhibitors do not protect against ZOL-induced apoptosis

To investigate the role of caspase activation in the ZOL-mediated apoptosis of human OS cell lines, the effect of caspase inhibitors on the induction of apoptosis in the ZOL-sensitive HOS cells was examined. Cells were incubated for 72 hours with 25 μM of ZOL, either alone or in the presence of the broad specificity caspase inhibitors, zVAD-fmk or BOC-D-fmk, or the specific caspase-3 specific inhibitor, zDEVD-fmk. Consistent with previous experiments, the HOS cell line showed a significant decrease in cell number in the presence of ZOL alone (seen in **Figure 3.7A**). However, none of the caspase inhibitors was able to prevent the ZOL-induced cell loss despite the complete and irreversible inhibition of caspase-3 activity achieved in their presence (**Figure 3.7B**).

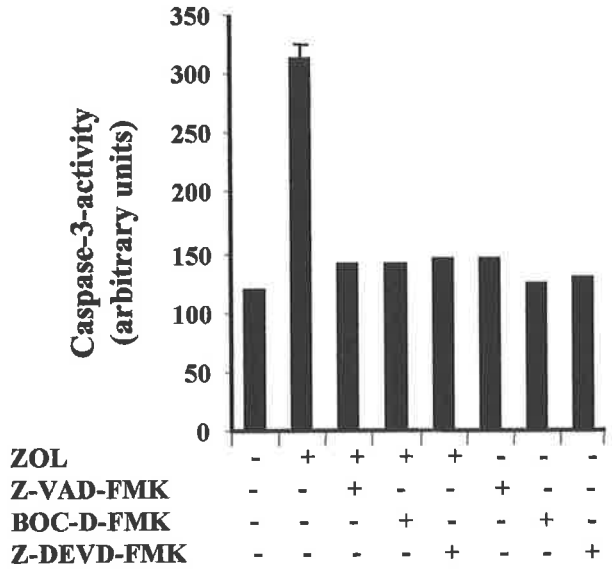
Similarly, caspase inhibition failed to protect HOS cells from ZOL-induced morphological changes. Representative photographic images of the cells following DAPI staining, with the percentage of apoptotic cells in each treatment, is shown in **Figure 3.8**. The effect of caspase inhibition on ZOL-induced apoptosis was quantified by counting the number of pyknotic nuclei in the absence or presence of the general caspase inhibitor zVAD-FMK (**Figure 3.8A**). The result was 3 apoptotic cells in the control untreated cells and 71 and 80 apoptotic cells in the treatment media containing

Figure 3.7 Caspase inhibitors do not protect against Zoledronic acid induced apoptosis. (**A**) HOS cells were treated for 72 hours with 25 μM ZOL alone, or were co-incubated with the broad specificity caspase inhibitors z-VAD-fmk (50 μM) or Boc-D-fmk (50 μM), or the caspase-3 specific inhibitor z-DEVD-fmk (50 μM). To investigate possible toxic effects of the inhibitors, cells were also treated with each inhibitor alone. Cell viability determined using the WST-1 cell proliferation reagent assay kit. The absorbance was measured using an ELISA reader at 420-480 nm and cell viability expressed as % of control. Data points show means of quadruplicate results from a representative experiment, repeated at least twice; *bars* ±SD. (**B**) Cell lysates were used to determine caspase-3-like activity, using the caspase-3 specific fluorogenic substrate, zDEVD-AFC.

A


Cell viability
(% of control)
120
100
80
60
40
20
0
ZOL - + + + + - - -
Z-VAD-FMK - - + - - + - -
BOC-D-FMK - - - + - - + -
Z-DEVD-FMK - - - - + - - +

B


Caspase-3-activity
(arbitrary units)
350
300
250
200
150
100
50
0
ZOL - + + + + - - -
Z-VAD-FMK - - + - - + - -
BOC-D-FMK - - - + - - + -
Z-DEVD-FMK - - - - + - - +

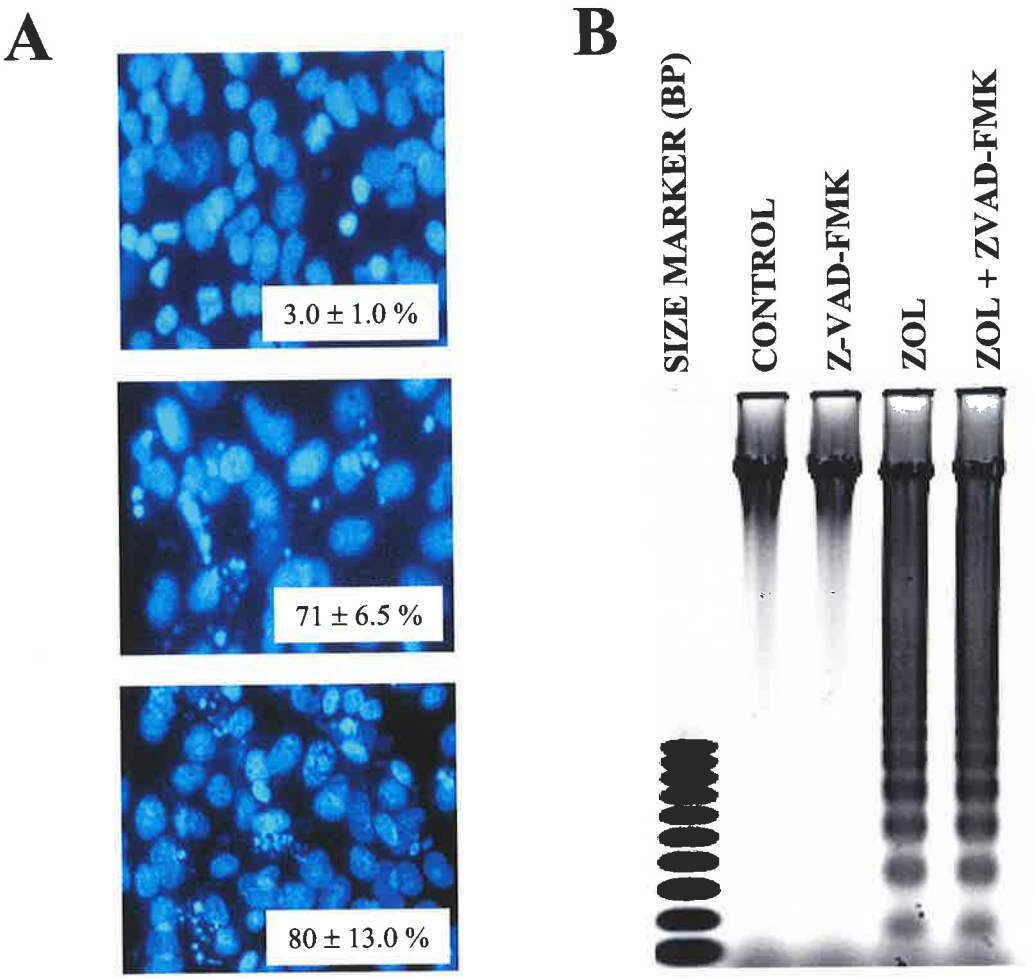


Figure 3.8 ***A.*** *Top image:* Control, untreated HOS cells showing fluorescent nuclei homogeneously stained. *Middle image*: Cells were treated for 72 hours with 25 μM ZOL. The DAPI nuclear fluorescence stain reveals changes in the nuclei consistent with induction of apoptosis. *Bottom image:* Cells were treated for 72 hours with 25 μM ZOL in combination with the general caspase inhibitor z-VAD-FMK (50 μM). Nuclear apoptotic bodies containing DNA condensations were seen, as for the ZOL-treated cells in the absence of z-VAD-FMK. The percentage of apoptotic cells from each treatment is indicated (mean ± SEM from three random fields of 200 cells each).

B. HOS cells were treated for 72 hours with 25 μM ZOL, either alone or in combination with the general caspase inhibitor z-VAD-FMK (50 μM). Genomic DNA was isolated and subjected to agarose gel electrophoresis. Intranucleosomal genomic DNA fragmentation, characteristic of apoptosis, was observed in the ZOL alone and in the ZOL + Z-VAD-FMK treated cells.

25μM ZOL and 25μM ZOL with 50μM zVAD-FMK respectively. In addition, caspase inhibition did not prevent ZOL-induced DNA fragmentation, as assessed by agarose gel electrophoresis (**Figure 3.8B**). These results with OS cell lines therefore differ from findings reported previously for breast cancer cells [Fromigue *et al.* 2000] and osteoclasts [Reszka *et al.* 1999], showing that bisphosphonate-induced apoptosis in these other cell types could be suppressed by the co-addition of the broad specificity caspase inhibitor z-VAD-fmk. Our results suggest that ZOL-induced apoptosis of OS cell lines is independent of caspase

3.3.5 Geranylgeraniol protects against ZOL-induced apoptosis

The N-BPs, including ZOL, inhibit the mevalonate pathway by inhibiting geranylgeranylation of small GTP-binding proteins such as Ras, Rho, Rac and Rab [Luckman *et al.* 1998; Coxon *et al.* 2000; Dunford *et al.* 2001; Fisher *et al.* 2000; Rodan 1998; Rogers *et al.* 2000]. These proteins are important for many cellular processes, including proliferation, cytoskeletal organization and intracellular signalling. To examine the involvement of the mevalonate pathway on the ZOL-mediated apoptosis of human OS cells, we assessed the ability of geranylgeraniol (GGO), an intermediate of the mevalonate pathway, to protect HOS and BTK-143 cells from ZOL-induced apoptosis. As seen in **Figure 3.9**, the addition of GGO protected these cells, in a dose dependent manner, from ZOL-induced apoptosis. For BTK-143 cells, complete protection was achieved with 50μM GGO, whereas 200μM GGO was required to achieve the same level of protection in the HOS cells. Concentrations of up to 500μM GGO, when used alone, had no effect on cell proliferation. Geranylgeraniol was able to protect all human OS cell lines from ZOL-induced apoptosis. Using a standard amount

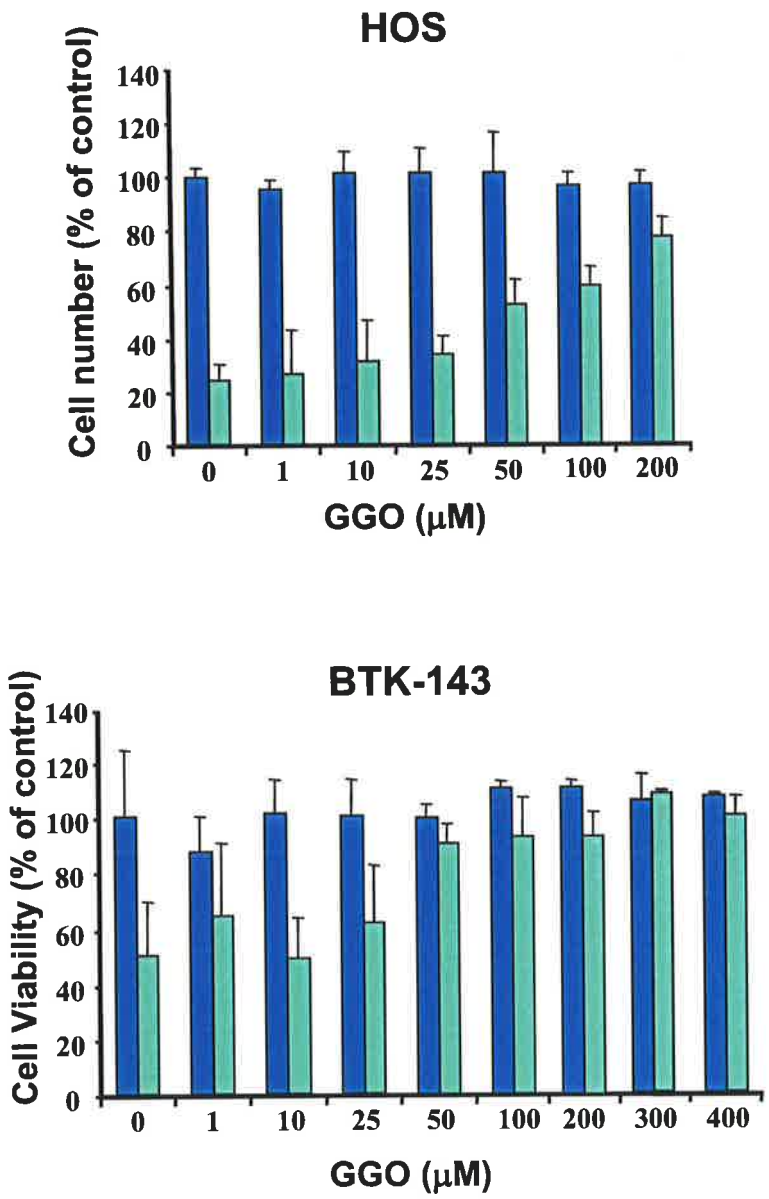


Figure 3.9 Geranylgeraniol dose dependently abrogates the effect of ZOL in OS cells. HOS and BTK-143 cells were treated for 72 hours with 25 μM ZOL alone (■), or were co-incubated with ZOL and increasing concentrations of geranylgeraniol (■), as indicated. Cell viability is represented as a percentage of control untreated cells. Data points show means of triplicate results from a representative experiment, repeated at least twice; *bars* ±SD.

of 300μM GGO, and 25μM ZOL, **Figure 3.10** shows protection from cell death in four cell lines; HOS, BTK-143, K-HOS and KRIB cells.

In addition, a dose-dependent increase of GGO added to BTK-143 and HOS cells, along with 25μM ZOL, inhibited an increase in caspase activation, as shown in **Figure 3.11**. These results are consistent with the importance of the mevalonate pathway as an intracellular target for the effects of ZOL on cell proliferation.

We compared the effects of ZOL on cell viability with its ability to inhibit protein prenylation. Lysates at each dose of treatment were analysed by western blotting for the presence of the non-prenylated form of the small GTPase, Rap1A. Our results show that there was a clear correlation between ZOL dose on cell viability and the ability to inhibit protein prenylation. For example, in the HOS cells, ZOL at a dose of 5 μM, which reduced cell viability by approximately 50% (see dose response curves, **Figure 3.1**), also effectively inhibited Rap 1A prenylation. By contrast, the same concentration of ZOL, which was less potent at reducing cell viability of MG-63 cells (~5%) failed to inhibit Rap 1A prenylation (**Figure 3.12**). In these cells, the presence of unprenylated Rap1 A was not obvious until the concentration of ZOL reached 10 μM. The levels of total Rap 1A did not change with treatment

3.3.6 ZOL treatment leads to anoikis

As seen in **Figure 3.3**, the induction of cell death in OS cells by ZOL resembled "anoikis', a special mode of apoptosis that occurs when adherent cells detach or lose the particular attachment contacts with the extracellular matrix that confer survival signals to the cells. To investigate whether anoikis may be the mechanism for induction of cell death in OS cells, experiments were carried out in which adhesion of HOS cells by their

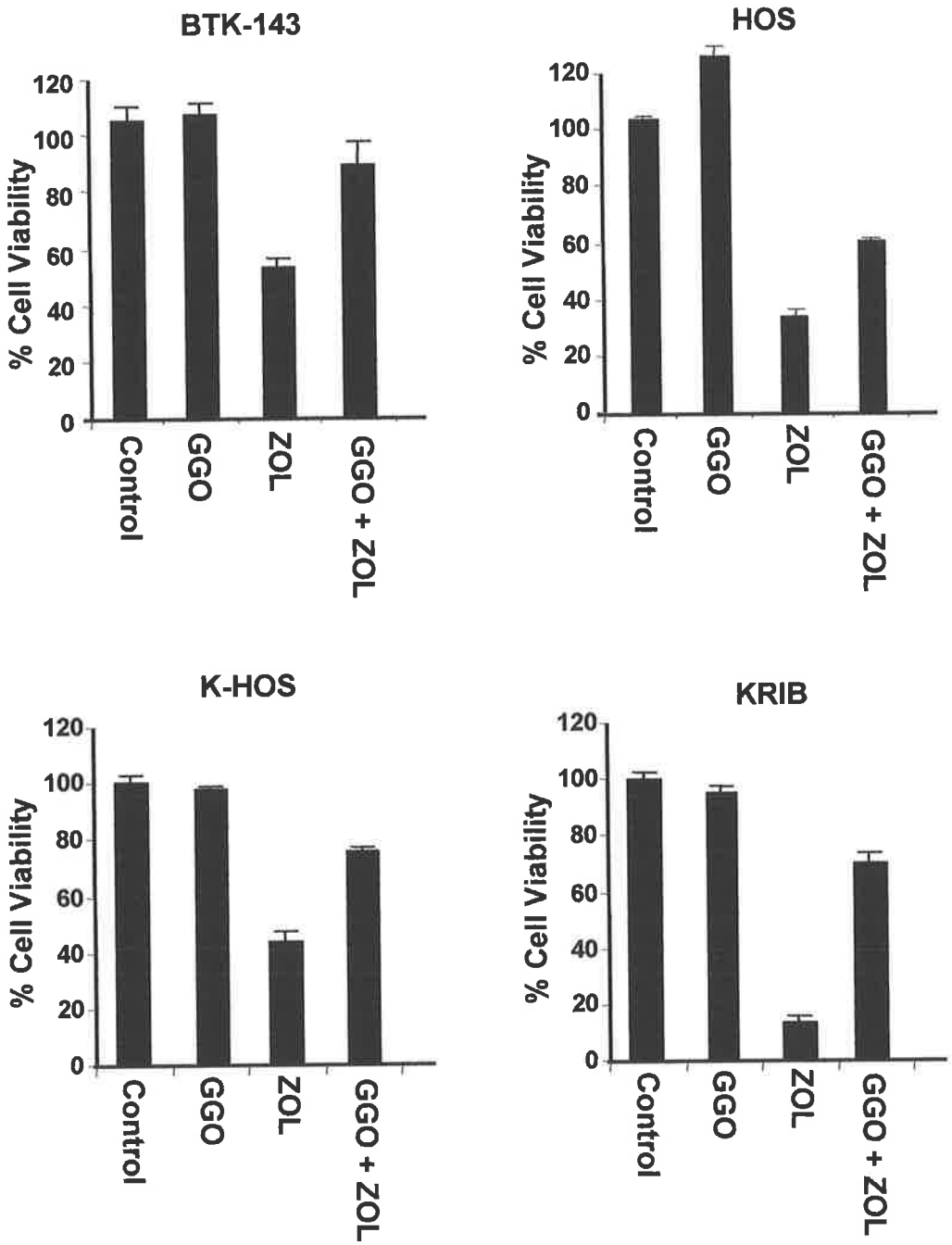


Figure 3.10 Geranylgeraniol protects against ZOL-induced apoptosis in OS cell lines. The co-addition of 300μM of GGO to HOS, BTK-143, K-HOS and KRIB cells was able to protect against the apoptotic effects of ZOL (25μM) when compared to cells treated with ZOL alone for 72 hours. *Columns*, Cell viability as a percentage of control untreated cells. Data points show means of triplicate results from a representative experiment, repeated at least twice; *bars* ±SD.

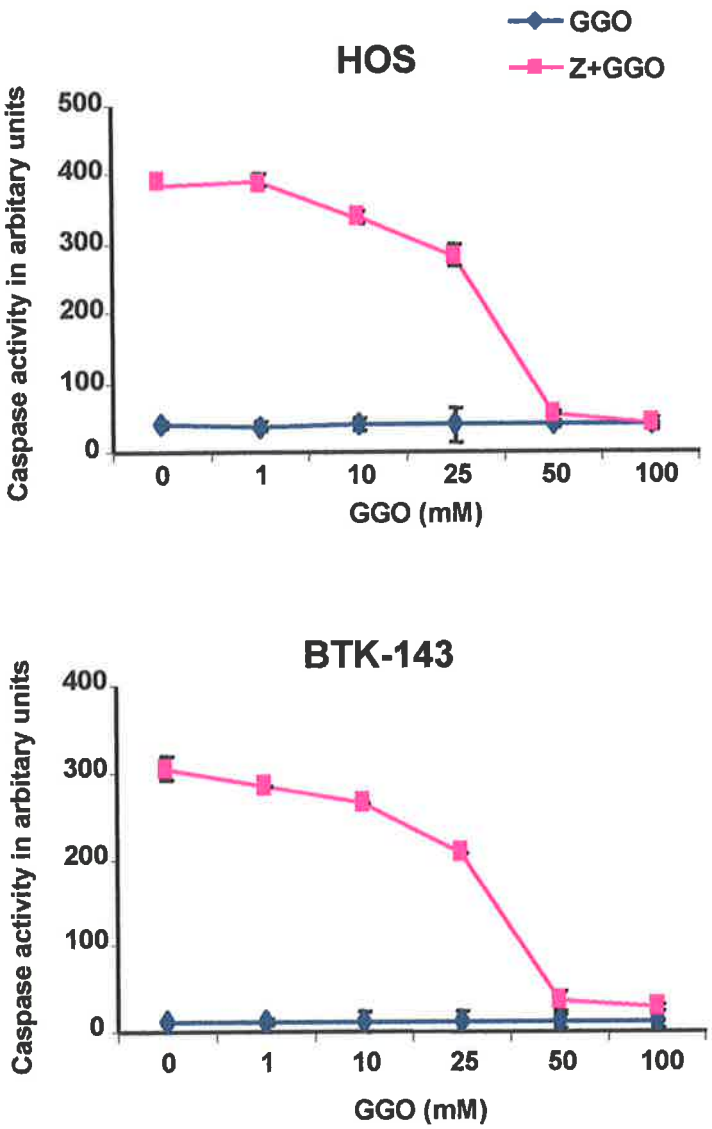


Figure 3.11 Geranylgeraniol (GGO) inhibits ZOL mediated increases in caspase activation. HOS and BTK-143 cells were treated with increasing concentrations of GGO (0, 1, 10, 25, 50, 100μM) either with (■) or without (◆) 25μM ZOL, for 72 hours. Cell lysates were used to determine caspase-3-like activity, using the caspase-3 specific fluorogenic substrate, zDEVD-AFC. Data points show means of quadruplicate results from a representative experiment, repeated at least twice; *bars* ±SD.

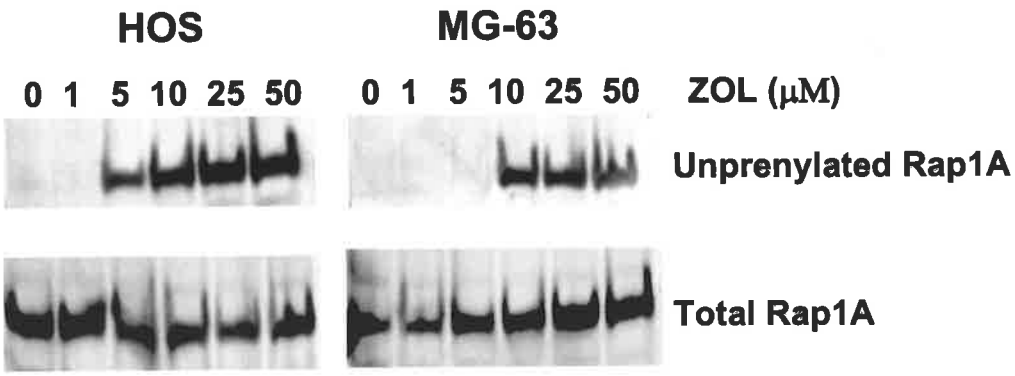


Figure 3.12 ZOL inhibits protein prenylation. HOS and MG-63 OS cells were seeded in 25cm^2 flask and incubated for 72 hours with media containing increasing concentrations of ZOL (1-50 μM) for 48 hours. Cell lysates were analysed on western blots for the presence of the unprenylated form of Rap 1A and total Rap 1A.

culture was prevented, by using flasks coated with polyHEMA. The non-ionic nature of polyHEMA prevents both matrix deposition and subsequent cell attachment. We found that the number of dead cells in the non-anchored cell population increased progressively with time from 15% at 8 hours to more than 35% at 24 hours, compared to anchored cells (see **Figure 3.13A**). In addition, the non-anchored HOS cells demonstrated a 3-fold increase in the level of caspase-3-like activity at 24 hours as shown in **Figure 3.13B** and displayed morphological changes characteristic of apoptosis, as seen in the ZOL treated cells (see **Figure 3.13C**).

3.3.7 ZOL induces S-phase arrest of the cell cycle

We next performed flow cytometric analysis of DNA content to identify cell cycle perturbations following treatment with ZOL over a 96 hour period. In the MG-63 cells, in which ZOL was cytostatic and did not cause a loss of cell viability, there was an accumulation of cells in S-phase (**Figure 3.14**). The effects of ZOL on cell cycle parameters in MG-63 cells were not obvious at 24 hours. However, by 48 hours, ZOL caused an approximate 2-fold increase in the number of cells arrested in S-phase (55% vs 25%), concomitant with a reduction of cells in G_0/G_1 and G_2/M phases. There were no cells in the apoptotic sub- G_0/G_1 peak at any time following treatment with ZOL in these cells, consistent with the results showing no loss of viability and confirming the absence of ZOL-induced apoptosis in MG-63 cells. In contrast, ZOL treatment resulted in S-phase arrest in the HOS cells, exerting its effects as early as 24 hours, with the number of cells in S-phase increasing from 25% in control untreated cells to 55% in the corresponding ZOL treated cells. At 48 hours the number of cells in S phase in the ZOL treated cultures increased to 69% of the total population. The proportion of hypodiploid

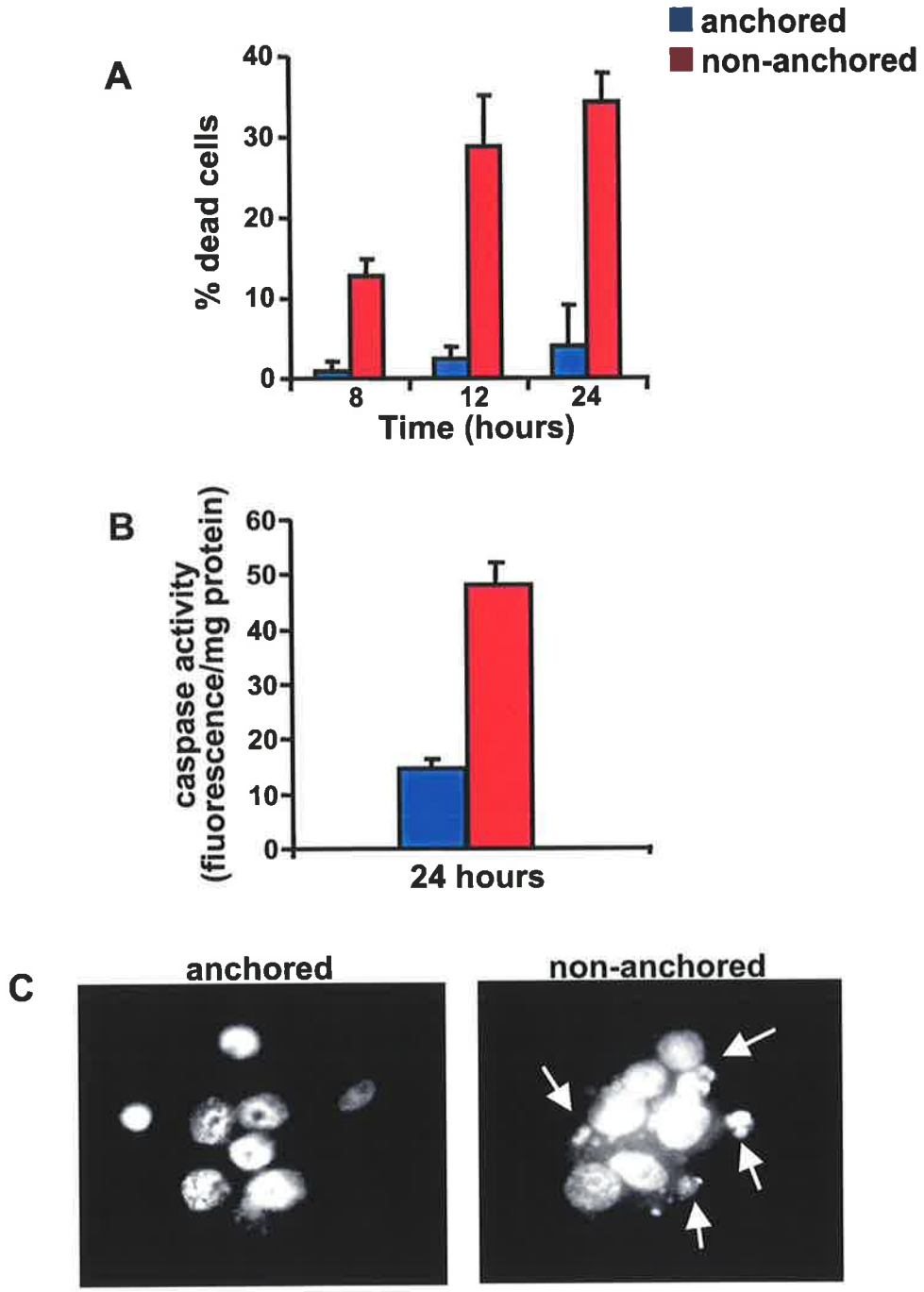


Figure 3.13 HOS cells were plated on polyHEMA-coated or control tissue culture wells and cultured for the indicated times. (**A**) Cell viability was assessed by trypan blue dye exclusion and the number of dead cells at each time point was expressed as a percentage of the total cells. (**B**) Cells grown on polyHEMA (non-anchored) for 24 hours demonstrated a three fold increase in caspase-3-like activity compared with the control (anchored) cells and (**C**) displayed morphological changes characteristic of apoptosis, as assessed by DAPI staining of their nuclei. Data points show means of triplicate results from a representative experiment, repeated at least twice; *bars* ±SD.

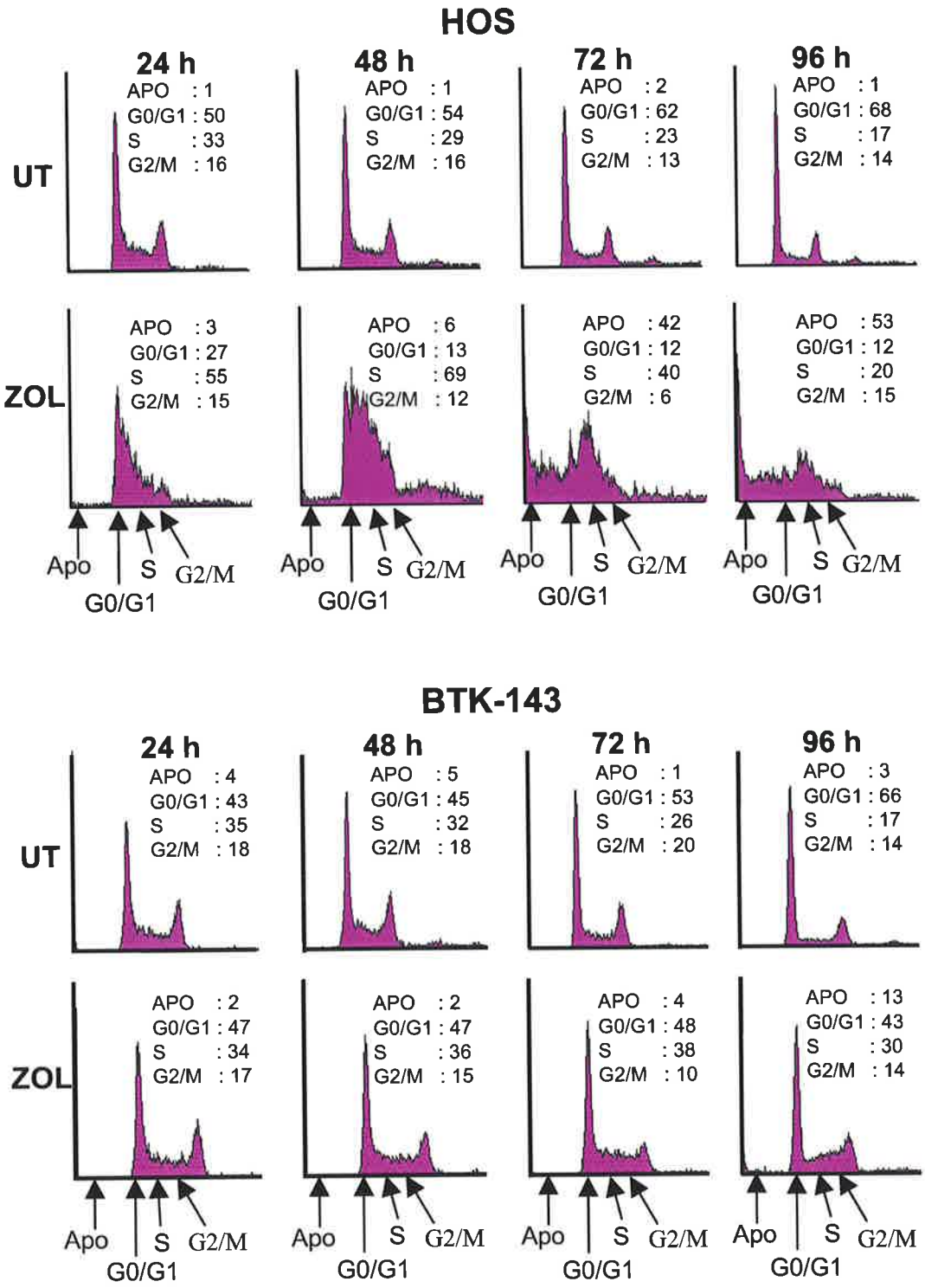


Figure 3.14 Zoledronic acid induces S-phase arrest of the cell cycle. HOS and BTK-143 cells were incubated for 24, 48, 72 and 96 hours in the absence (control) or presence of 25μM ZOL. At each time point, cells were harvested, fixed and stained with propidium iodide. The positions on the histograms of the hypodiploid Apo (sub-G_0/G_1), G_0/G_1, S, G2/M peaks and the percentage of cells in each of the cycle phases, in a representative experiment, are indicated.

cells found in the apoptotic sub-G_0/G_1 peak, consistent with the onset of apoptosis, increased progressively to 6%, 42% and 53% at 48, 72 and 96 hours, respectively, as shown in **Figure 3.14**.

3.4 DISCUSSION

In this study we have shown that ZOL treatment of OS cells induced a dose- and time-dependent decrease in proliferation in all of the cell lines tested, although it was variably effective in reducing cell number in the different cell lines. The anti-proliferative effect of ZOL in the SaOS-2, SJSA-1 and MG-63 cell lines was the result of inhibition of cell cycle progression associated with a time-dependent increase in the proportion of cells arrested in S-phase. In contrast, the decrease in number of the HOS, BTK-143, G-292, K-HOS and KRIB cells was the result of increased cell death. Flow cytometric analysis of these cells demonstrated a time-related increase in the proportion of cells in the apoptotic sub Go/G_1 peak of the cell cycle. However, the increase in apoptotic cells was preceded by an increase in the proportion of cells arrested in S-phase. Other N-BPs have been shown to also cause S-phase arrest [Iguchi *et al.* 2003; Forsea *et al.* 2004]. The reasons for the observed differences in activity and potency of ZOL in the different cell lines are unclear but may reflect differences in bioavailability, cellular uptake or intracellular effects of ZOL in different cell types. Nonetheless, our results indicate that the underlying mechanisms of the ZOL effect on cell proliferation involve the inhibition of cell cycle progression, mainly due to S-phase arrest, and the induction of apoptosis.

In those OS cell lines, in which ZOL decreased cell viability, the effects were not immediate and were seen after a lag period of 48 to 72 hours. This is consistent with previous published studies showing similar delayed effects of BPs in other cancer cells, as well as osteoclasts [Ito *et al.* 1999; Fromigue *et al.* 2000; Senaratne and Colston 2002]. As suggested previously [Rodan 1997], this lag period may represent the time required for the turnover of prenylated proteins already present in the cells, before the inhibition of prenylation of newly synthesised proteins by BPs, in this case ZOL, takes

effect. The concentrations, at which ZOL was effective in our experimental model (1-50 μM), are unlikely to be reached in the circulation using current *in vivo* treatment regimens. The maximum concentration of ZOL in human plasma after a 4 mg dose is only 2 μM [Rogers *et al.* 2000]. However, it has been suggested that because of the high affinity of BPs for bone mineral, the local concentration of BPs in the bone microenvironment will be much higher than serum levels [Sato *et al.* 1991; Sahni *et al.* 1993]. It is generally accepted that, *in vivo*, it is the osteoclasts that are most likely to be exposed to high levels of BP during active resorption of bone. In the absence of any convincing evidence to suggest that BPs can exert direct effects on cancer cells themselves within the bone microenvironment, we can only speculate that the close proximity of tumour cells and actively resorbing osteoclasts within osteolytic lesions will result in the sufficient release of the BP required to induce tumour cell apoptosis. It is therefore crucial to test the effectiveness of BPs against OS *in vivo*. This will be discussed further in chapter 6 and 7. Whether the effects of ZOL on growth and survival of OS cells in bone occurs through its direct actions or *via* secondary effects mediated by osteoclast inhibition remains to be determined. The positive effects of inhibiting osteoclast activity may include preventing the release of factors which inhibit osteoblast differentiation and/or promote tumour cell growth from the osteoclasts themselves and the bone mineral and ultimately lead to the inhibition of bone loss, which may be adequate treatment in itself if direct effects are unattainable *in vivo* (**Figure 3.15**).

Treatment of the ZOL-sensitive OS cells, for example HOS cells, with ZOL caused significant cell detachment, as viewed by light microscopy. In addition, staining of nuclei with the nuclear fluorescent stain DAPI demonstrated morphological changes characteristic of apoptosis, including chromatin condensation, nuclear fragmentation and the formation of dense rounded apoptotic bodies. Induction of intranucleosomal

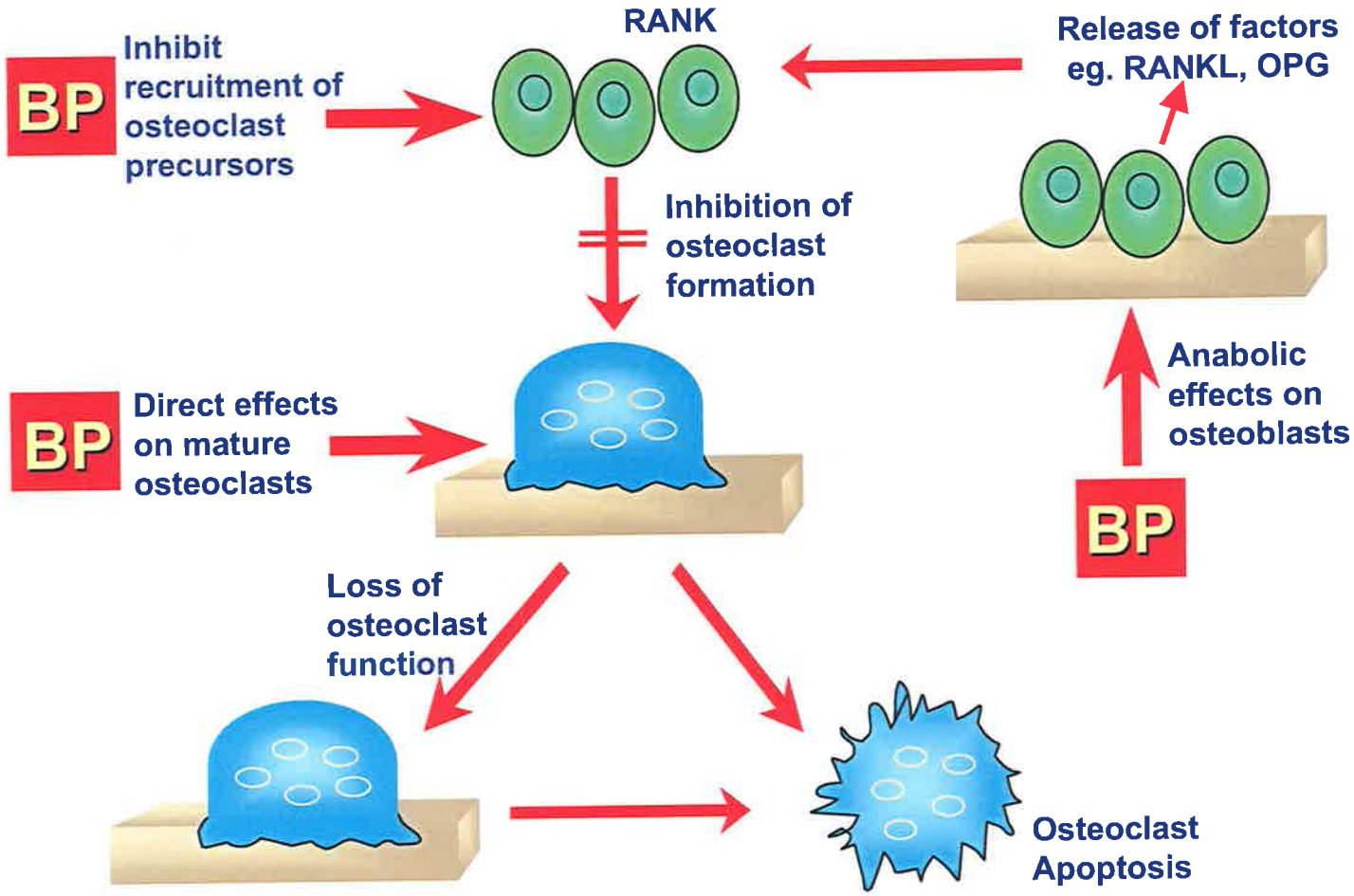


Figure 3.15 Inhibition of bone resorption by N-BPs. N-BPs act *via* multiple mechanisms to reduce osteoclast number and activity and thus inhibit bone resorption. This can occur directly by inducing loss of function and apoptosis, or indirectly by stimulating osteoblast proliferation and influencing changes in osteoblastic gene expression and the secretion of soluble factors involved in regulating osteoclast differentiation and activity.

DNA fragmentation, as assessed by agarose gel electrophoresis, confirmed the induction of apoptosis by ZOL. We investigated the induction of apoptosis of OS cells by ZOL, by monitoring the activation of caspase-3. We found that caspase activation following treatment with ZOL paralleled the decreased cell viability in a dose- and time-dependent manner. Thus, ZOL induced the largest increase, and earliest activation, of caspase-3 in the HOS, BTK-143 cells and G-292, which were the cell lines that were most sensitive to the effects of ZOL on cell survival. In contrast, the increase in caspase activity in the ZOL-resistant SaOS-2, SJSA-1 and MG-63 cells was relatively small and delayed. The general specificity caspase inhibitors, Boc-D-fmk and zVAD-fmk, and the specific caspase-3 inhibitor z-DEVD-fmk, blocked the ZOL-induced activation of caspases but did not prevent cell detachment or cell death. Our results therefore differ from findings previously reported for breast cancer cells [Fromigue *et al.* 2000] or [Reszka *et al.* 1999] showing that BP-induced apoptosis in these other cell types could be suppressed by the co-addition of the broad specificity caspase inhibitor z-VAD-fmk. It is more likely that cell detachment is the critical event in the induction of cell death by ZOL, with the activation of caspases a consequence of loss of critical cell attachments, rather than a primary cause of cell death. The induction of cell death in OS cells by ZOL therefore resembled "anoikis" [Frisch and Francis 1994], a special mode of apoptosis that occurs when adherent cells detach or lose the particular attachment contacts with the extracellular matrix that confer survival signals to the cells (**Figure 3.16**). Evidence supporting anoikis as the means of inducing cell death in OS cells was their increased apoptosis in the absence of cell-matrix contacts by plating on polyHEMA. These data are consistent with a ZOL-specific induction of cell apoptosis that involves cell detachment as a prerequisite (anoikis), and in which caspase activation occurs secondarily and is redundant as a mediator of cell death.

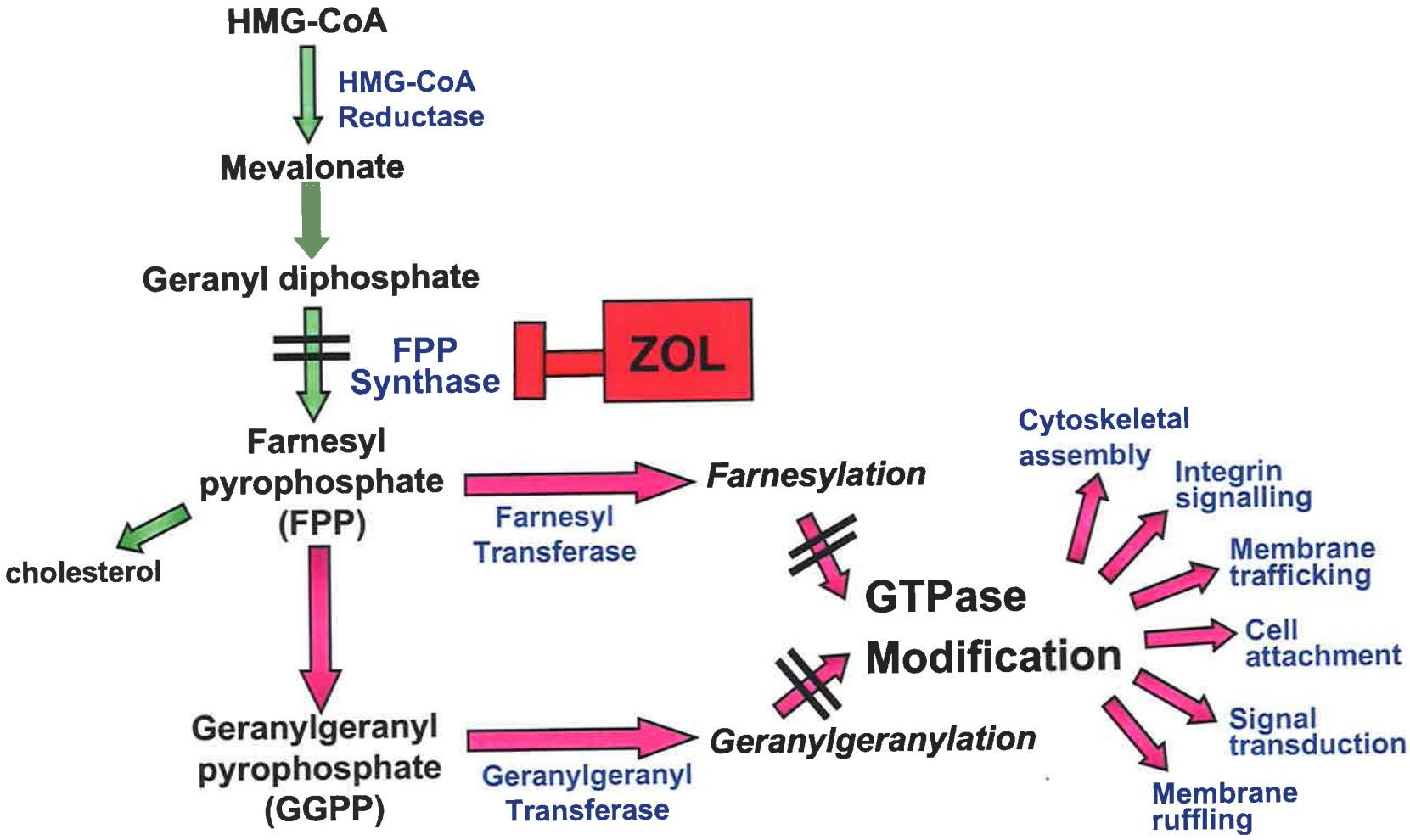


Figure 3.16 ZOL inhibits protein prenylation. N-BPs such as ZOL inhibit an enzyme of the mevalonate pathway, preventing downstream processes including farnesylation and geranylgeranylation, required for post-translational modifications of GTPase proteins. These GTPases are involved in numerous roles vital for cell survival.

Consistent with several previous reports in osteoclasts [Coxon *et al.* 2000; Reszka *et al.* 1999], and in other cancer cell types [Shipman *et al.* 1997], we have shown that the effects of ZOL on cell proliferation of OS cells are mediated mainly through the inhibition of the mevalonate pathway. These effects of ZOL were reversed in the presence of geranylgeraniol (GGO), an intermediate of the mevalonate pathway (**Figure 3.17**). Therefore, the effects of ZOL on cell detachment are likely to be mediated by changes in cytoskeletal integrity brought about by the disruption of cell-cell and cell-extracellular matrix interactions. Further studies investigating the prenylation of small GTPases that regulate these processes will be required to delineate further the molecular mechanisms involved.

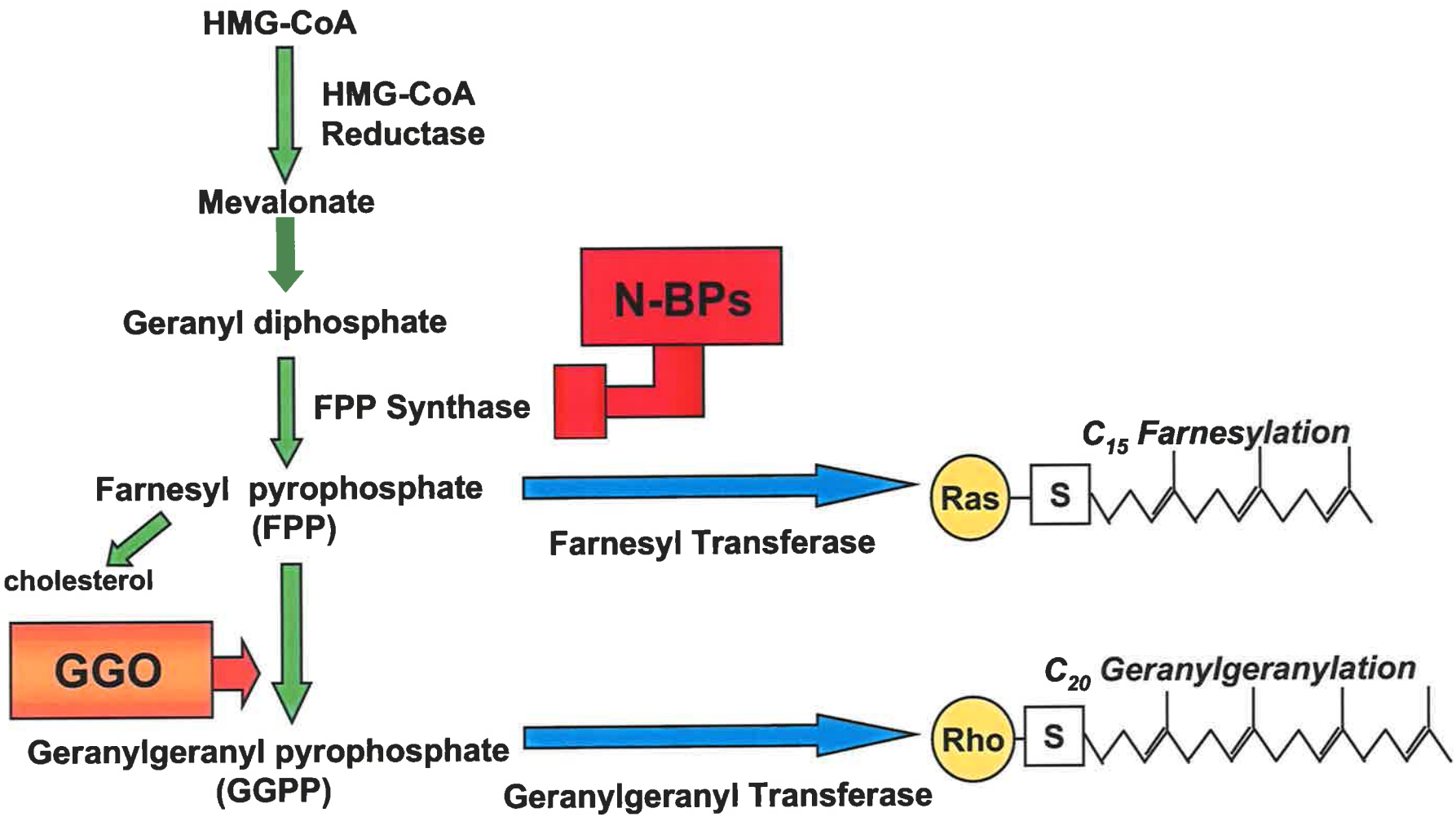


Figure 3.17 The mevalonate pathway. N-BPs inhibit the enzyme as indicated above, preventing downstream processes including farnesylation and geranylgeranylation. Geranylgeraniol (GGO) acts as an intermediate for geranylgeranylation in the presence of N-BPs.

CHAPTER FOUR

ZOLEDRONIC ACID SENSITISES HUMAN OSTEOSARCOMA CELLS TO TRAIL INDUCED APOPTOSIS:

Are dual treatments more effective?

4.1 INTRODUCTION

The chief objective of anticancer strategies is the selective induction of apoptosis in cancer cells but not normal cells. Investigating combinations of agents with synergistic or additive activity is appealing because this approach potentially enables the use of lower drug doses and therefore reduced toxic side effects.

BPs and TRAIL induce apoptosis in cancer cells, at least *in vitro*. Cytostatic and cytotoxic effects due to TRAIL have been reported in transformed cell lines including colon, lung, breast, central nervous system, kidney, brain and skin [Ashkenazi and Dixit 1999; Pitti *et al.* 1996; Wiley *et al.* 1995]. Sensitivity to TRAIL differs in various cell lines and the reason for this is unknown, although sensitivity appears not to depend solely on TRAIL receptor expression. TRAIL was only weakly apoptotic in a panel of human OS cell lines [Evdokiou *et al.* 2002]. However, treatment of TRAIL-resistant cell lines with chemotherapeutic agents resulted in their sensitization to TRAIL, suggesting that TRAIL-induced apoptosis can be enhanced [Griffith *et al.* 1998]. The mode of action of sensitizing agents may be *via* the stimulation of either or both the extrinsic or intrinsic apoptotic signalling pathways. Both *in vivo* and *in vitro* studies have confirmed the effect of chemotherapeutic agents to enhance the antitumor activity of TRAIL in many cell lines, including human OS [Griffith *et al.* 1998; Thomas and Hersey 1998; Gliniak and Le 1999; Ashkenazi *et al.* 1999; Keane *et al.* 1999; Mizutani *et al.* 2001; Gibson *et al.* 2000; Clayer *et al.* 2001; Evdokiou *et al.* 2001]. Conversely, TRAIL does not seem to affect normal cells *in vivo,* unlike its TNF-ligand superfamily counterparts, Fas and TNF-α, which are cytotoxic, making TRAIL a potential anticancer agent [French and Tschopp 1999; Ashkenazi *et al.* 1999].

There is a pressing need to specifically target cancer cells within the bone. The highly selective localisation and retention of BPs in bone is the basis for its use in skeletal disorders. The most potent BP currently available is ZOL and its use in animal

models ensures that the highest possible doses can be achieved *in vivo* [Green *et al.* 1994]. *In vitro* experiments have shown that ZOL induces apoptosis in a variety of cancer cell types including breast, prostate, myeloma, colon and OS cell lines [Fromigue *et al.* 2000; Jagdev *et al.* 2001; Evdokiou *et al.* 2003; Lee *et al.* 2001]. It has been reported that the apoptotic effect of ZOL is far greater when combined with some chemotherapeutic drugs such as paclitaxel and dexamethasone than with either agent alone [Jagdev *et al.* 2001; Tassone *et al.* 2000]. Studies have also demonstrated that the effect of ZOL was not enhanced when combined with chemotherapeutic drugs such as cisplatin, etoposide and doxorubicin. This may be due to the particular chemotherapeutic agent and cell types examined [Evdokiou *et al.* 2002]. Many chemotherapeutic agents are cytotoxic mainly to actively cycling cells. Some agents are phase-specific; that is, they are cytotoxic to cells in a particular phase of the cell cycle and others are capable of exerting cytotoxicity at any phase of cell cycle [Chabner and Longo 1996]. In this case it is the depletion of prenylated proteins that leads to the apoptotic efforts of ZOL, thus hindering this process by halting cell cycling may not provide a combined apoptotic effect. For this reason it may be more efficacious to combine ZOL with agents that act by different mechanisms.

The work described in this chapter investigated the combination treatment of TRAIL, which acts *via* the TRAIL receptors to activate caspase-mediated apoptosis, and ZOL, which acts to inhibit the mevalonate pathway, in a panel of human OS cell lines.

4.2 MATERIALS AND METHODS

4.2.1 Cells and cell culture

The osteosarcoma cell lines and the osteoblast-like cells from donors of normal human bone used in this chapter are listed in section 2.1.1. The methods for cell preparation and propagation are described in section 2.1.2 and 2.1.3.

4.2.2 Measuring cell viability of ZOL and TRAIL treated cells

The method used to assess cell viability after zoledronic acid and/or TRAIL treatment is described in section 2.4.2. and 2.4.3.

4.2.3 Measuring caspase activity

The caspase inhibitors used are listed in section 2.2.4. Caspase activity protocols used in this chapter are described in section 2.4.4.

4.2.4 Cell attachment dependent survival using polyHema

The preparation of polyHema plates and the method used to assess cell attachment dependent survival is described in section 2.4.8.

4.2.5 Effect of geranylgeraniol on ZOL and TRAIL combination treatment in OS Cells

The method used to assess cell viability after treatment is described in section 2.4.2. and 2.4.3.

4.2.6 Western blot analysis

The antibodies used in the western blots are listed in section 2.2.5. The method used is described in section 2.4.6. and 2.4.9.

4.2.7 Cell surface receptor analysis after treatment

The antibodies used in cell surface receptor analysis are listed in section 2.2.5. The method used is described in section 2.4.10.

4.2.8 Cell cycle analysis after treatment

The method used is described in section 2.4.11.

4.2.9 Statistical analysis

Microsoft excel was used to obtain mean standard deviations of experimental data. Data shown in each case are from a representative experiment, which was repeated at least three times.

4.3 RESULTS

4.3.1 ZOL augments TRAIL-induced apoptosis of human OS cells

It has been reported previously that ZOL can exert cytotoxic or cytostatic effects on OS cells [Evdokiou *et al.* 2003], and that, while most OS are poorly sensitive to TRAIL as a single agent, relevant chemotherapeutic agents often used in the clinic for OS treatment were shown to be highly effective in sensitising these cells to the apoptotic effects of TRAIL [Evdokiou *et al.* 2002]. To investigate the effect of ZOL on TRAIL-induced cytotoxicity, a panel of OS cells (HOS, BTK-143, G-292, Saos2, SJSA-1, MG-63, K-HOS and KRIB) were exposed to increasing concentrations of ZOL in the absence or presence of TRAIL, as shown in **Figure 4.1**. The apoptotic effects of ZOL alone varied between cancer cell lines; sensitive cell lines were HOS, BTK-143, K-HOS, G-292 and KRIB and resistant cell lines were MG-63, SJSA-1 and Saos2. However, the concurrent treatment of ZOL plus TRAIL for 72 hours induced more apoptosis than single agent alone, in all OS cell lines to varying degrees. Combination treatment significantly augmented the apoptotic effect seen with ZOL alone, with as little as 10 μM ZOL in HOS, BTK-143 and G-292 and 25 μM ZOL in K-HOS resulting in 30%, 30%, 40% and 65% greater cell death, respectively, in these cell lines compared with ZOL treatment alone. Saos2, SJSA-1, MG-63, and KRIB required 10, 25, 25 and 25 μM of ZOL to induce 13%, 18%, 20% and 15% greater cell death, respectively, in combination with TRAIL. The cell lines that were highly sensitive to ZOL as a single agent gave the highest potentiated apoptotic effect when combined with TRAIL (**Figure 4.2**). This suggests that the role of ZOL is important for the augmented apoptotic effect of the combination treatment, however, the differences in sensitivity highlight the complexity of the mechanisms involved.

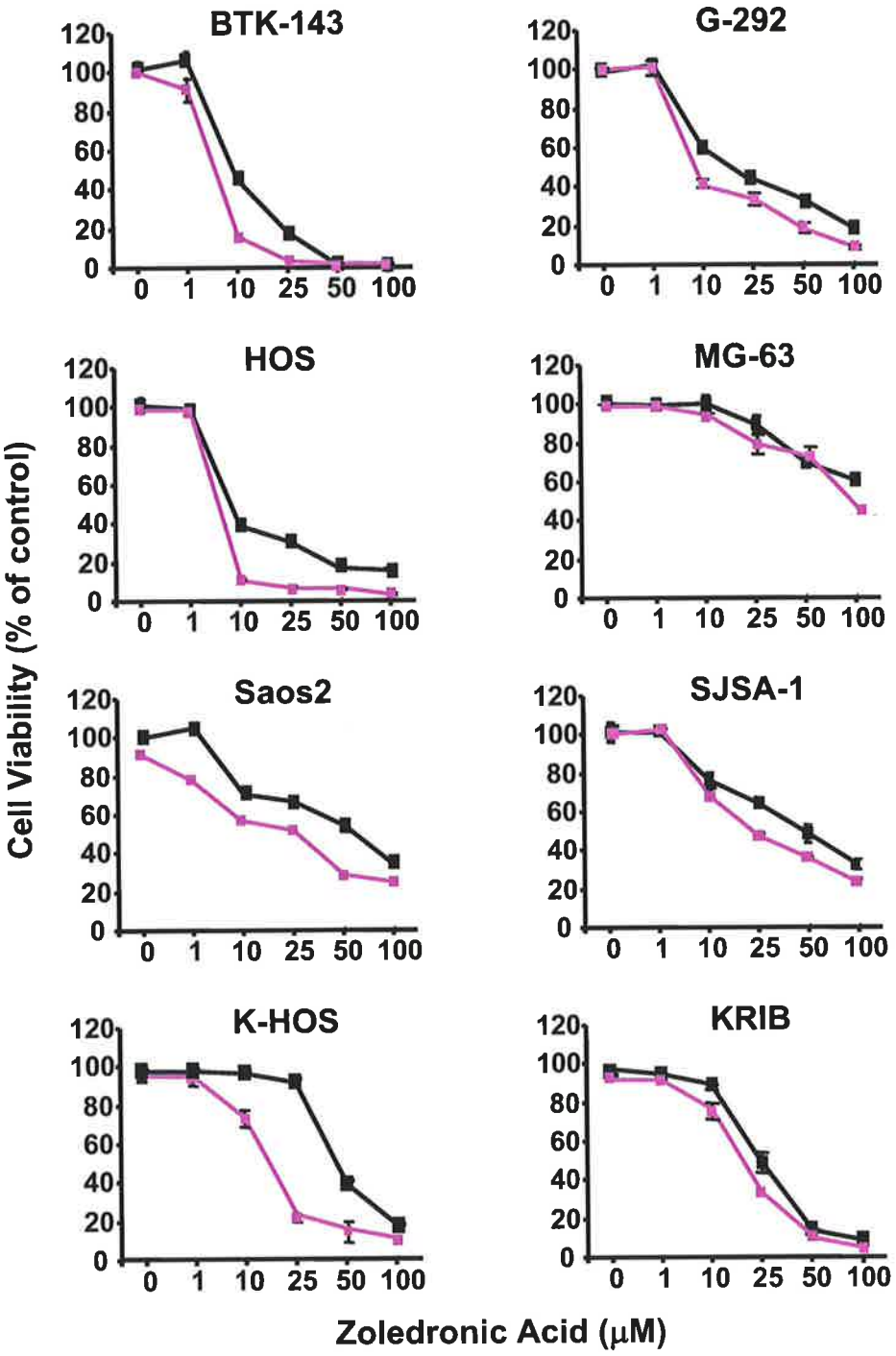


Figure 4.1 Zoledronic Acid augments TRAIL-induced apoptosis in OS cells. Cells were seeded at 1 x 10^4 cells per well in 96 well microtiter plates and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of ZOL (1-100 μM), either with (■) or without (■) 100ng/ml TRAIL. Cell viability was determined using the crystal violet assay (as described in chapter 2) and is expressed as % of control. Data shown in each case are from a representative experiment which was repeated at least three times: points are means ±SD.

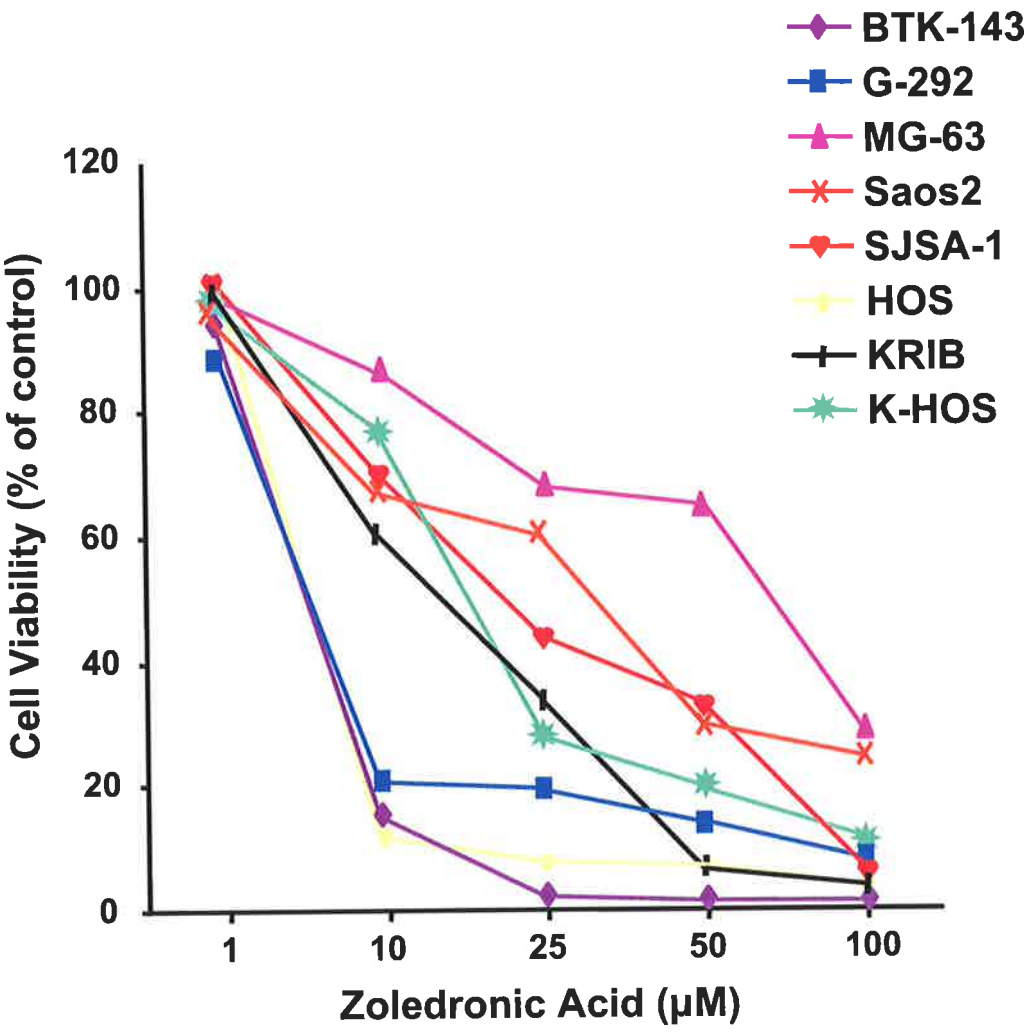


Figure 4.2 Differential response of OS cells to combined treatment with ZOL and TRAIL. 1 x 10^4 cells per well were seeded in 96 well microtiter plates and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing 100ng/ml TRAIL and increasing concentrations of ZOL (1-100 μM). Cell viability was determined using the WST-1 cell proliferation reagent assay kit, as described in chapter 2. Data is expressed as a % of control cells and are from a representative experiment which was repeated at least three times. Error bars have been omitted for better clarity.

4.3.2 The augmented apoptotic effect of ZOL and TRAIL is time-dependent

Experiments were designed to monitor any significant changes in the time required for the onset of apoptosis in the combination of ZOL and TRAIL treated OS cells compared with cells treated with ZOL alone (as shown in **Figure 4.3**). This suggests that the time required for ZOL to exert its effects is critical to the observed synergistic activity with TRAIL co-treatment. From **Figure 4.3,** augmentation is clearly evident for BTK-143 cells and moderately evident for HOS and G-292 cells. It is not known what differences exist between cell lines, to cause differential sensitivity to ZOL, however if these could be defined, treatments could be adjusted based on different cell characteristics of OS cells.

Morphological changes characteristic of apoptosis, including chromatin condensation, nuclear fragmentation and the formation of dense rounded apoptotic bodies, were clearly evident in a high percentage of cells from all cell lines, following treatment for 72 hours with ZOL and TRAIL (**Figure 4.4**). Analysis by agarose gel electrophoresis showed that combination treatment of HOS cells also resulted in an increase in the level of intranucleosomal genomic DNA fragmentation (**Figure 4.5A**), a key feature of apoptosis that arises from activation of endogenous endonucleases. Western Blot analysis detected PARP cleavage, an early specific marker of apoptosis. At 48 hours, PARP cleavage was more apparent in the combination treated cells than in cells treated with either agent alone (**Figure 4.5B**).

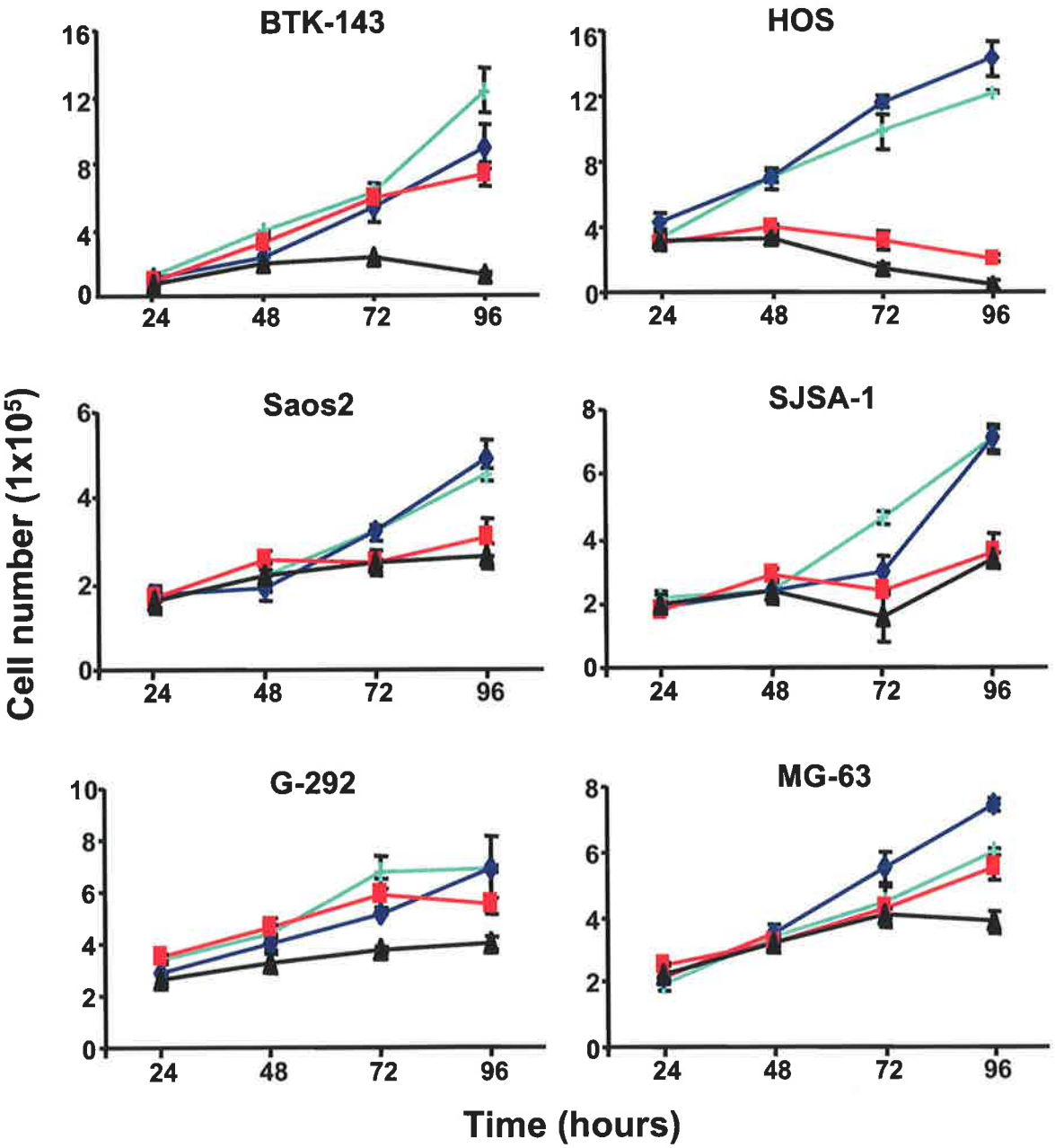


Figure 4.3 The augmented apoptotic effect of Zoledronic acid and TRAIL is time-dependent. Cells were incubated for the times indicated with media alone (❙), media containing 100ng/ml TRAIL (◆), 25 μM ZOL (■) and combination 100ng/ml TRAIL and 25 μM ZOL (▲). The number of viable cells was quantitated, using a heamocytometer. Data shown in each case are from representative experiments repeated at least three times: bars, ±SD.

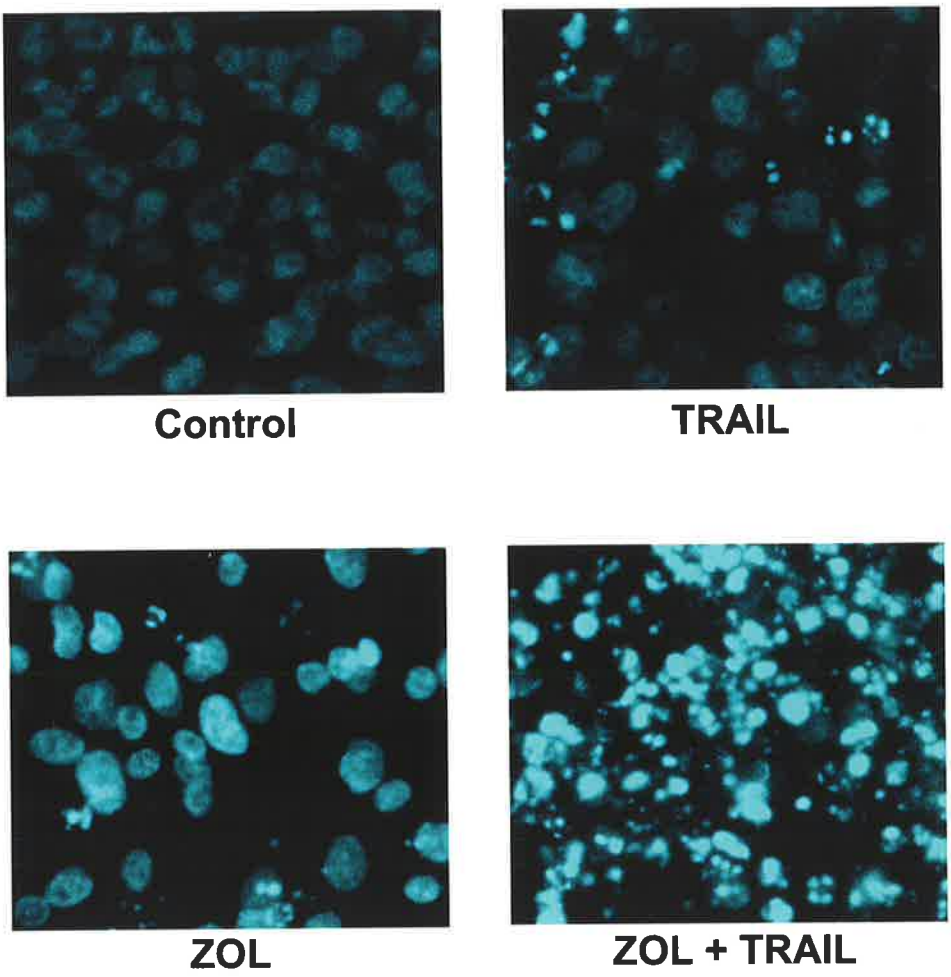


Figure 4.4 Morphological characteristics of apoptosis in HOS cells treated with 25μM ZOL and/or 100ng/ml TRAIL, as assessed by DAPI staining of their nuclei.

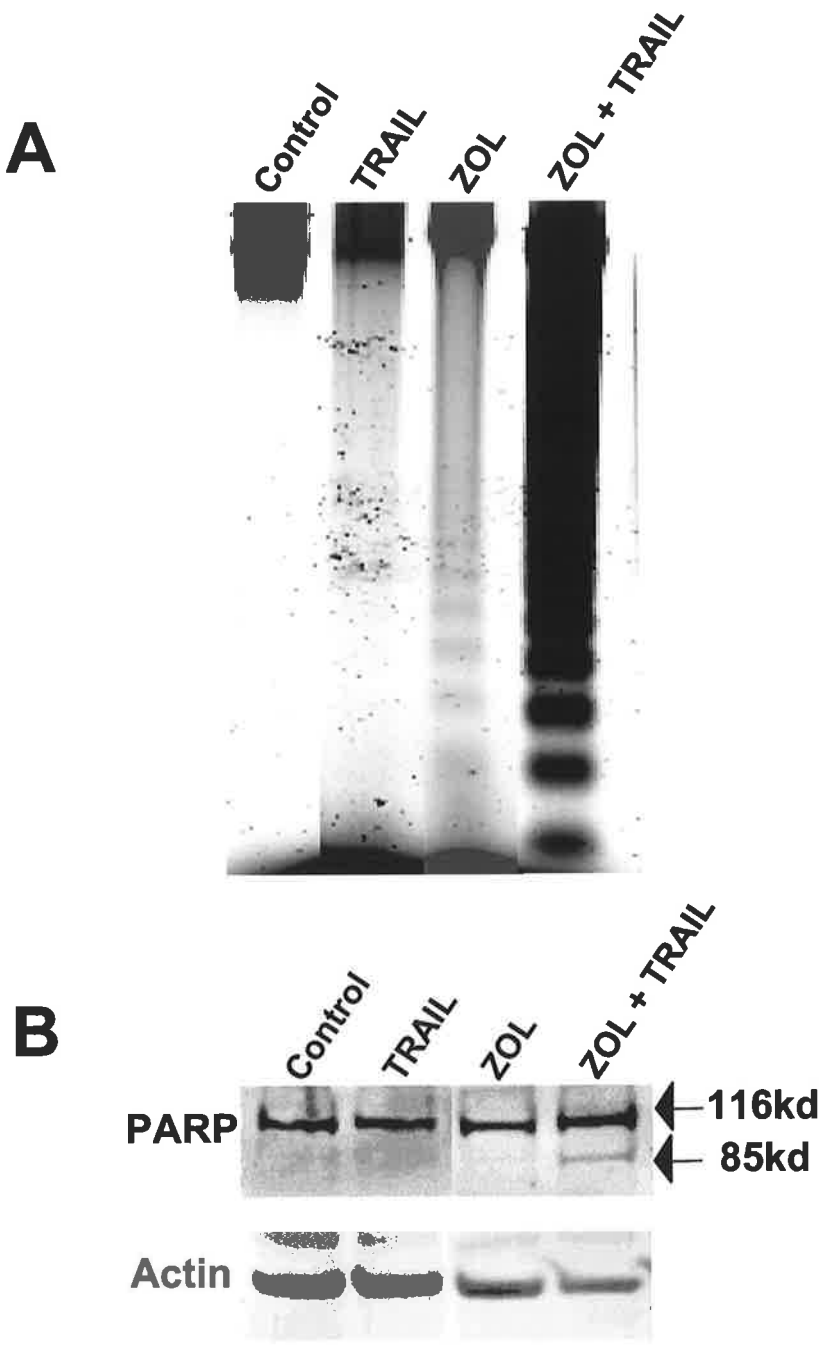


Figure 4.5 HOS cells were incubated for 72 hours with 25 μM ZOL either alone or in combination with 100ng/ml TRAIL. ***A.*** Genomic DNA was isolated and subjected to agarose gel electrophoresis. Intranucleosomal genomic DNA fragmentation was observed in the ZOL alone and in the ZOL + TRAIL treated cells. ***B.*** Cell lysates were analysed by western blot for the presence of PARP cleavage, a common characteristic of apoptosis. Actin was used as a control to assess overall level of protein in lysates.

4.3.3 ZOL-induced apoptosis in OS cells is associated with caspase activation

Treatment of OS cells with ZOL leads to the activation of the caspase cascade, although the addition of caspase inhibitors failed to prevent ZOL-induced cell loss [Evdokiou *et al.* 2003]. Treatment of HOS and Saos2 cells with ZOL and TRAIL resulted in greater activation of caspase-3 in the combination treated cells than the ZOL alone, regardless of the dose of ZOL (**Figure 4.6A**). In HOS cells the level of caspase-3 activity increased as early as 24 hours and remained higher at all time points in the combination treatment, when compared to the single agent treated cells (**Figure 4.6B**). In Saos2 cells the level of caspase-3 activity in the combination treated cells only exceeded that in ZOL treated cells at 72 hours and only marginally (**Figure 4.6B**). As HOS cells are more sensitive to ZOL than Saos2 cells it is perhaps not surprising that these results are also reflected in the combination treatments.

The addition of the caspase inhibitor z-VAD-fmk failed to protect BTK-143R and K-HOS cells from ZOL-induced cell death as shown in **Figure 4.7**. Consistent with previous results, caspase inhibition did protect the cells from the augmented apoptotic effect, resulting in cell loss equal to the level induced by ZOL alone. This suggests that ZOL may sensitise OS cells to the effects of TRAIL *via* the activation of the caspase cascade and that the addition of the caspase inhibitor z-VAD-fmk protects against this sensitisation by irreversibly inhibiting caspase-3 activity.

4.3.4 Geranylgeraniol prevents sensitisation to TRAIL by ZOL

The addition of geranylgeraniol (GGO) with ZOL treatment alone was able to protect BTK-143 and K-HOS cells from ZOL-induced apoptosis (**Figure 4.8**). Cells

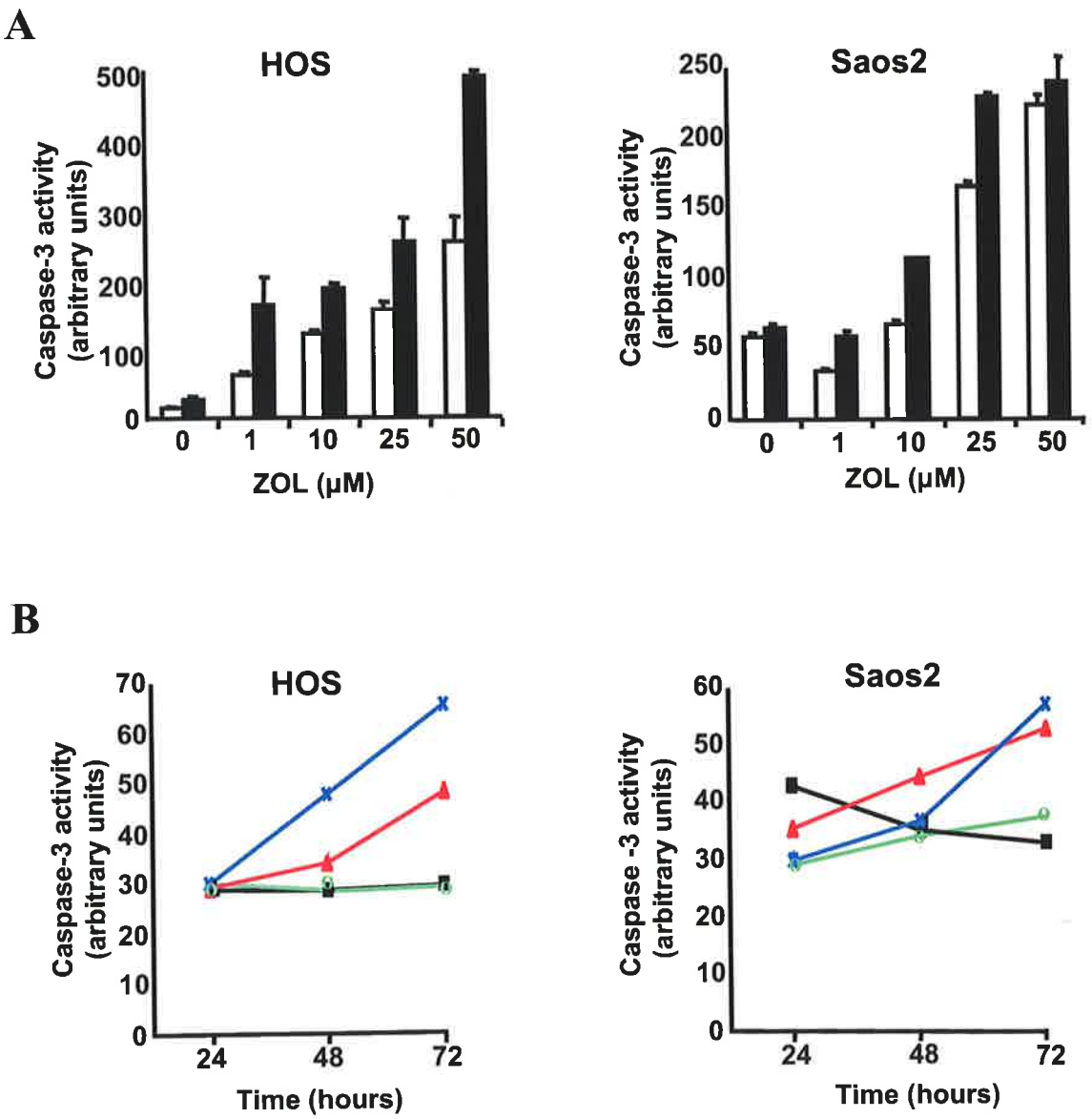


Figure 4.6 Caspase activation in OS cells treated with ZOL and TRAIL is dose and time dependent. ***A.*** HOS and Saos2 OS cells were incubated for 72 hours with increasing concentrations of ZOL (1-50 μM) and 100ng/ml TRAIL, either alone (unfilled bars) or in combination (filled bars). ***B.*** HOS and Saos2 cells were either untreated (■) or treated with a fixed concentration of ZOL (25 μM) (▲), TRAIL (100ng/ml) (o) or combination (x) for 24, 48, or 72hours. Cell lysates were collected and used to determine caspase-3-like activity, using the caspase-3 specific fluorogenic substrate, zDEVD-AFC, as described in chapter 2. Data are from representative experiments, which were repeated at least three times.

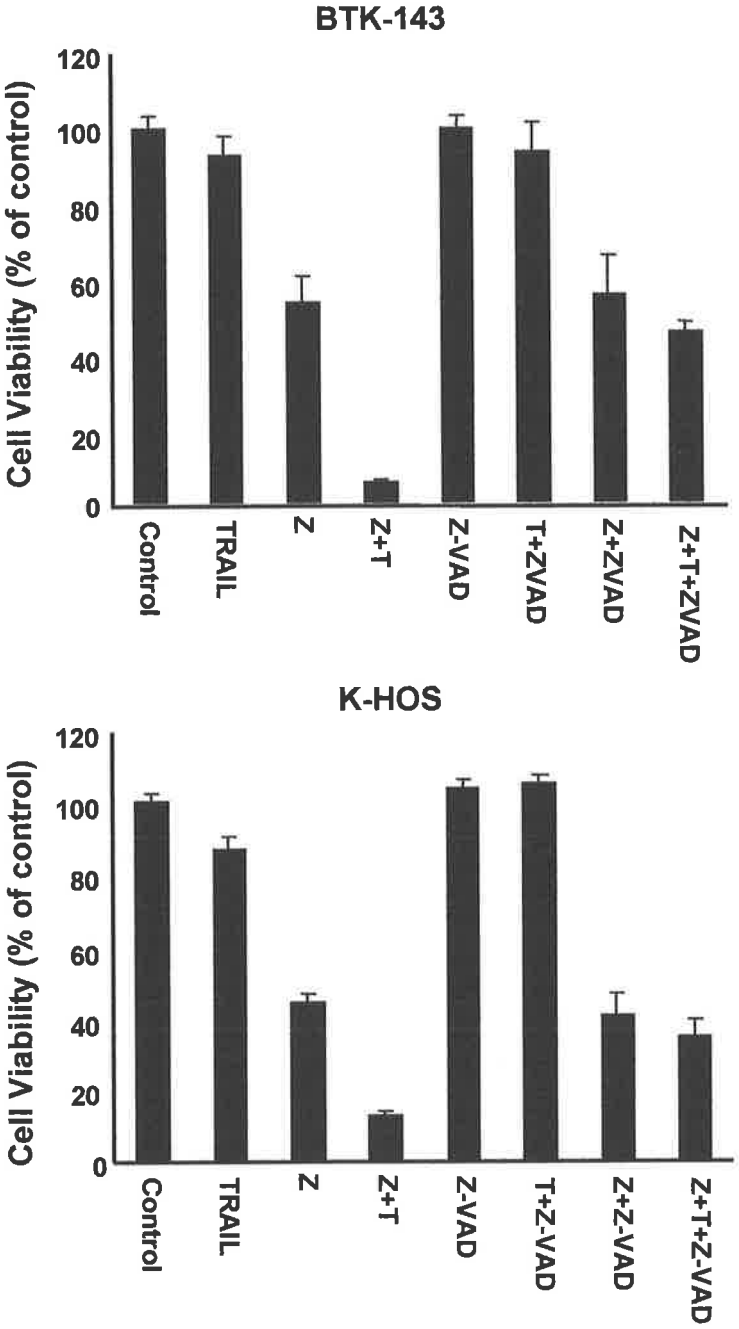


Figure 4.7 Caspase inhibition prevents augmentation of cell death by TRAIL in combination treatment with ZOL. BTK-143 and K-HOS cells were incubated for 72 hours with 25 μM ZOL, 100ng/ml TRAIL or the broad specificity caspase inhibitors z-VAD-fmk (50 μM), either alone or in combination. Cell viability was determined using the crystal violet assay. The absorbance was measured using an ELISA reader at 420-480 nm and cell viability is expressed as % of control. Data points show means of quadruplicate results from a representative experiment, which was repeated at least twice; *bars* ±SD.

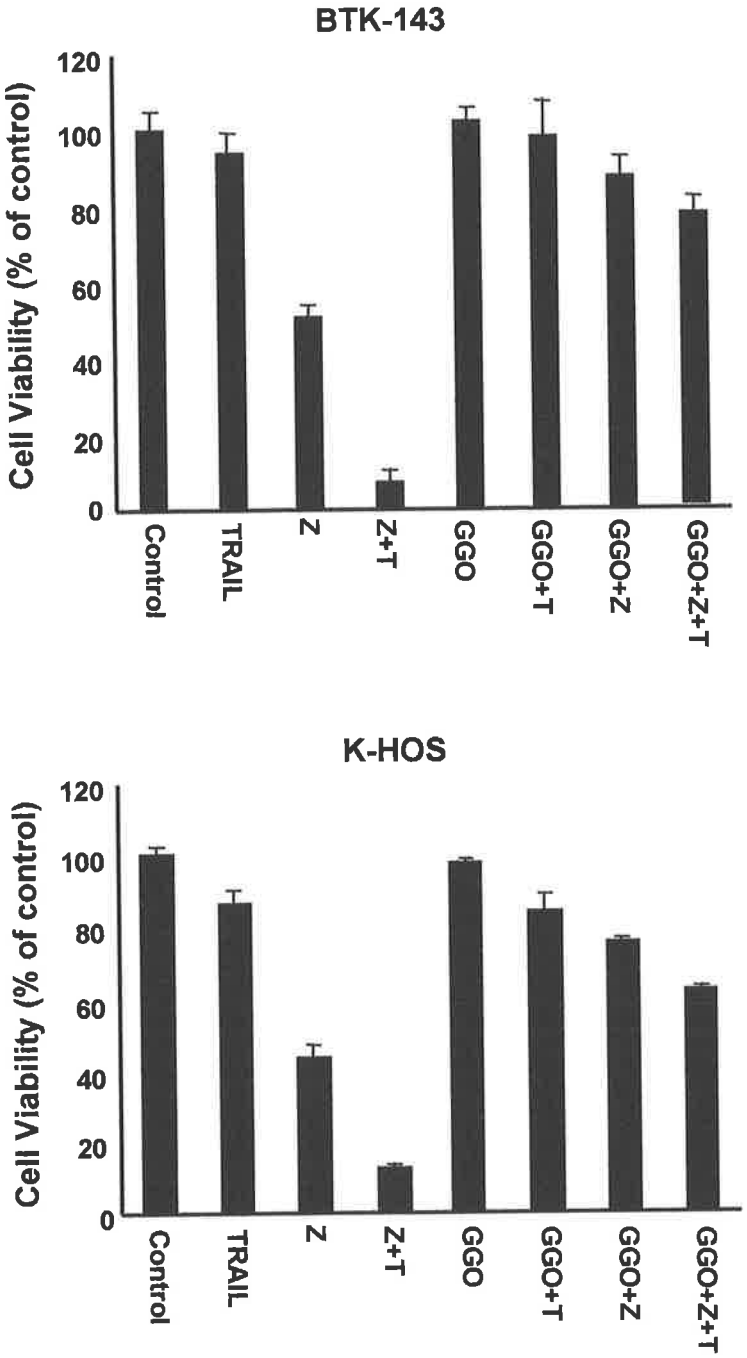


Figure 4.8 Geranylgeraniol (GGO) prevents sensitisation to TRAIL in combination treatment with ZOL. The co-addition of 300 μM of GGO to BTK-143 and K-HOS cells was able to protect against ZOL (25 μM) sensitisation of the OS cells to the apoptotic effects of TRAIL (100ng/ml). *Columns*, Cell viability is expressed as a percentage of control untreated cells. Data points show means of triplicate results from a representative experiment, which was repeated at least twice; *bars* ±SD.

treated with ZOL, TRAIL and GGO exhibited cell death similar to that seen with TRAIL alone. GGO alone, or in combination with TRAIL, had no effect on cell number in either cell line. These results support the significance of inhibition of the mevalonate pathway by ZOL, as a factor in the sensitisation of OS cells to the effects of TRAIL.

Western blot analysis was employed to measure inhibition of protein prenylation using lysates from cells treated with ZOL alone, TRAIL alone, or ZOL and TRAIL together. The non-prenylated form of Rap 1A was detected specifically in ZOL treated cells, confirming the role of ZOL in inhibiting protein prenylation (**Figure 4.9**). Total Rap 1A protein levels did not change in any of the treatments. Western Blot analysis of Bcl-2 and Bax resulted in no changes between cells treated with ZOL and TRAIL both alone and in combination (**Figure 4.9**).

4.3.5 Loss of cell anchorage sensitises cells to TRAIL-induced apoptosis

As stated in chapter 3, the addition of ZOL to OS cells leads to cell detachment prior to apoptosis by a process resembling anoikis [Evdokiou *et al.* 2003; Frisch and Francis 1994]. A comparison of anchored versus non- anchored cells, either untreated or treated with TRAIL, revealed a large proportion of cell death of 40% at 8 hours and up to 75% at 12 hours in the TRAIL treated non- anchored cells, compared to 20% cell death in the untreated anchored cells (**Figure 4.10A**). The morphological changes were extremely apparent between the anchored and non- anchored cells at both time points. Almost no apoptotic cells were identified in the anchored cells with TRAIL treatment. The non-anchored cells showed typical apoptotic characteristics including cell shrinkage, cell surface blebbing and fragmentation (**Figure 4.10B**). These results suggest that cell detachment is an important factor in sensitising the cells to TRAIL.

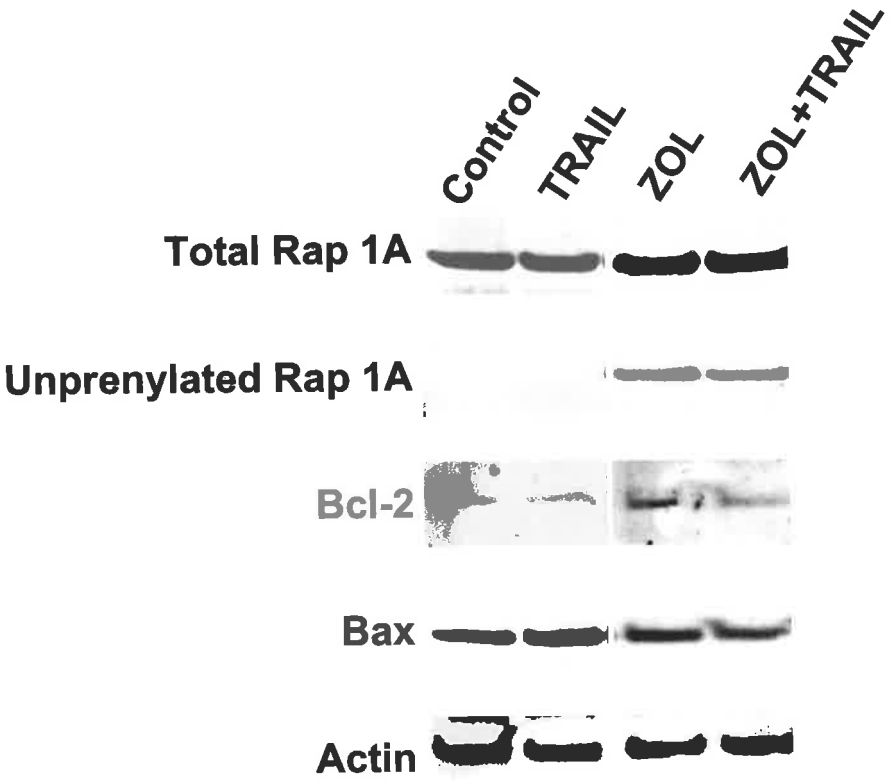


Figure 4.9 Western Blot analysis of HOS cells treated with ZOL and TRAIL either alone or in combination. HOS cells were seeded in 25cm^2 flask and incubated for 48 hours with media containing 25 μM ZOL, 100ng/ml TRAIL or combination of both. Cell lysates were analysed by western blot using antibodies for the unprenylated form of Rap 1A, total Rap 1A, Bcl-2, Bax and actin (see chapter 2 for details).

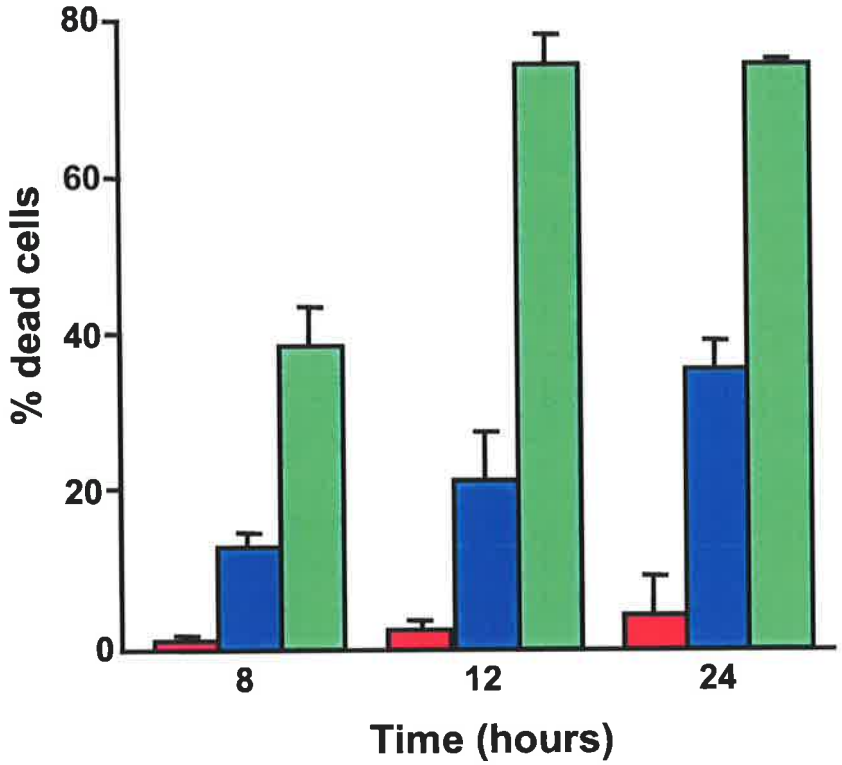


Figure 4.10 *A.* Loss of cell anchorage sensitises HOS cells to TRAIL-induced apoptosis. HOS cells were added to standard culture flasks (■ anchored) or polyHEMA-coated flasks (non-anchored) either with (■) or without (■) TRAIL (100ng/ml). The number of dead cells was counted at 8, 12 and 24 hours and and expressed as percent of total cells. Data points show means of triplicate results from a representative experiment, which was repeated at least twice; *bars* ±SD.

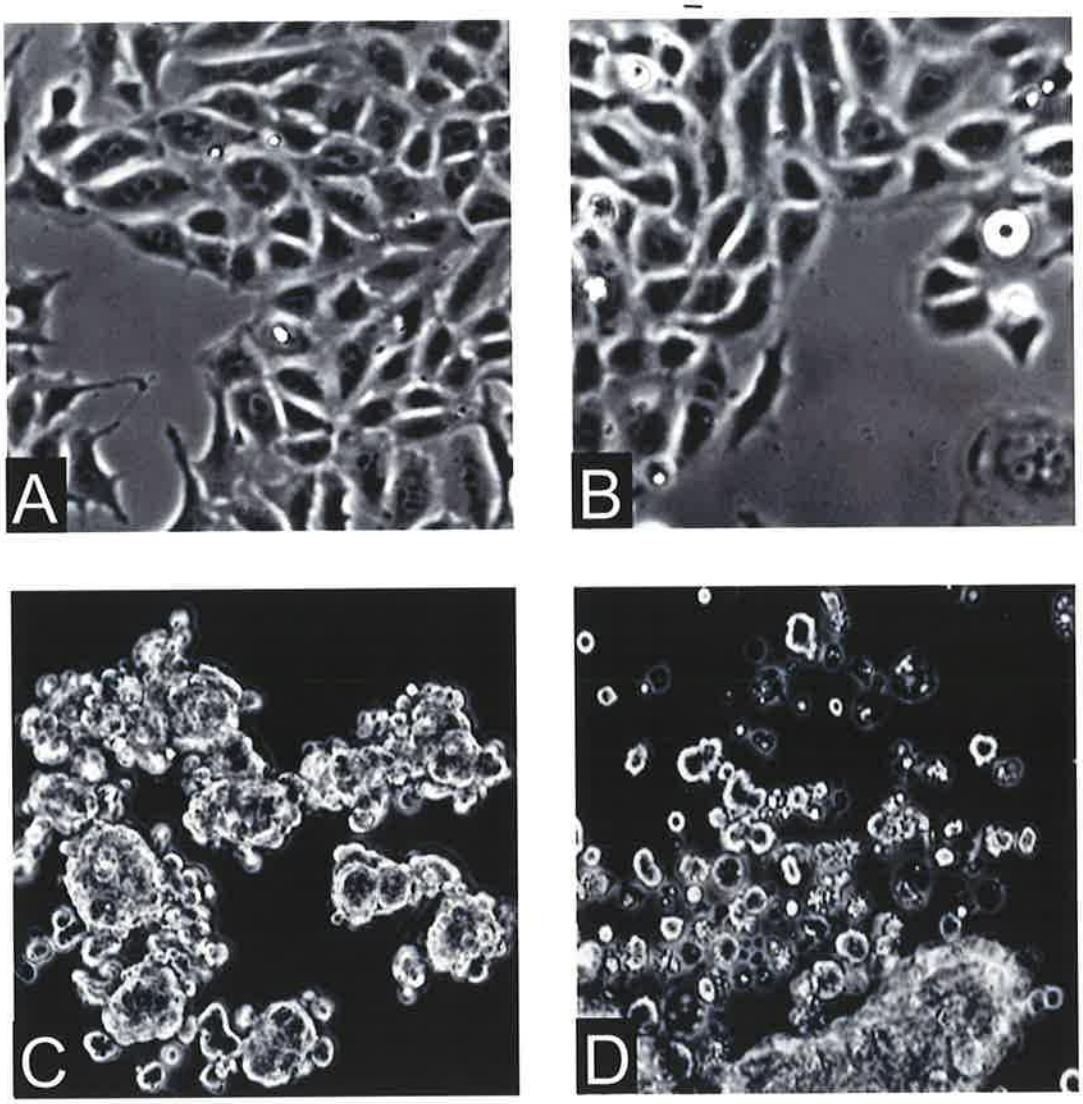


Figure 4.10 ***B.*** Comparison photographic images of HOS cells from anchored (plastic flasks) versus non-anchored (polyHEMA-coated flasks). (**A**) anchored cells treated with 100ng/ml TRAIL for 8 hours (**B**) anchored cells treated with 100ng/ml TRAIL for 12 hours; (**C**) non-anchored cells treated with with 100ng/ml TRAIL for 8 hours; (**D**) non-anchored cells treated with with 100ng/ml TRAIL for 12 hours.

4.3.6 Cell cycle analysis of ZOL treated OS cells leads to S-phase arrest whereas combination with TRAIL leads to apoptosis

Cell cycle perturbations were characterised in HOS and BTK-143 cells following co-treatment with ZOL and TRAIL, using flow cytometric analysis. TRAIL treatment alone had no effect on the cell cycle, even after 4 days of treatment (**Figure 4.11**). As described previously, ZOL leads to a cytostatic response in OS cells, with an accumulation of cells in S-phase, as early as 24-48 hours [Evdokiou *et al.* 2003]. In the present experiments, the proportion of HOS cells in S-phase decreased progressively with ZOL and TRAIL treatment, from 55, 38, 23 and 10%, compared with ZOL alone with 55, 69, 40 and 20% at 24, 48, 72 and 96 hours, respectively (**Figure 4.11**). The decrease in S phase correlated with an increase in the apoptotic sub-G_0/G_1 peak to 5, 44, 65 and 83% at 24, 48, 72 and 96 hours, respectively. Similar trends were observed in the BTK-143 cell line. By 72 hours ZOL treatment, the proportion of BTK-143 cells in the S-phase peaked at 38% compared with 26% in control cells. The proportion of BTK-143 cells in S-phase after treatment with ZOL and TRAIL decreased progressively from 30, 24, 16 and 10% at 24, 48, 72 and 96 hours, respectively (**Figure 4.11**), correlating with an increase in the apoptotic sub-G_0/G_1 peak to 12, 27, 59 and 66% at 24, 48, 72 and 96 hours, respectively. These trends were also identified in the G-292, and Saos2, but not the MG-63 and SJSA-1 cell lines, at least at these concentrations of agents (data not shown).

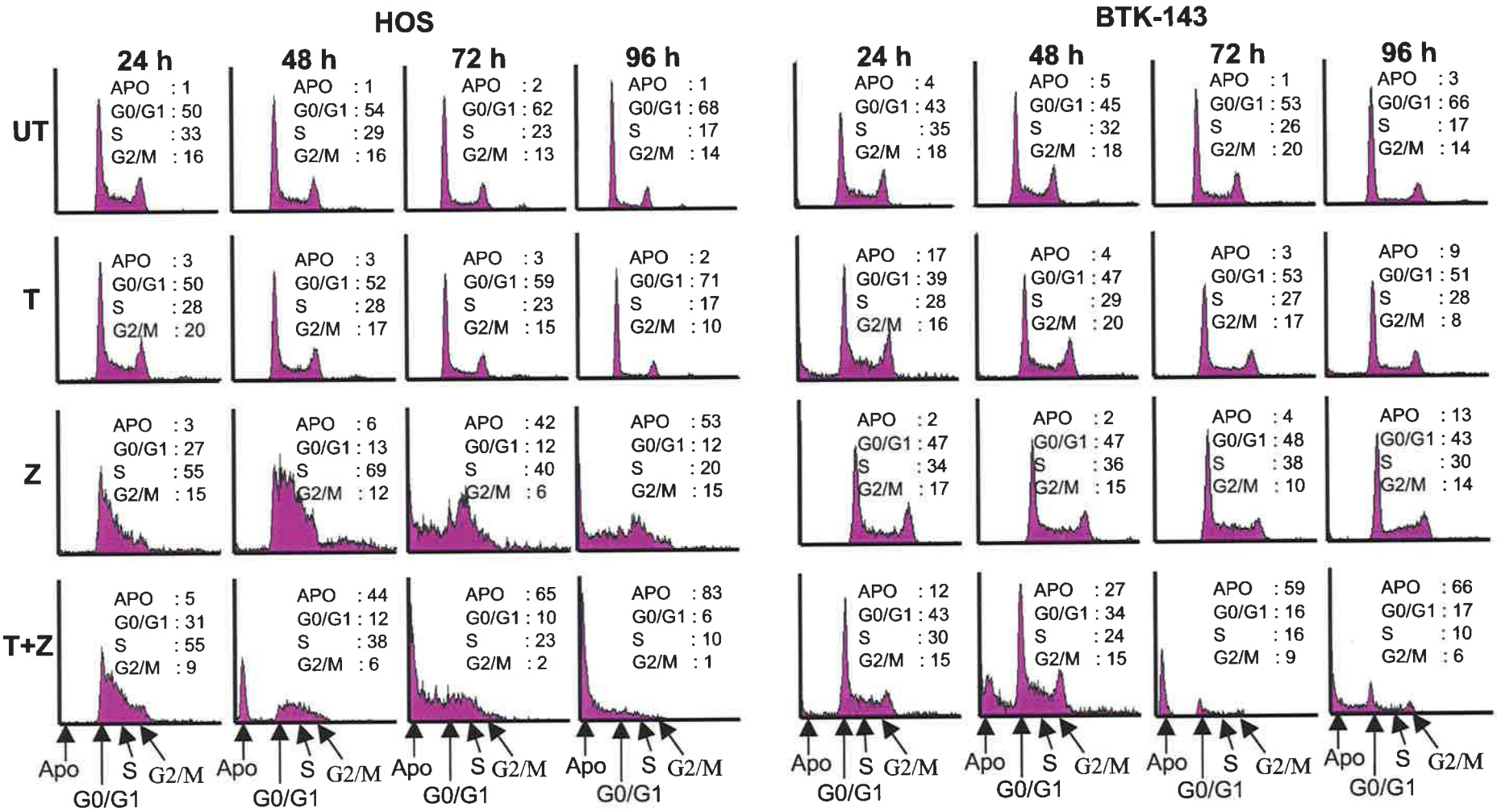


Figure 4.11 ZOL leads to S-phase arrest in OS cells whereas combination with TRAIL leads to apoptosis. HOS and BTK-143 cells were incubated with fresh media containing TRAIL (100ng/ml) in the absence (control) or presence of 25 μM ZOL for 24, 48, 72 and 96 hours. At each time point, cells were harvested, fixed and stained with propidium iodide for cell cycle analysis using the flow cytometer. The positions on the histograms of the hypodiploid Apo (sub-G_0/G_1), G_0/G1, S, G2/M peaks and the percentage of cells in each of the cycle phases, in a representative experiment, are indicated.

4.3.7 Normal human bone donor cells are not sensitised to TRAIL-induced apoptosis by ZOL

NHB cells obtained from patients at the time of joint replacement surgery were used to assess the effect of ZOL and TRAIL combination treatment. NHB cells did not exhibit augmented apoptotic effects with co-treatment of ZOL and TRAIL, even at the highest concentrations of ZOL (100μM), (**Figure 4.12**). Although there was a reduction in the overall survival of cells at the higher concentrations, cell survival still exceeded that seen in any of the human OS cell lines. Apart from the dose experiments, all other experiments conducted throughout chapter 3 and 4 used ZOL at a concentration of 25μM, at which no augmentation was observed in any of the NHB cell donors tested.

4.3.8 ZOL upregulates TRAIL cell surface death receptors

In HOS cells it was clearly evident that ZOL treatment lead to the upregulation of both DR4 and DR5 death receptors, while no changes were observed in the expression of DcR1, DcR2 and OPG decoy receptors (**Figure 4.13**). Consistent with the lack of effect of ZOL and TRAIL on NHB viability, these treatments did not affect expression of death and decoy receptors on NHB cells (**Figure 4.13**). These data suggest that ZOL may act to sensitise OS cells to TRAIL *via* upregulation of the death receptors.

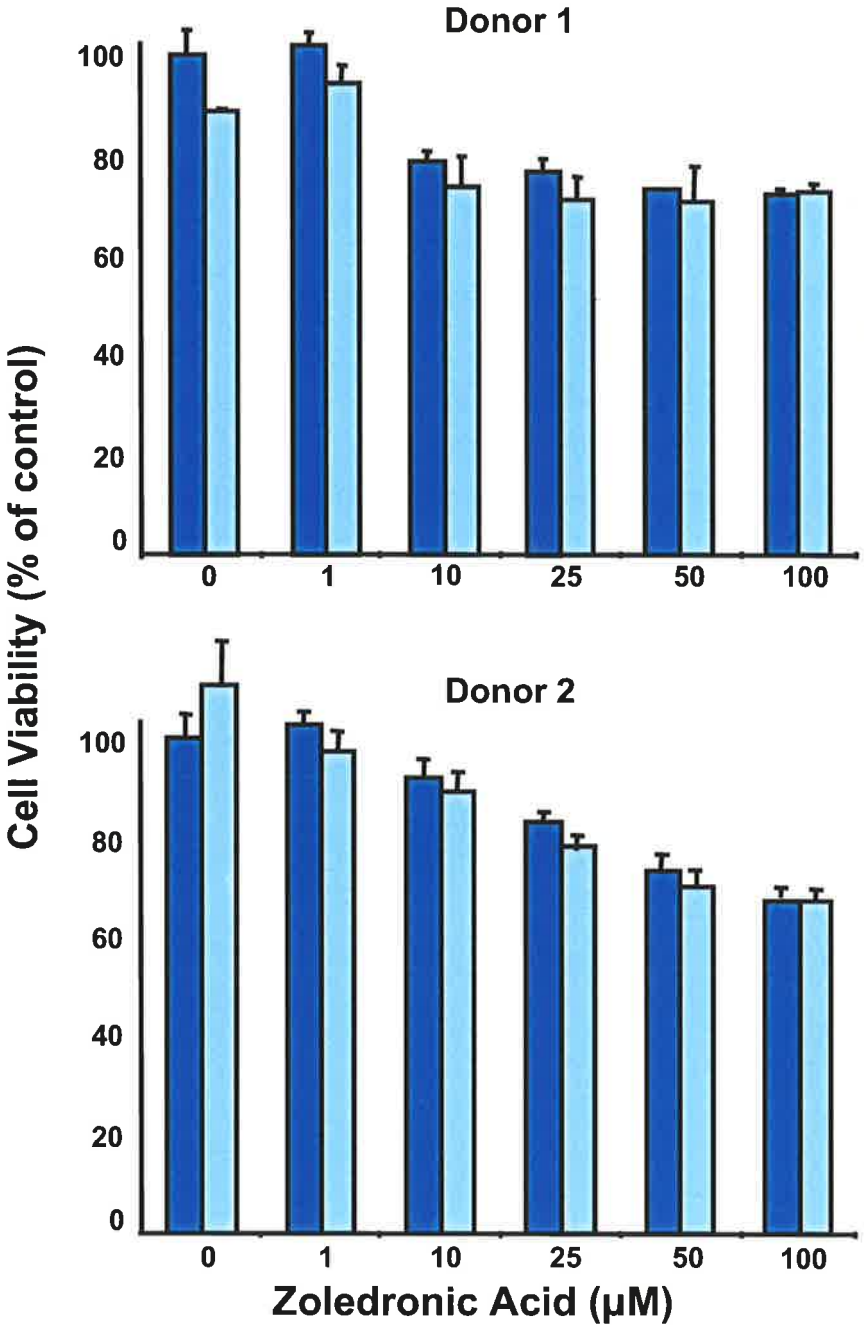


Figure 4.12 Normal human osteoblast-like cells derived from two different donors, were seeded into plates and allowed to adhere before treatment for 72 hours with increasing doses of ZOL (■) or a fixed concentration of 100ng/ml TRAIL with increasing ZOL doses (□). Cell viability was determined using the crystal violet assay, as described in the chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times: points are means ±SD.

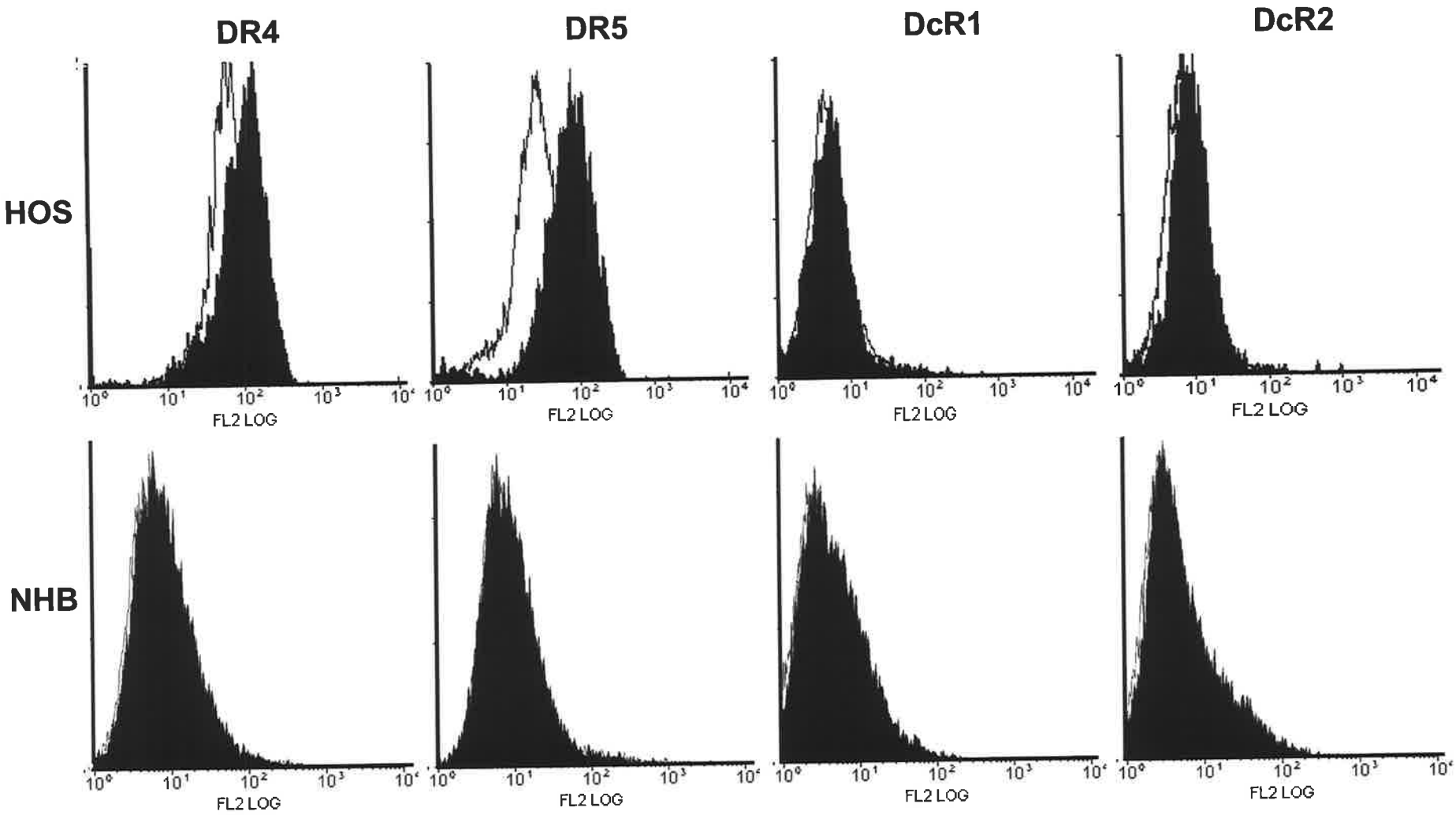


Figure 4.13 Zoledronic acid upregulates TRAIL death receptors on the cell surface of OS cells. HOS and NHB cells were left untreated or treated with 25 μM ZOL for 48 hours. Monoclonal antibodies specific for human TRAIL receptors, DR4, DR5, DcR1, DcR2 were used to analyse cell surface receptor expression in intact cells by FACS. Filled histograms represent Zol–treated cells.

4.4 DISCUSSION

In this study the combination treatment with ZOL and TRAIL led to an augmented apoptotic effect in OS cell lines but not in normal human bone cells (NHB). Combination treatment with ZOL and TRAIL resulted in varied degrees of augmented cell death, which reflected the sensitivity of cells to ZOL treatment alone. While combination treatment led to a greater apoptotic effect than either agent alone, the duration of this effect was not notably swifter than ZOL alone, suggesting the depletion of geranylgeranylated proteins as a crucial factor in sensitisation to TRAIL. Flow cytometric analysis of cells given the combination treatment demonstrated a time-related decrease in the proportion of cells in the apoptotic sub-G0/G1 peak of the cell cycle. Unlike treatment with ZOL alone, combination treatment did not cause S-phase arrest, but instead led to direct apoptosis. It remains unclear why there is variation in the response to ZOL between cell lines but may be due to differences in bioavailability, cellular uptake (although the mode of transport has yet to be identified) and intracellular differences such as altered FPP synthase expression [Green 2003; Salomo *et al.* 2003]. Nonetheless, although these differences exist, it is evident that some cell lines, which are normally resistant to TRAIL induced cell death, are strongly sensitised by the co-addition of ZOL.

Morphological changes characteristic of apoptosis, including chromatin condensation, nuclear fragmentation and the formation of dense rounded apoptotic bodies, were clearly evident in OS cells stained with the nuclear fluorescent stain, DAPI, following ZOL and TRAIL treatment. Apoptosis, confirmed by agarose gel electrophoresis, showed that combination treatment also resulted in an increase in the level of intranucleosomal genomic DNA fragmentation, a key feature of apoptosis that arises from activation of endogenous endonucleases. PARP cleavage was detected as an early specific marker of apoptosis. Further investigation of caspase activation revealed a

higher level of caspase activity in the combination treated cells than cells treated with ZOL alone, regardless of ZOL concentration and cell type. This was not surprising as TRAIL acts *via* the extrinsic and intrinsic pathway to directly induce apoptosis *via* caspase activation. The amount of caspase activity between the single and dual treatments was greater in HOS than Saos2 cells but this may reflect the sensitivity differences to ZOL, which were observed between the two cell lines. This may also be the reason for the early onset of caspase activation in the combination treated cells seen at 48 hours in HOS cells, compared to 72 hours in Saos2 cells. The addition of the caspase inhibitor zVAD-fmk failed to protect the cells from ZOL-induced cell loss, as described previously [Evdokiou *et al.* 2003]. The presence caspase inhibitor prevented the augmented apoptotic effect in cells treated with the combination of ZOL and TRAIL and the degree of cell death was equal to that observed with ZOL treatment alone. The prevention of cell attachment and the subsequent exposure to TRAIL resulted in considerably more cell death than attached cells exposed to TRAIL. This suggests that cell detachment, is a potential mechanism for the sensitisation of OS cell lines by ZOL and supports the importance of prenylation of proteins required for cell attachment and integrity [Zhang and Casey 1996; Ridley and Hall 1992].

The use of intermediates of the mevalonate pathway such as geranylgeraniol (GGO) to reverse the effects of ZOL was shown previously [Reszka *et al.* 1999] and when administered to cells with combination treatment it prevented the augmented apoptotic effect and prevented ZOL–induced cell loss, again confirming that ZOL sensitises cells to the effects of TRAIL. This was further confirmed by the presence of unprenylated Rap 1A protein found only in ZOL treated cell lines. No changes were observed in the levels of Bcl-2 and Bax protein between treatments of HOS cells. This conflicts previous reports of down-regulation of the Bcl-2 protein with ZOL treatment [Oades *et al.* 2003; Senaratne *et al.* 2000]. Changes in other Bcl-2 family proteins may

be involved and further investigation is needed to better understand the involvement of the intrinsic pathway during ZOL-induced apoptosis. Other changes observed in ZOL treated cells was the upregulation of the cell surface receptors DR4 and DR5, commonly referred to as death receptors, due to their ability to elicit apoptosis *via* caspase activation. No change in receptor expression was observed in normal cells. It is possible that a depletion of prenylated proteins would lead to cell stress and upregulation of death receptors at the cell surface, which may be one mechanism, by which ZOL sensitises OS cells to TRAIL.

The use of ZOL in the treatment of OS has exciting potential however it may be unrealistic to believe that high enough concentrations can be achieved *in vivo,* despite suggestions that there is an elevated localised concentration in the bone microenvironment due to the high affinity of ZOL for bone, compared to serum levels. The use of combination treatment with synergistic or additive activity however, reduces the concentration of ZOL required to achieve significant apoptosis, improving the chances of attaining sufficient concentration levels *in vivo* with reduced toxic side effects. [Sato *et al.* 1991].

CHAPTER FIVE

OTHER INHIBITORS OF THE MEVALONATE PATHWAY: STATINS AND PRENYL TRANSFERASE INHIBITORS INDUCE APOPTOSIS IN OSTEOSARCOMA CELLS– PRELIMINARY STUDIES.

Are there effective alternatives to Zoledronic Acid?

5.1 INTRODUCTION

BPs have a high affinity for the hydroxyapatite mineral in bone and are selectively taken up and adsorbed to mineral surfaces at sites of increased bone turnover where they exert their effects. The statins and prenyl transferase inhibitors (PTIs), like the N-BPs, act by targeting an enzyme of the mevalonate pathway. However, unlike N-BPs, which preferentially localise to the bone, both the statins and PTIs are systemically distributed *in vivo*. This makes them very appealing as alternative agents to ZOL not only for tumours localised to the bone, but also for soft tissue tumours.

Much like N-BPs, agents such as statins and prenyl transferase inhibitors (PTIs), also hinder the progress of the mevalonate pathway by inhibiting key enzymes. Statins, principally used for the treatment of hypercholesterolemia, inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is required for cholesterol biosynthesis [Goldstein and Brown 1990]. The PTIs inhibit the enzymes farnesyl transferase and geranylgeranyl transferase and thus prevent farnesylation and geranylgeranylation, respectively. Both the statins and the PTIs have similar effects as N-BPs, such as anti-proliferation and apoptosis in human cancer cells [Newman *et al.* 1994; Wong *et al.* 2002; Ashar *et al.* 2001; Bolick *et al.* 2003; Morgan *et al.* 2003; van de Donk *et al.* 2003; Vogt *et al.* 2004].

N-BPs, statins and PTI have all been shown to exhibit anti-cancer properties that are amplified in combination with chemotherapeutic agents [Sun *et al.* 1999; Feleszko *et al.* 2002; Feleszko *et al.* 1999; Jagdev *et al.* 2001; Tassone *et al.* 2003]. The high affinity of ZOL for bone makes it potentially useful for the treatment of OS because its bioavailability may be greater in proximity to bone than in other tissues, whereas the use of statins and PTIs may be more useful in OS metastases and other soft tissue sarcomas. More importantly, like ZOL, clinical trials using statins and FTIs have shown these to be well tolerated and safe, with little overt toxicity [Thibault *et al.* 1996; Larner

et al. 1998; Brunner *et al.* 2003; Karp *et al.* 2001; Gotlib *et al.* 2002]. These characteristics make them worth considering for cancer therapy.

In this chapter the effect of statins and PTIs in combination with TRAIL was investigated. Such an outcome would bring the hope of achieving substantial apoptotic response both in this tumour type and soft tissue tumours, without the toxic side effects of current therapies.

5.2.1 MATERIALS AND METHODS

5.2.1 Cells and cell culture

The osteosarcoma cell lines and the normal human bone donors used in this chapter are listed in section 2.1.1. The methods for cell preparation and propagation are described in section 2.1.2. and 2.1.3.

5.2.2 Measuring cell viability

The method used to assess cell viability is described in section 2.4.2. and 2.4.3.

5.2.3 Measuring caspase activity

The caspase inhibitor used is listed in section 2.2.4. Caspase activity protocols used in this chapter are described in section 2.4.4.

5.2.4 Western blot analysis

The antibodies used in the western blots are listed in section 2.2.5. The method used is described in section 2.4.6. and 2.4.9.

5.2.5 Statistical analysis

Microsoft excel was used to obtain mean standard deviations of experimental data. Data shown in each case are from a representative experiment, which was repeated at least three times.

5.3 RESULTS

5.3.1 Statins induce dose-dependent apoptosis

The cytotoxic effects of the statins, Lovostatin (LOV) and Mevastatin (MEV), on cell proliferation were tested against a panel of six human OS cell lines (HOS, BTK-143, G-292, Saos2, SJSA-1, MG-63). Cells were treated for 72 hours with increasing concentrations of LOV or MEV at 0, 1, 10, 25, 50 μM. Treatment with LOV or MEV dose-dependently resulted in fewer cells, compared with untreated control cultures, in all six OS cell lines, (**Figure 5.1** and **5.2**). LOV was most potent in the G-292, BTK-143, Saos2 and HOS cells, with IC_{50}'s of 6 μM, 7 μM, 15 μM, and 18 μM, respectively. In contrast, there was a weaker response to LOV in the MG-63 and SJSA-1 cell lines, with IC_{50}'s of 22 μM and 52 μM, respectively. The sensitivity to LOV was reflected in the MEV treatments, with the most sensitive cell lines being G-292, BTK-143, Saos2 and HOS cells, with IC_{50}'s of 8 μM, 10 μM, 20 μM, and 32 μM respectively. The more resistant cell lines were MG-63 and SJSA-1, with IC_{50}'s of 55 μM and 100 μM, respectively.

As observed with ZOL in chapter 3 and 4, differential sensitivity between cell lines was also observed with statin treatment. Furthermore, the same relative order of sensitivity was preserved for the two types of agents. Comparison of **Figure 3.1** (showing ZOL dose-response) and **Figure 5.1** and **5.2** (statin dose-response) shows a similar pattern of responsiveness between treatments of the same cell line.

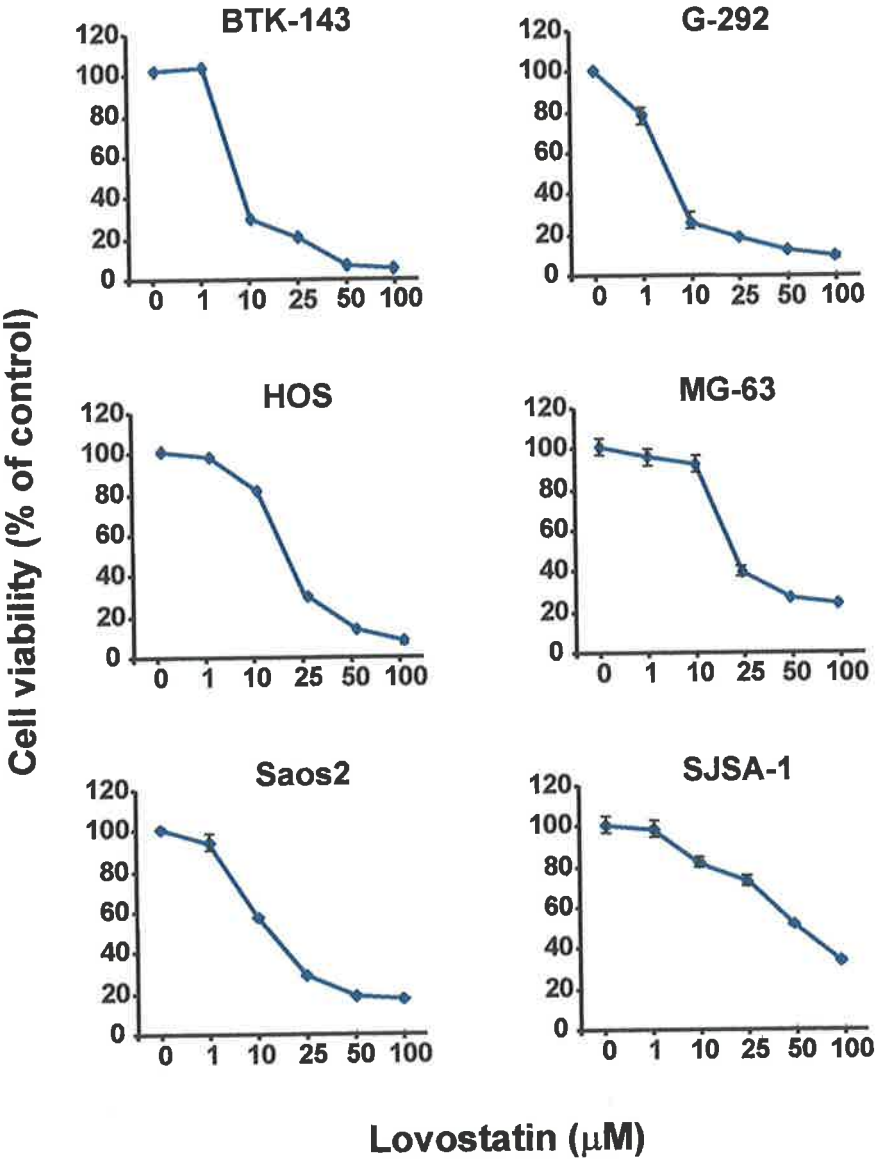


Figure 5.1 The effect of Lovostatin on OS cells. Cells were seeded at 1 x 10^4 cells per well in 96 well microtiter plates and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of LOV (1-100μM). Cell viability was determined using crystal violet staining, as described in the chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times: points are means ±SD.

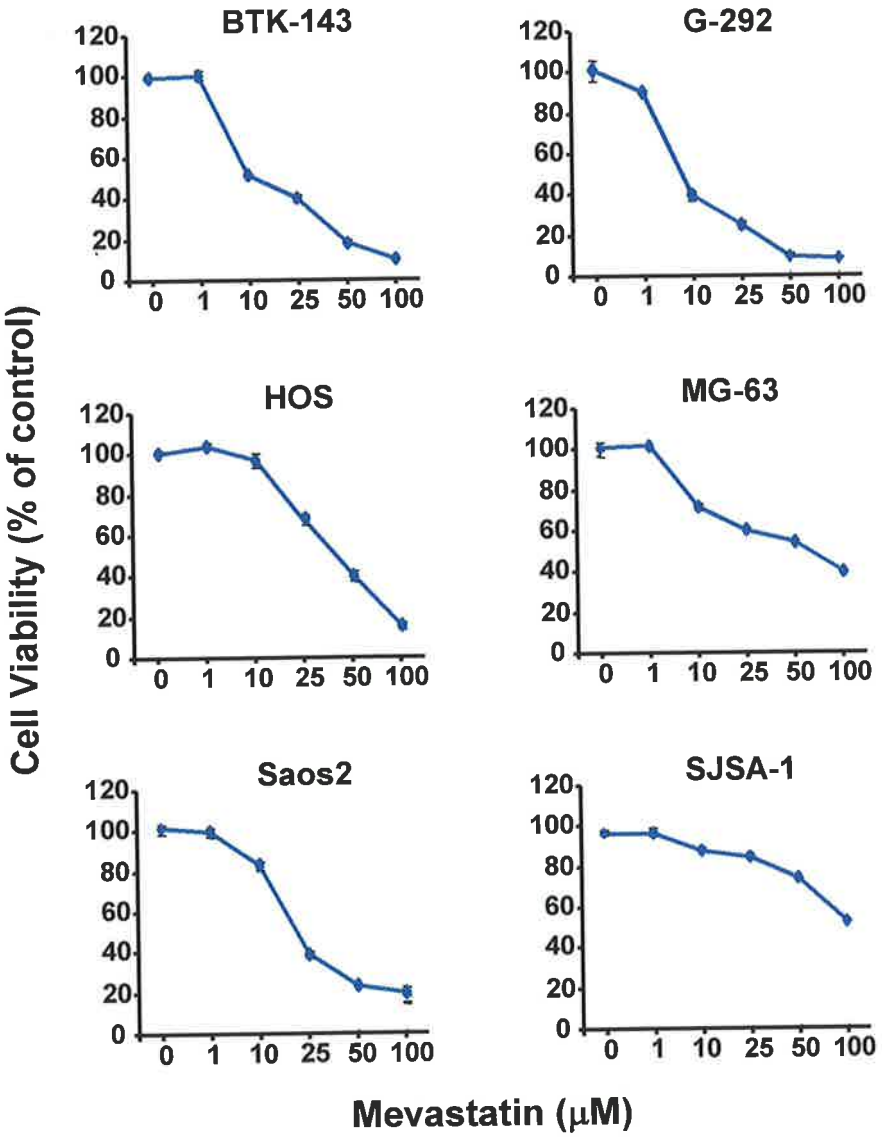


Figure 5.2 The effect of Mevastatin on OS cells. Cells were seeded at 1 x 10^4 cells per well in 96 well microtiter plates and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of MEV (1-100μM). Cell viability was determined using crystal violet staining, as described in chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times: points are means ±SD.

5.3.2 Statins augment TRAIL-induced apoptosis in a dose-dependent manner

Experiments were performed to determine whether the augmented apoptotic response observed previously with combination of ZOL and TRAIL treatment (chapter 4), could also be seen with TRAIL in combination with the statins. HOS, BTK-143, G-292, Saos2, SJSA-1 and MG-63 OS cells were exposed to increasing concentrations of LOV or MEV (1-50 μM) in the absence or presence of TRAIL. As described for ZOL in chapter 4, the concurrent treatment of LOV or MEV plus TRAIL for 72 hours induced more apoptosis than single drug alone, in all OS cell lines, although to varying degrees (**Figure 5.3** and **5.4**).

Lovostatin and TRAIL combination treatment reduced the IC_{50} in all the OS cell lines when compared to Lovastatin alone, as shown in **Table 5.1**. In the G-292, BTK-143, Saos2, HOS, MG-63 and SJSA-1 cell lines respectively, up to 8, 19, 12, 25, 18 and 15% greater cell death respectively, was observed. Likewise MEV and TRAIL also reduced the in all the cell lines with G-292, BTK-143, Saos2, HOS, MG-63 and SJSA-1 cell lines, resulting in up to 14, 22, 26, 40, 15 and 8% increased cell death respectively (**Table 5.1**).

Cell morphology prior to cell death in the statin treated cells resembled that seen in ZOL treated cells, with loss of cell attachment a common factor. The time taken for apoptosis to occur was also similar, with most cell death occurring at 48-72 hours (data not shown).

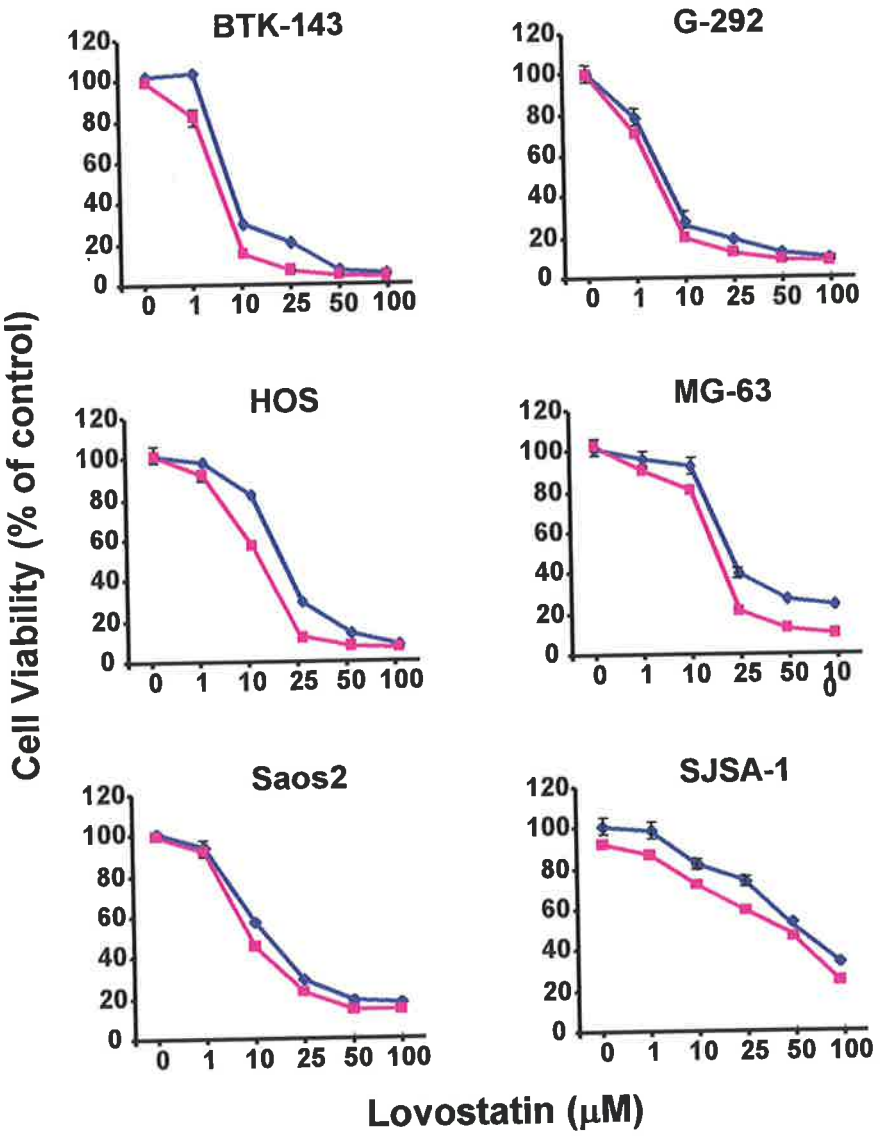


Figure 5.3 The effects of Lovostatin and TRAIL combination treatment. OS cells were seeded at 1 x 10^4 cells per well in 96 well microtiter plates and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of LOV 1-100μM, with (■) and without (●) 100ng/ml TRAIL. Cell viability was determined using crystal violet staining, as described in the chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times: points are means ±SD.

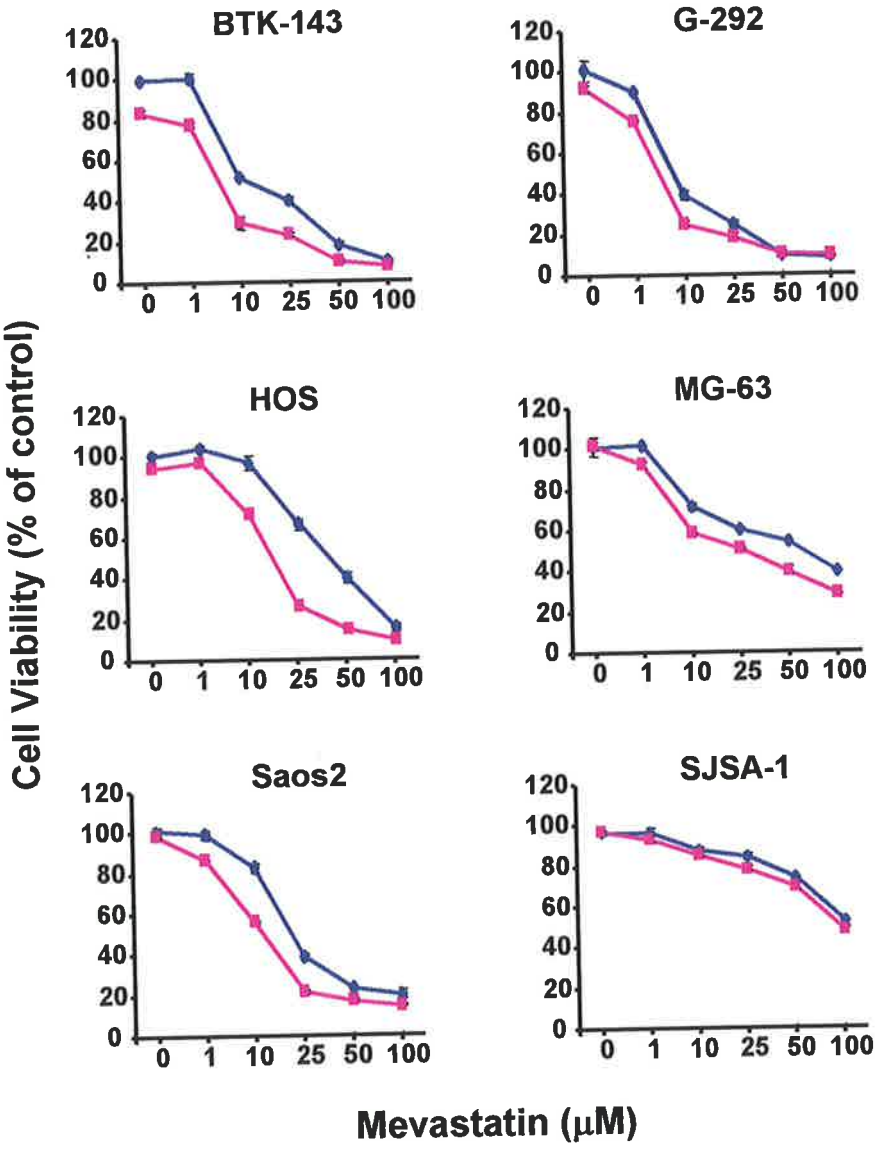


Figure 5.4 The effect of Mevastatin and TRAIL combination treatment in OS cells. Cells were seeded at 1 x 10^4 cells per well in 96 well microtiter plates and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of MEV 1-100μM, (■) with and without (●) 100ng/ml TRAIL. Cell viability was determined using crystal violet staining, as described in t.he chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times: points are means ±SD.

	IC50 MEV	IC50 MEV+T	% Increase in cell death	IC50 LOV	IC50 LOV+T	% Increase in cell death
BTK-143	10	5	22% at 10μM	7	4	19% at 1μM
G-292	8	5	14% at 10μM	6	4	8 % at 1μM
HOS	32	16	40% at 25μM	18	13	25% at 10μM
MG-63	55	25	15% at 50μM	22	16	18% at 25μM
Saos2	20	13	26% at 10μM	15	9	12% at 10μM
SJSA-1	100	90	8% at 25μM	51	41	15% at 25μM

Table 5.1 The IC_{50} was determined for all OS cell lines either with Mevastatin (MEV) or Lovostatin (LOV) treatment alone or in combination with TRAIL. The maximum percent increase in cell death observed in the combination treatment is also shown for each cell line. IC_{50} values are represented in μM.

5.3.2 Prenyl transferase inhibitors induce dose-dependent apoptosis

To investigate the effect of PTIs on OS cells, HOS and BTK-143 cells were seeded into plates and allowed to adhere prior to the addition of fresh media containing increasing concentrations (1-50 μM) of FTI and GGTI (see section 2.2.2 for details of PTIs used). These cells were shown to respond well to both ZOL and statins, and for this reason were chosen for the experiments described in this chapter. A dose-dependent decrease in cell viability was observed in both the FTI and GGTI treated cells, as shown in **Figures 5.5** and **5.6**. The IC_{50}'s of HOS and BTK-143 cells were 50 and 20 μM, respectively, with FTI treatment, and 20 and 23 μM, respectively, with GGTI treatment. The onset of cell death was similar to that observed with ZOL and statin treatment, as was the appearance of the cells prior to apoptosis.

5.3.4 Prenyl transferase inhibitors augment TRAIL-induced apoptosis in a dose-dependent manner

The addition of TRAIL to GGTI and FTI treated cells was investigated in HOS and BTK-143 cell lines and compared to single treatments to determine if combination treatment leads to an augmented apoptotic effect. Cells were treated for 72 hours with increasing concentrations of FTI and GGTI (1-50 μM), in the presence or absence of TRAIL. Both FTI and GGTI in combination with TRAIL led to augmented cell death compared with single treatment alone, as shown in **Figures 5.7** and **5.8**. HOS cells showed up to 23% greater cell death in the combination treatments with TRAIL and both FTI and GGTI. An increase of 13% and 40%, respectively, greater cell death was observed in the BTK-143 cells in the combination treatments with FTI and GGTI.

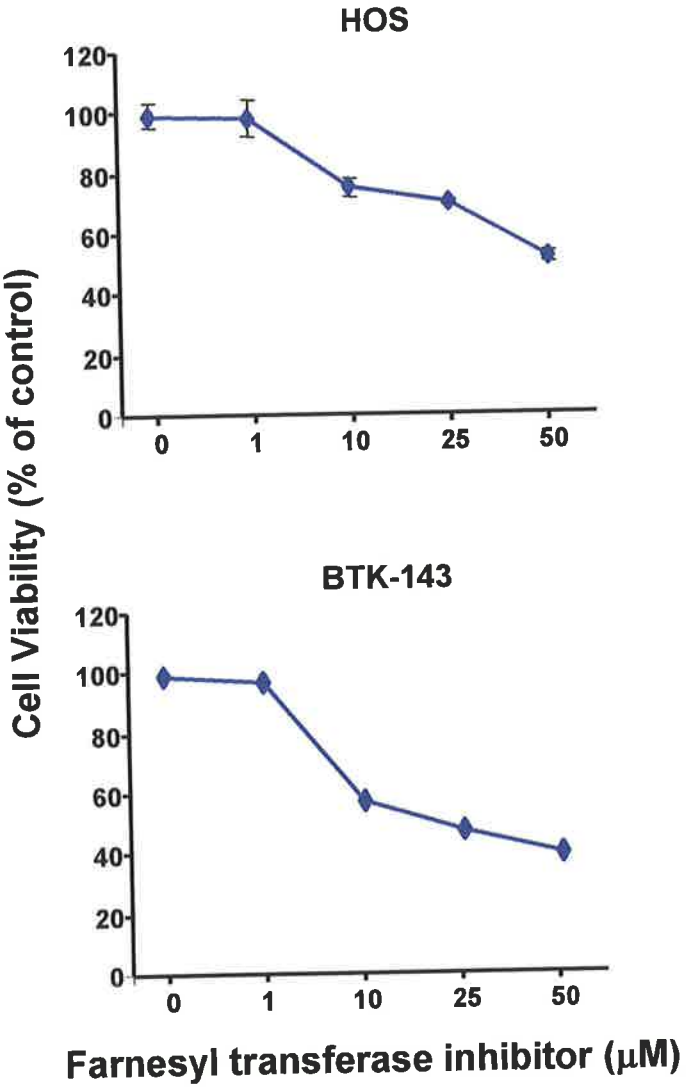


Figure 5.5 The effect of Farnesyl transferase inhibitor on viability of OS cells. HOS and BTK-143 cells were seeded at 1 x 10^4 cells per well in 96 well microtiter plates and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of FTI (1-50μM). Cell viability was determined using crystal violet staining, as described in the chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times. Results are expressed as % of control: points are means ±SD.

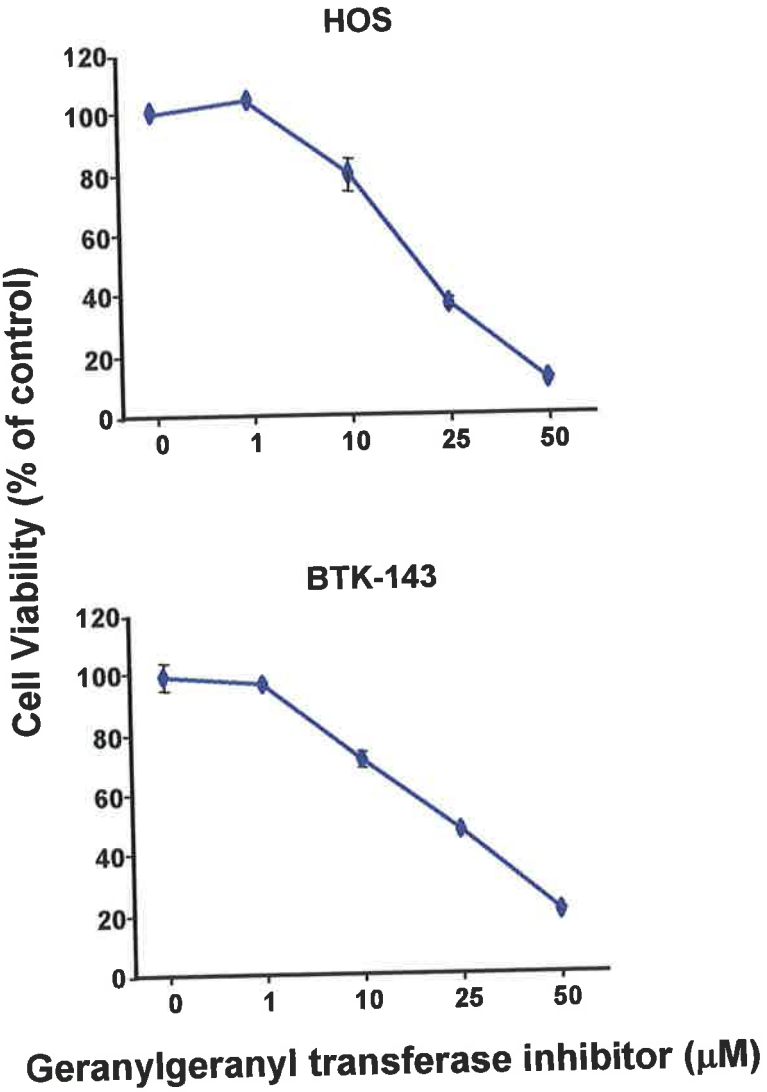


Figure 5.6 The effect of Geranylgeranyl transferase inhibitor on viability of OS cells. HOS and BTK-143 cells were seeded at 1 x 10^4 cells per well and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of GGTI (1-50μM). Cell viability was determined using crystal violet staining, as described in chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times. Results are expressed as % of control: points are means ±SD.

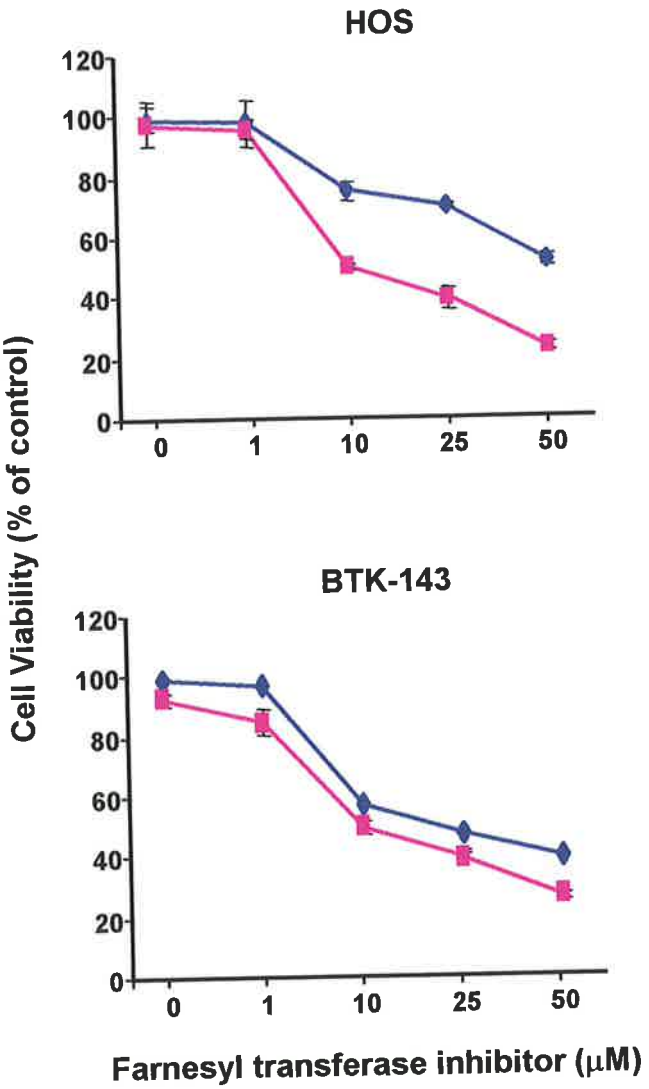


Figure 5.7 The effect of Farnesyl transferase inhibitor and TRAIL in combination. HOS and BTK-143 cells were seeded at 1 x 10^4 cells per well in 96 well microtiter plates and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of FTI 1-50μM, with (■) and without (●) 100ng/ml TRAIL. Cell viability was determined using crystal violet staining, as described in chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times. Results are expressed as % of control: points are means ±SD.

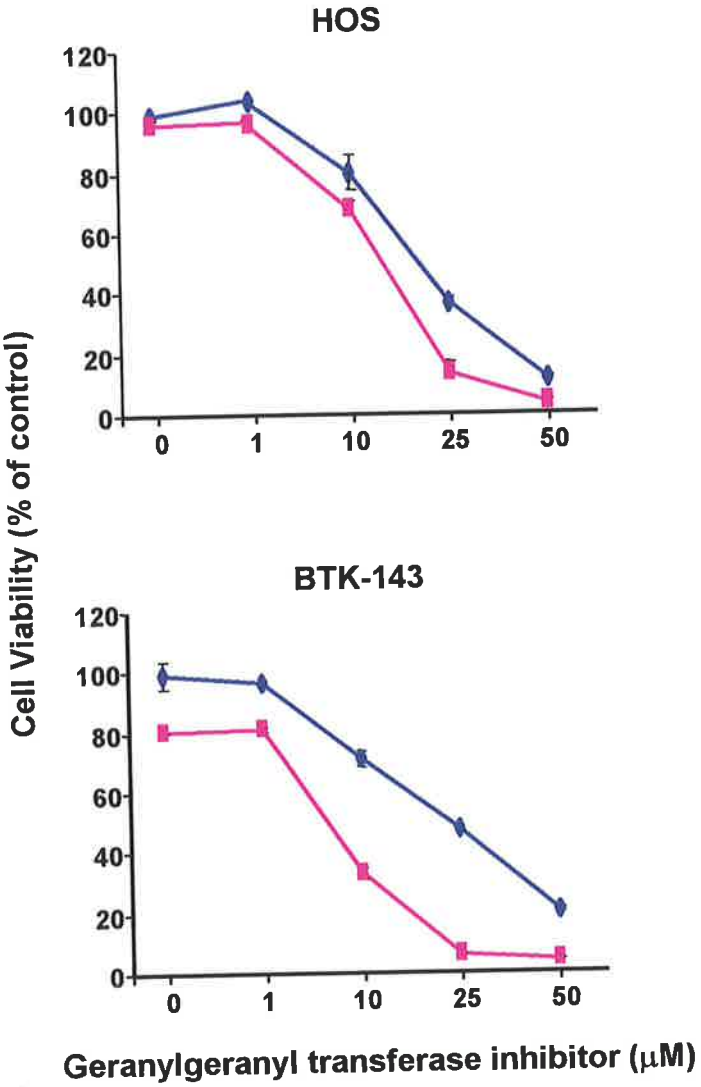


Figure 5.8 The effect of Geranylgeranyl transferase inhibitor and TRAIL combination on OS cells. HOS and BTK-143 cells were seeded at 1 x 10^4 cells per well and allowed to adhere. Cells were then incubated for 72 hours with fresh media containing increasing concentrations of GGTI 1-50μM, with (■) and without (●) 100ng/ml TRAIL. Cell viability was determined using crystal violet staining, as described in chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times. Results are expressed as % of control: points are means ±SD.

5.3.5 Normal human bone donor cells are not sensitised to TRAIL-induced apoptosis by statins or prenyl transferase inhibitors

Experiments were performed to determine whether normal bone cells succumb to the effects of statins and PTIs either with or without TRAIL much like the OS cells, or whether they behave in a similar pattern to ZOL-treated NHB cells, that is, they are not sensitised to the combination treatments. Osteoblast-like cells derived from two donors were used to assess the effect of combination treatments of TRAIL with either statins or PTIs. The addition of either 25 μM LOV, MEV, GGTI or FTI, with 100ng/ml TRAIL, to normal human bone (NHB) cells failed to exhibit augmented death in cells from either donor, for any of the combination treatments (shown in **Figure 5.9**). Therefore, NHB cells were not sensitised to TRAIL-induced apoptosis by statins or PTIs as were most of the OS cell lines. Although there was a slight reduction in the overall survival of cells, probably due to a small degree of inhibition to the mevalonate pathway, cell survival still exceeded that seen in any of the human OS cell lines.

5.3.6 Caspase inhibitors do not protect against apoptosis induced by inhibitors of the mevalonate pathway

The effect of the caspase inhibitor, z-VAD-fmk, on the induction of apoptosis in cells treated with a combination of statins or PTIs and TRAIL was examined. HOS cells were treated with the statins LOV or MEV, TRAIL and z-VAD-fmk, either alone or in combination. Addition of the caspase inhibitor z-VAD-fmk failed to protect these cells from statin-induced cell loss (**Figure 5.10A** and **5.10B**). However, the co-addition of the caspase inhibitor with TRAIL did prevent the augmented apoptotic effect, resulting in cell loss equal to the level induced by statin alone. Similar results were also observed

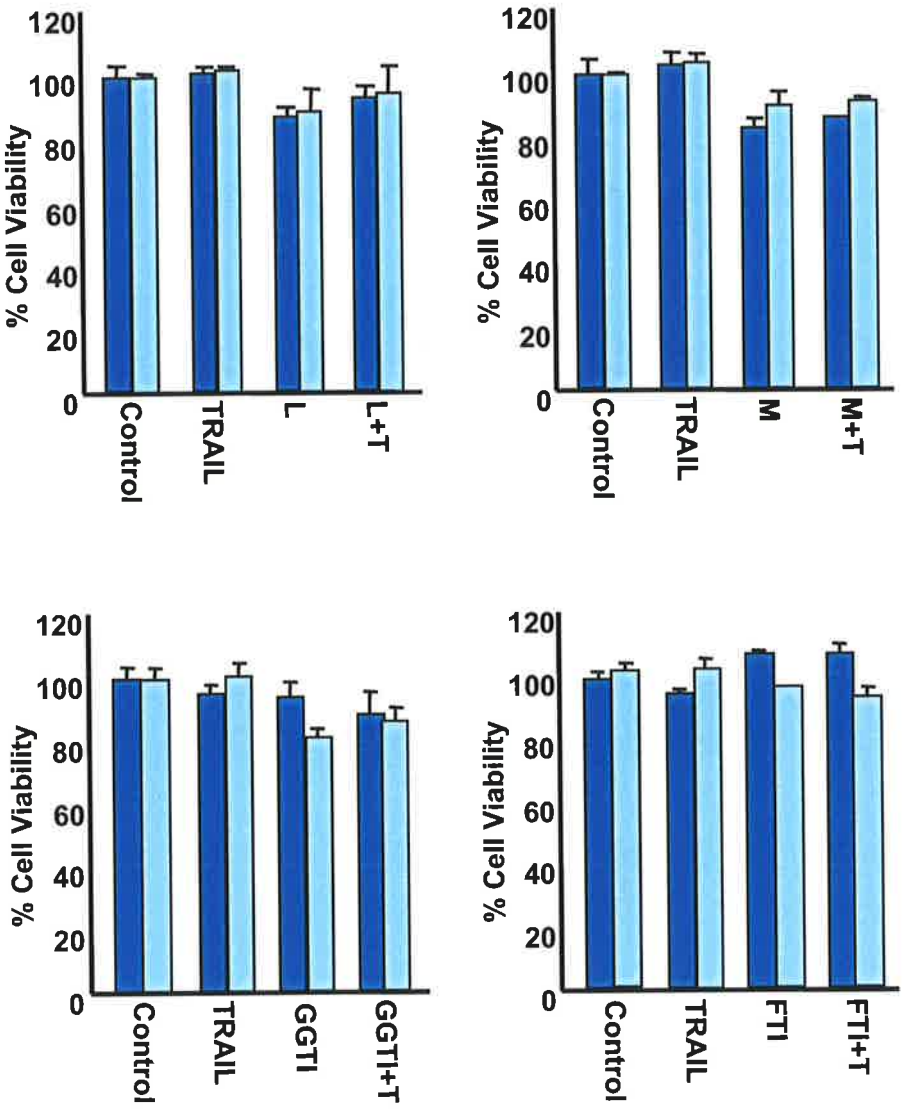


Figure 5.9 The inhibitors of the mevalonate pathway do not affect the viability of NHB cells. Cells from two donors (■) and (□)were seeded into plates and allowed to adhere before treatment for 72 hours with 25μM LOV, MEV, GGTI or FTI and a fixed concentration of 100ng/ml TRAIL. Cell viability was determined using the crystal violet assay and expressed as % of control, as described in chapter 2. Data shown in each case are from a representative experiment, which was repeated at least three times: points are means ±SD.

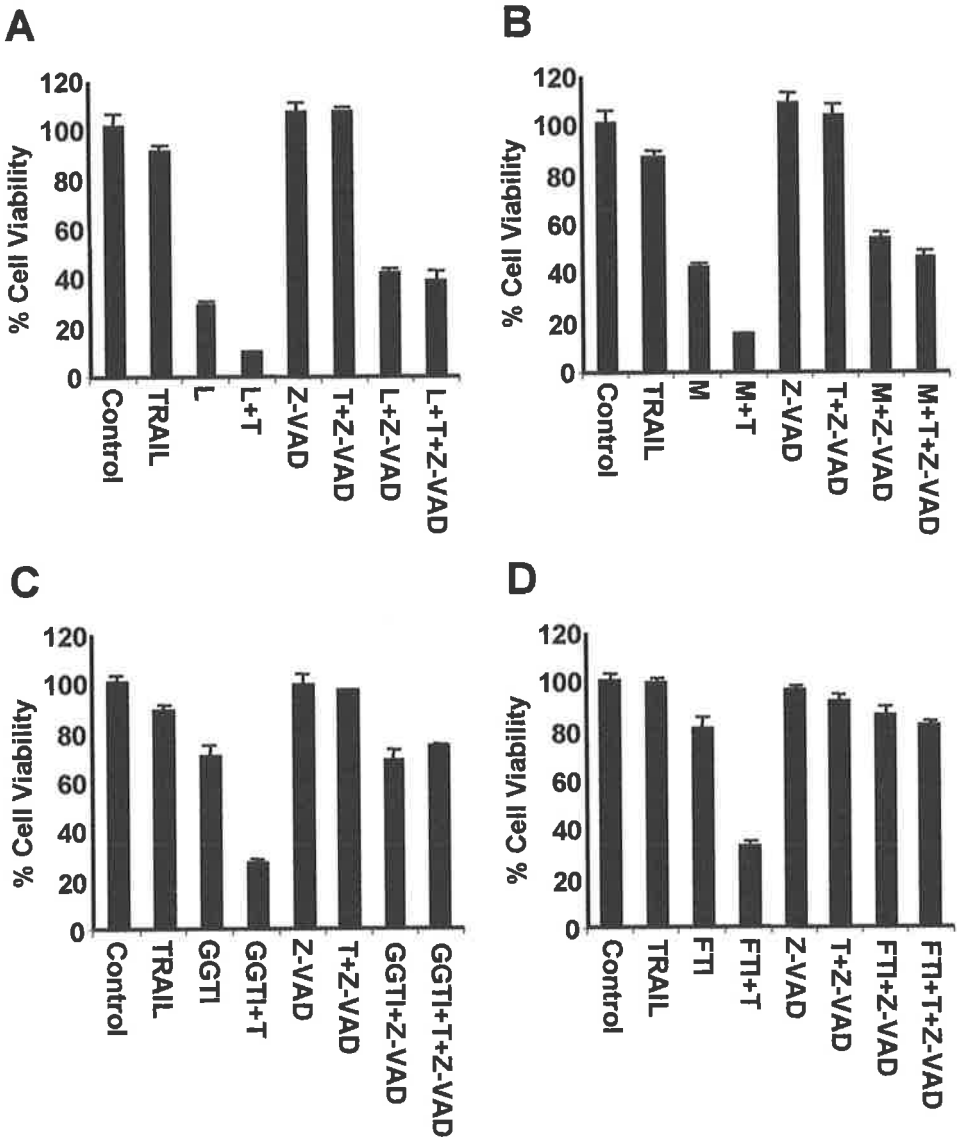


Figure 5.10 Caspase inhibitors do not protect against apoptosis induced by inhibitors of the mevalonate pathway. HOS cells were treated for 72 hours with (**A**) 10μM LOV, (**B**) 10μM MEV, (**C**) 10μM GGTI, or (**D**) 10μM FTI, 100ng/ml TRAIL, and the broad specificity caspase inhibitors z-VAD-fmk (50 μM), either alone or in combination. Cell viability was determined using the crystal violet assay and cell viability is expressed as % of control. Data points show means of triplicate results from a representative experiment, which was repeated at least twice; *bars* ±SD.

using GGTI or FTI with TRAIL and z-VAD-fmk (**Figure 5.10C** and **5.10D**). This supports the previous suggestion that statins and PTIs, like ZOL, may sensitise OS cells to the effects of TRAIL *via* activation of the caspase cascade and that the addition of the caspase inhibitor z-VAD-fmk protects against this sensitisation by irreversibly inhibiting caspase-3 activity.

5.3.7 Geranylgeraniol is able to rescue cells from the effects of statins but not prenyl transferase inhibitors

Geranylgeraniol was added to combination treatments of statins or PTIs and TRAIL, to examine its effect on the induction of apoptosis. HOS cells were treated with GGO, TRAIL, or together with LOV, MEV, GGTI or FTI either alone or in combination. The addition of GGO to the statin treated cells was able to protect the cells from statin-induced apoptosis, as shown in **Figure 5.11A** and **5.11B**. Combination treatment with LOV, TRAIL and GGO led to a 60% increase in cell viability, when compared to the corresponding LOV and TRAIL treated cells. Similarly MEV, TRAIL and GGO treated cells showed 45% improvement when compared to MEV and TRAIL treated cells.

In contrast to the N-BPs and statins, GGO did not protect the cells from PTI-induced apoptosis. This was also observed in the combination of GGO, PTI, and TRAIL treated cells, with cell death equal to that observed with PTI and TRAIL alone (**Figure 5.11C** and **5.11D**). This suggests that GGO is unable to act as an intermediate of the mevalonate pathway and therefore unable to rescue the cells from apoptosis. This will be discussed further in section 5.4.

Western blot analysis revealed PARP cleavage in all combination IMP and TRAIL treated cell lysates (**Figure 5.12**). At 48 hours however, there was no PARP

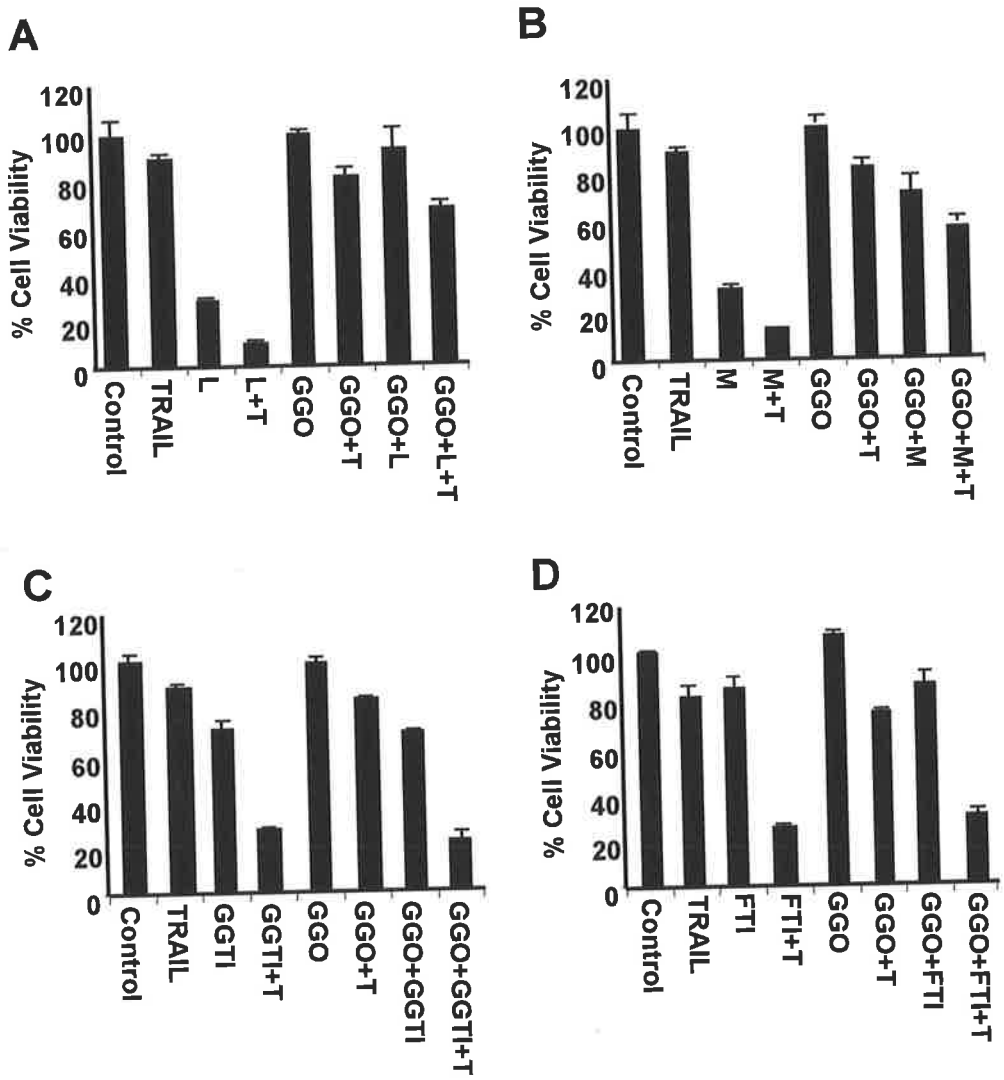


Figure 5.11 Geranylgeraniol (GGO) is able to rescue cells from the effects of statins but not prenyl transferase inhibitors. HOS cells were treated for 72 hours with (**A**) 10μM LOV, (**B**) 10μM MEV, (**C**) 10μM GGTI, or (**D**) 10μM FTI, 100ng/ml TRAIL, and 300 μM GGO, either alone or in combination. Cell viability was determined using the crystal violet assay and cell viability is expressed as % of control. Data points show means of triplicate results from a representative experiment, which was repeated at least twice; *bars* ±SD.

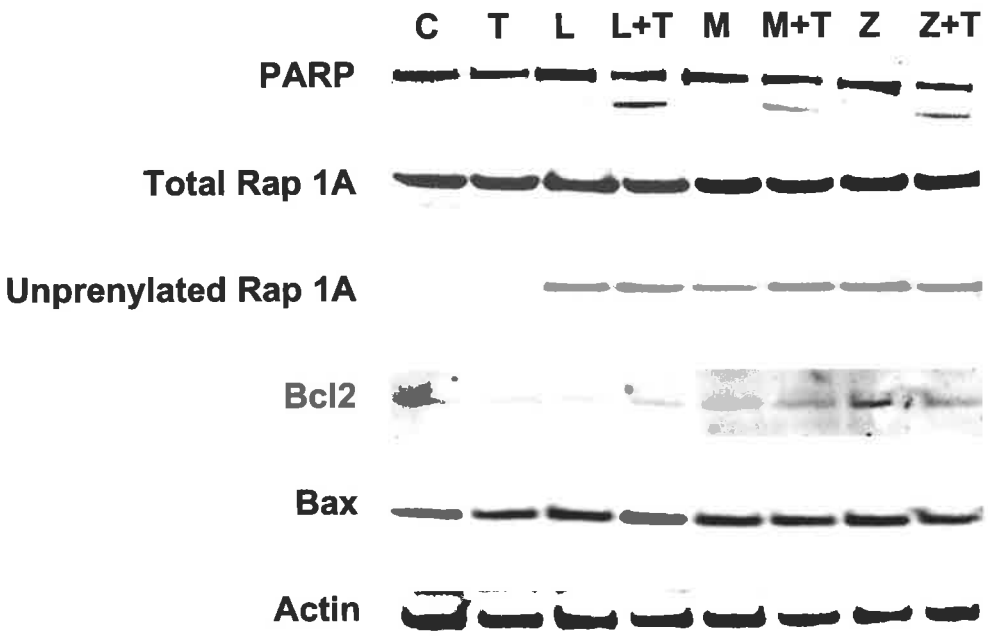


Figure 5.12 Western blot analysis of HOS cells after 48 hours treatment showing *(i)* increase in PARP cleavage when TRAIL (T) is combined with 25 μM ZOL (Z) or LOV (L) or MEV (M). *(ii)* ZOL, LOV and MEV inhibit protein prenylation as assessed by the presence of unprenylated Rap 1A. These are all compared to control untreated cells (C). *(iii)* No significant changes in the levels of Bcl-2 and Bax are detected.

cleavage with IMP treatment only. Detection of the unprenylated form of RAP 1A was identified in all cell lysates given IMP treatment but not detected in control or TRAIL treated cells (**Figure 5.12**). As observed with ZOL treated cells, no significant changes in the levels of Bcl-2 or Bax were detected (**Figure 5.12**).

5.4 DISCUSSION

In this study, statins and PTIs were used, either alone or in combination with TRAIL, to assess their effectiveness as alternative inhibitors of the mevalonate pathway to ZOL. Like ZOL, both statins (MEV and LOV) and PTIs (FTI and GGTI) exhibited direct effects on proliferation and survival of OS cells *in vitro* in a dose-dependent manner, supporting previous reports in other tumour types [Dimitroulakos *et al.* 1999; Dimitroulakos *et al.* 2001; Newman *et al.* 1994; Rubins *et al.* 1998; Jones *et al.* 1994; Muller *et al.* 1998; Wong *et al.* 2002; Chan *et al.* 2003; Bolick *et al.* 2003; Barrington *et al.* 1998; Sun *et al.* 1998; Lobell *et al.* 2001].

The augmentation of TRAIL induced apoptosis was not restricted to ZOL but appears to be a general phenomenon of inhibitors of the mevalonate pathway, with statins and PTIs capable of augmenting TRAIL-induced apoptosis in a similar manner to that observed with ZOL. A similar pattern of reduced cell viability was reflected between ZOL, statin and PTI treatments of each cell line. Comparison of the IC_{50}'s between different treatments demonstrates modest augmentation in all cell lines (**Table 5.2**). Single treatment of the sensitive cell lines HOS, BTK-143 and G-292 with either IMP shows a similar pattern of sensitivity. The BTK-143 cell line is the most sensitive to all five agents with the lowest values seen with LOV, slightly better than ZOL and MEV. Combination treatment of all agents with TRAIL in the BTK-143 cells reduced the IC_{50} value to between 4-11μM for all treatments. IMP treatments in HOS cells revealed ZOL to be more potent followed by the statins and the PTIs, However combination treatment reduced the IC_{50} values to between 5 and 16 μM. In contrast the statins had a more potent effect in the G-292 cells than ZOL, however dual treatments with TRAIL reduced the IC_{50} values to between 4 and 8 μM. The SJSA-1, Saos2 and MG-63 cell lines, considered to be more resistant to ZOL, also required greater amounts of statin to achieve 50% cell death. However MG-63 and Saos2 cells were more

	IC_{50}		IC_{50}		IC_{50}		IC_{50}		IC_{50}	
	Z	Z+T	M	M+T	L	L+T	F	F+T	G	G+T
BTK-143	10	5	10	5	7	4	21	11	25	7
HOS	8	5	32	16	18	13	50	10	19	13
G-292	15	8	8	5	6	4	-	-	-	-
MG-63	>100	90	55	25	22	16	-	-	-	-
Saos2	50	27	20	13	15	9	-	-	-	-
SJSA-1	35	23	100	90	51	41	-	-	-	-
K-HOS	45	16	-	-	-	-	-	-	-	-
KRIB	25	18	-	-	-	-	-	-	-	-

Table 5.2 Combined table of IC_{50} values for each cell line. Treatments are listed as T (100ng/ml TRAIL), Z (25μM ZOL), M (25μM MEV), L (25μM LOV), F (25μM FTI) and G (25μM GGTI). IC_{50} values are represented in μM.

susceptible to statin than ZOL with much lower IC_{50} values. This was not observed for the SJSA-1 cell line. All three cell lines resulted in reduced IC_{50} values with the combination TRAIL treatments, with the Saos2 values within the same range as that seen in the sensitive cell lines. The possible reasons for the differences observed between agents may depend on various factors, such as cell type, which may lead to variations in the expression of proteins involved in apoptotic or survival pathways. Other factors include variations in the chemical structure of the agents, which can reflect potency, as well as differences in efficient uptake of the agent into the cell [Lennernas and Fager 1997]. Further analysis of a greater range of statins and PTIs may prove beneficial in identifying a more potent alternative to ZOL. The concentration of statins used in this study to induce apoptosis compare with achievable concentrations *in vivo,* especially when given in combination with TRAIL. Phase I clinical trials administering LOV at a dose of 25 mg/kg/day, led to plasma concentrations of up to 3.92 μM, which corresponds to the dose range that can trigger apoptosis of sensitive tumour cells *in vitro* [Dimitroulakos *et al.* 2001; Dimitroulakos *et al.* 1999]. Since then phase II and III trials have used LOV at 40mg/kg/dose four times daily, increasing plasma concentrations further [Minden *et al.* 2000]. As there are many types of statins and PTIs available, further investigation using a greater number of each type of agent, may identify a more potent agent capable of inducing greater cell death. However, the degree of cell death induced by both the statins and PTIs chosen for this study is significant and warrants further investigation as potential anticancer agents.

An important goal of anticancer strategies is the selective induction of apoptosis in cancer cells but not normal cells, that is, to specifically inhibit malignant growth with minimal systemic toxicity. Both statin and PTI treatment of NHB cells resulted in reduced cell death than that observed in human OS cells, even with the co-addition of TRAIL, supporting previous results obtained with ZOL.

Caspase inhibitors failed to protect cells from statin and PTI induced apoptosis, suggesting that their mode of action mimics that of ZOL, that is, apoptosis does not rely on the activation of the caspase cascade directly. On the other hand, the addition of GGO, an intermediate of the mevalonate pathway, was able to rescue the cells treated with statins but not PTIs. The addition of GGO to cells treated with statins and TRAIL, protected them from the augmented apoptotic effect observed with statin and TRAIL treatment alone. Although protection was not equal to that of TRAIL treatment alone, cell viability in the GGO/statin/TRAIL treated cells was double that of the statin/TRAIL treated cells for both MEV and LOV. Combination of FTI or GGTI and TRAIL treated cells were not protected by GGO and cell death was equal to that observed in the dual combination treatments. This difference is due to the effect of GGO within the mevalonate pathway. As statins inhibit an enzyme required early in the mevalonate pathway, the addition of GGO rescues the cell as it is downstream, therefore acts as an intermediate **(Figure 5.13)**. FTI and GGTI inhibit the enzymes responsible for prenylation itself and GGO is unable to rescue the cells at this point. Thus it is not surprising that this difference exists.

Consistent with the ZOL and TRAIL combination treated cells, HOS cells treated with both MEV and LOV in combination with TRAIL also exhibited PARP cleavage, confirming the increased apoptotic efficacy of the combination treatments. Similarly all cells treated with IMP displayed the presence of the unprenylated form of RAP 1A after western blotting, confirming that the mode of action of these agents is via inhibition of the mevalonate pathway.

It is possible that ZOL-induced anoikis, or prevention of attachment, regulates the levels of pro and anti-apoptotic factors below a threshold required for sensitisation of TRAIL-resistant OS cells to TRAIL-induced apoptosis. It was recently demonstrated that anoikis leads to the translocation of the pro-apoptotic protein, Bmf, to the

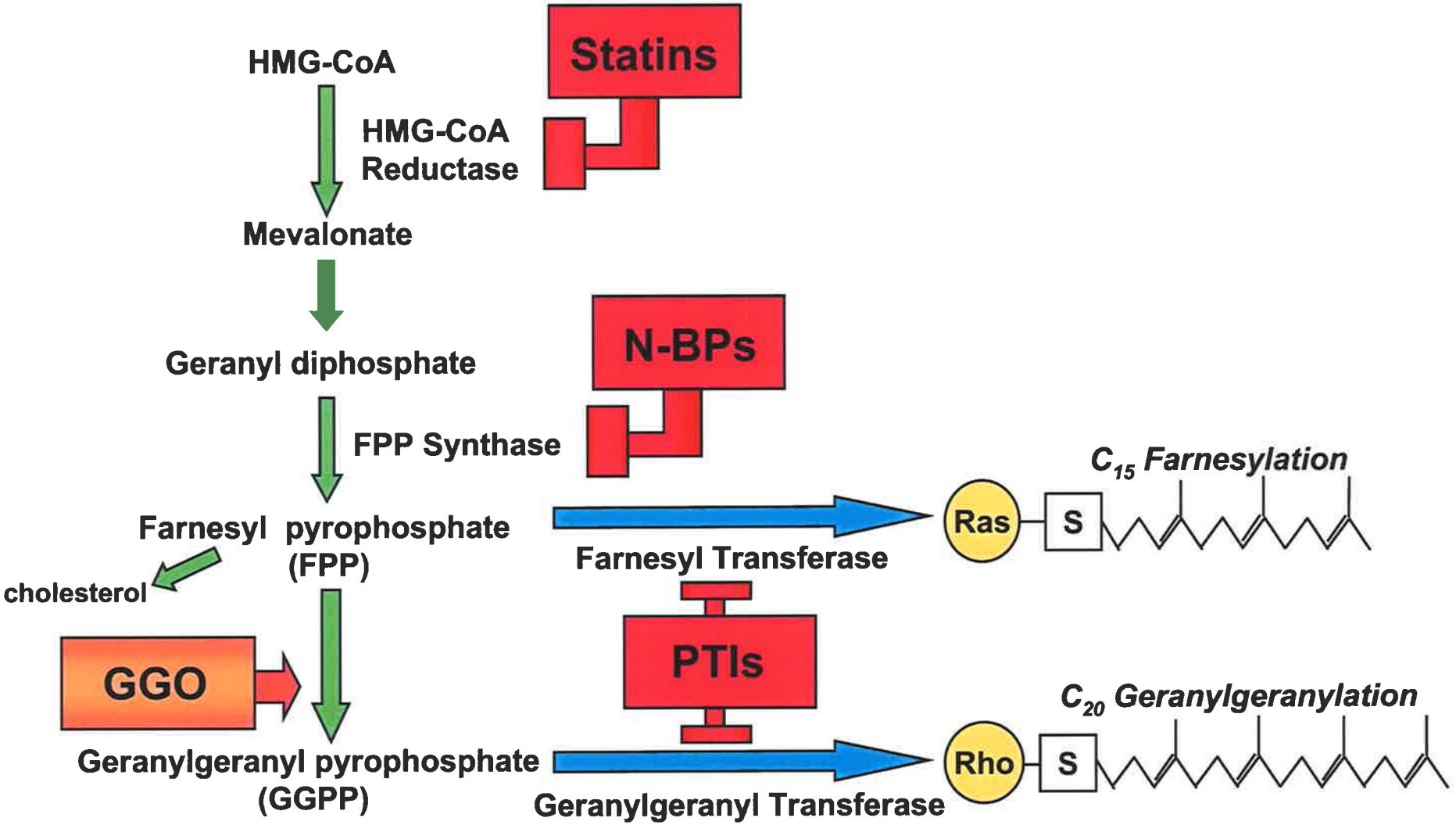


Figure 5.13 Geranylgeraniol (GGO) acts as an intermediate of the mevalonate pathway. Nitrogen-containing bisphosphonates (N-BPs), statins and Prenyl Transferase Inhibitors (PTIs) inhibit the enzymes indicated above, preventing downstream processes including farnesylation and geranylgeranylation., GGO allows geranylgeranylation to occur if enzymes upstream are inhibited.

mitochondria where it binds to, and antagonises the activity of, the pro-survival protein Bcl-2 [Puthalakath *et al.* 2001]. The extrinsic and intrinsic pathways communicate with each other, and death receptors can trigger activation of the *intrinsic* pathway by caspase 8 mediated cleavage of Bid, which interacts with other members of the Bcl-2 superfamily including Bax, leading to activation of caspase 9. No changes in Bcl-2 and Bax protein expression were observed for both ZOL and statin treated cells. This conflicts reports for ZOL and statins which have been shown to down-regulate Bcl-2 expression [Blanco-Colio *et al.* 2002; Wong *et al.* 2002; Senaratne *et al.* 2000; Oades *et al.* 2003]. In some cells the anti-apoptotic effects are partly attributed to the regulation of several inhibitors of apoptosis including c-IAP1, c-IAP2 and XIAP [Wang *et al.* 1998; Deveraux and Reed 1999]. A more exhaustive analysis is required to identify expression changes in proteins of the Bcl-2 superfamily as well as inhibitors of apoptosis, which may be involved [Wang *et al.* 1998; Deveraux and Reed 1999].

The use of statins and PTIs in *in vivo* models may prove more beneficial than ZOL, as they do not have an affinity for bone, thus their widespread systemic distribution may also be used for the treatment of other non-osseous malignancies including lung metastases, often associated with increased mortality in OS. Most importantly the lack of effect on normal bone cells by the IMP make them very desirable anti-cancer agents however further investigation is required to fully understand the mechanism of augmentation with TRAIL and why they are not uniformly effective in all cell lines. One way to overcome this problem may be in the form of a predictive test to determine the best course of therapy for OS patients.

CHAPTER SIX

ESTABLISHMENT OF *IN VIVO* OSTEOSARCOMA MOUSE MODEL

6.1 INTRODUCTION

Thus far there have been no reports investigating the potential anti-tumour activity of TRAIL alone, or in combination with BPs, statins or chemotherapy, in bone tumours *in vivo*. The aim of the work described in this chapter and the next was to evaluate these potential anti-tumour agents in an *in vivo* model. It was also considered important to test the ability of these agents to suppress the metastatic spread of human OS.

To begin investigating these agents in an *in vivo* environment, an OS animal model was established. This model was based on a simple technique, by which human OS cells are transplanted orthotopically into the tibia of athymic mice, as described by Berlin and colleagues [Berlin *et al.* 1993]. Berlin used the v-Ki-ras-oncogene-transformed human osteosarcoma cell line (KRIB) to develop a spontaneous metastasis model, in which a small number of tumour cells are injected into the tibial bones of athymic mice. Four weeks after intra-tibial transplantation, the KRIB cell line was shown to develop bone tumours, which were radiographically and histologically similar to primary human osteosarcoma [Berlin *et al.* 1993]. Furthermore, as in the human disease, cells from these primary tumours efficiently colonize the lungs of athymic nude mice, resulting in reproducible and quantifiable pulmonary metastases, evident both upon gross inspection of the lungs and histologically 6 weeks after tumour inoculation.

A pilot study was set up to identify a suitable cell line and an appropriate cell number for use in animal experiments. As noted in earlier chapters, KRIB OS cells are refractory to the apoptotic effects of TRAIL as a single agent. In addition, the presence of ZOL *in vitro* has little effect in sensitising KRIB cells to TRAIL-induced apoptosis. Other human OS cell lines, such as K-HOS, are also unaffected to TRAIL treatment alone, however, are strongly sensitised to the effects of TRAIL with co-treatment of ZOL. For this reason K-HOS cells were also chosen as a comparison to KRIB cells, for the pilot study.

6.2 MATERIALS AND METHODS

6.2.1 Cells and cell culture

The osteosarcoma cell lines used in this chapter are listed in section 2.5.2. The methods for cell preparation and propagation are described in section 2.1.3 and 2.5.1. Cell suspensions of K-HOS and KRIB cells in sterile PBS, at 2 X 10^3, 5 X 10^3 or 1 X 10^4 cells/20µl, were implanted into the proximal tibia. Mice were weighed twice weekly and progress of the tumour was monitored using radiography. At time of sacrifice, organs were collected for histology and tibiae were removed for micro-CT analysis.

6.2.2 Mouse specifications and maintenance

Animal specifications and housing are detailed in section 2.5.3.

6.2.3 Intra-tibial implantation: delivery and schedule

For detailed explanation of the intra-tibial implantation method please refer to section 2.5.4.

6.2.4 Histology and image analysis

Histological sections were prepared according to the protocol detailed in section 2.5.6. Radiography and micro-CT analysis were performed as listed in section 2.5.7 and 2.5.8 respectively.

6.3 RESULTS

6.3.1 Lytic lesions identified using radiograph analysis

The pilot study consisted of a total of 6 animals. Three animals were injected with varying concentrations of 2 X 10^3, 5 X 10^3 or 1 X 10^4 cells/20µl using KRIB cells. The remaining animals were injected with K-HOS cells at the same concentrations. After a minimum lag time of 4 weeks from intra-tibial injection of both KRIB and K-HOS cells, macroscopic tumours were evident in the tibiae of athymic mice as shown in **Figure 6.1**. Assessment by radiography at 2 weekly intervals revealed the development of osteolytic lesions that also progressively increased with time. The animal injected with 1 X 10^4 cells/20µl of KRIB cells exhibited some evidence of osteolysis at 3 weeks while all KRIB-injected animals showed clear osteolytic lesions by 5 weeks. None of the animals inoculated with K-HOS cells showed any evidence of osteolysis at 3 weeks using radiography but osteolysis was evident at 5 weeks. Animals were sacrificed if they developed large tumours with associated soft-tissue swelling and had some difficulty with movement or when they reached 8 weeks post tibial-inoculation. All animals injected with KRIB OS cells were sacrificed by 6 weeks due to the significant degree of osteolysis observed by radiography, however only one of the K-HOS-injected animals was sacrificed prior to 8 weeks because it also exhibited significant osteolysis. Radiographs were taken on the day of sacrifice and compared (**Figure 6.2** and **6.3**). All 3 animals injected with KRIB OS cells demonstrated considerably more osteolysis than the animals injected with K-HOS OS cells. However, the animal injected with 5,000 K-HOS cells exhibited significant osteolysis and a decision was made to use K-HOS cells for the study of the anti-cancer potential of ZOL and TRAIL *in vivo*. As animals were sacrificed at varying time points depending on the degree of osteolysis, the cell number only gives an indication of the time required for lesions to occur.

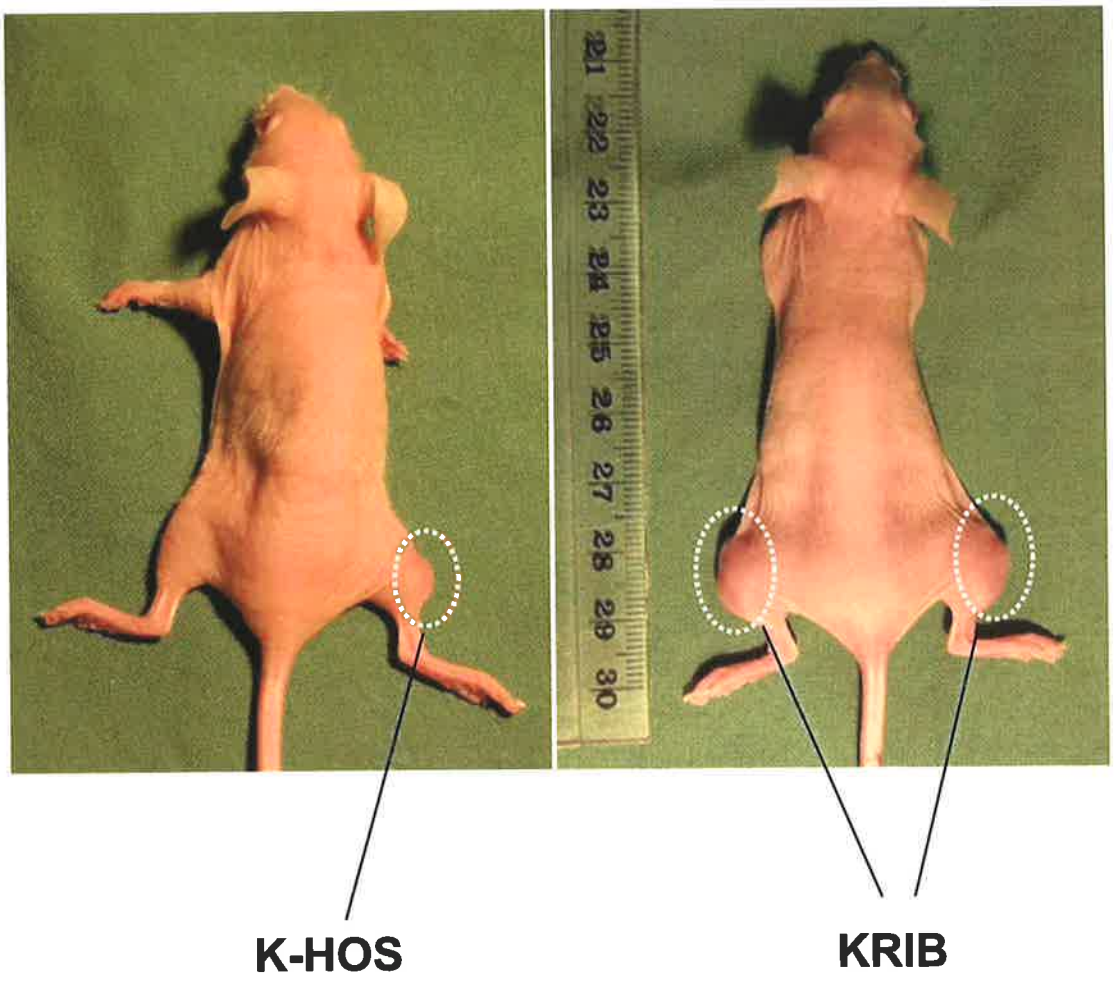


Figure 6.1 Intra-tibial injection of human OS cells resulted in the development of tumours. Photos were taken 6 weeks post intra-tibial injection. Clearly defined tumours were evident in mice injected with either K-HOS or KRIB cells.

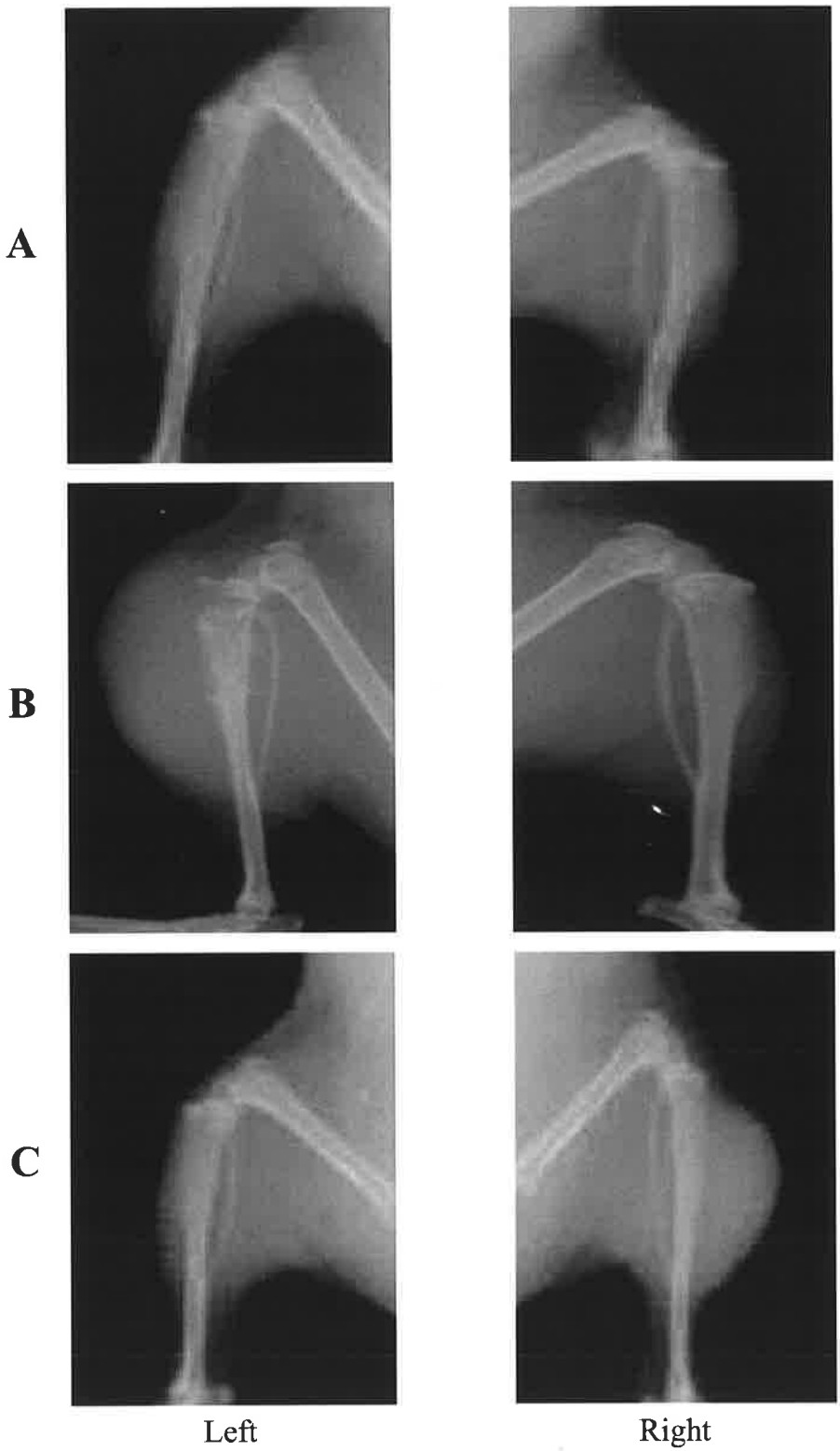


Figure 6.2 Radiographic analysis of mice tibiae from the K-HOS Pilot study. Mice were injected with **(A)** 2 X 10^3, **(B)** 5 X 10^3 or **(C)** 1 X 10^4 cells/20µl into the left tibia for **A** and **B** and right tibia for **C**. Radiographs of both tibiae were taken at 4 weeks post inoculation.

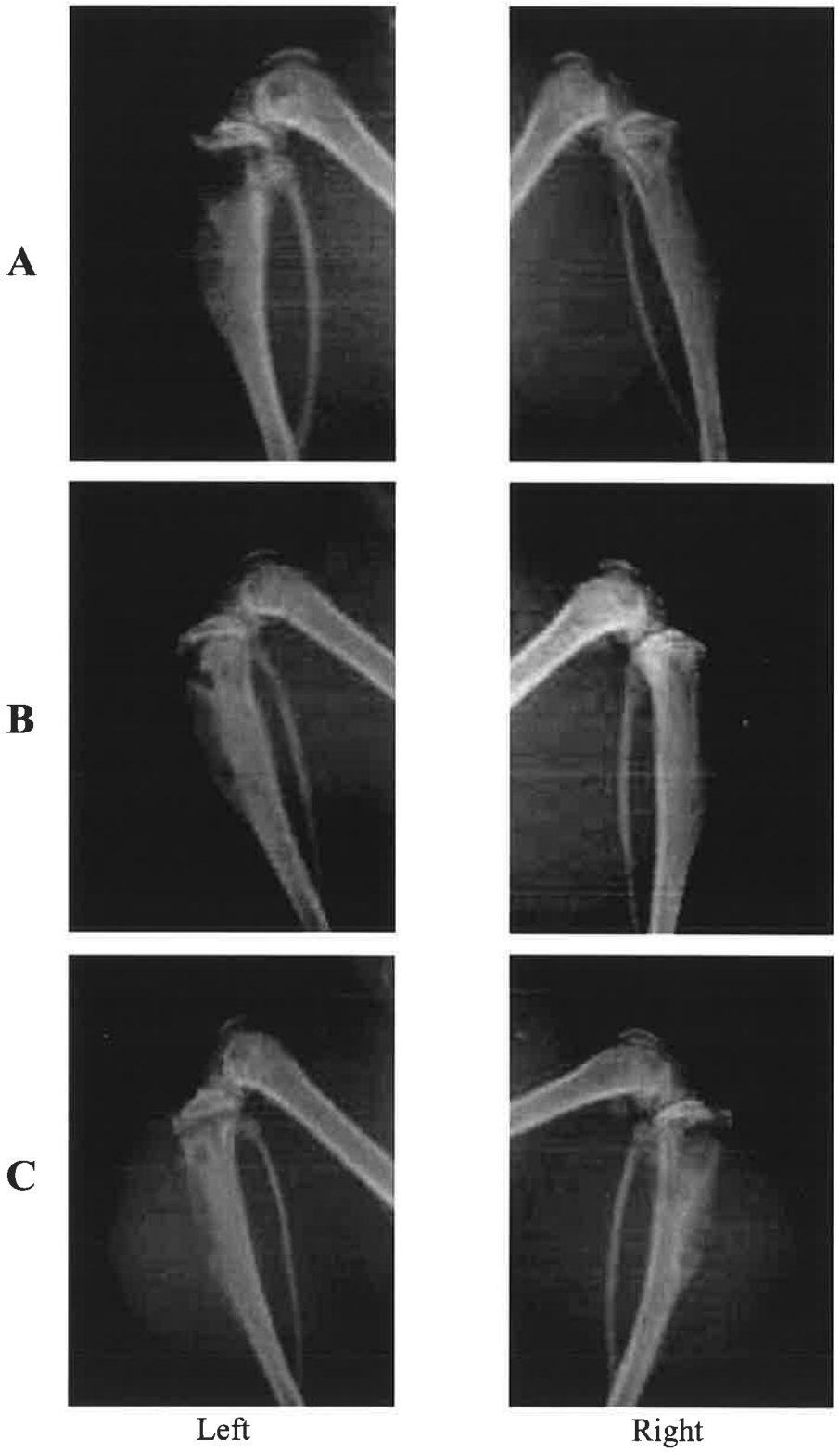


Figure 6.3 Radiographic analysis of mice tibiae from the KRIB Pilot study. Mice were injected with **(A)** 2 X 10^3, **(B)** 5 X 10^3 or **(C)** 1 X 10^4 cells/20µl into the left tibia for **A** and **B** and both right and left tibia for **C**. Radiographs of both tibiae were taken at 4 weeks post inoculation.

6.3.2 Improved imaging of lytic lesions using micro-CT analysis

Micro-computer tomography (μCT) was performed on the tibias of these animals to generate images of superior quality as compared to the radiograph. For micro-tomographical reconstruction, transmission X-ray images are acquired from 200 rotation views over 180 degrees of rotation. Each rotation view contains 1,004 lines, which can produce a two-dimensional (2-D) section representing 17.36 microns, when the same line from each rotation view is collated. The use of sophisticated software allows further image reconstruction of these 2-D sections leading to the generation of a three dimensional (3-D) image, which is far superior in detail and provides more information to that obtained by standard radiography. The 3D images can be rotated in any direction offering improved analysis of osteolytic destruction than two-dimensional images. These images can also be manipulated so that the tibias can be dissected, to allow investigation of inner bone structure and the degree of osteolysis from within the tibia. In addition the use of μCT and the associated computer programs, allows bone volume quantification to be assessed.

The μCT images obtained from mice injected with either K-HOS (**Figure 6.4**) or KRIB (**Figure 6.5**) cells both exhibited bone destruction however the extent of osteolysis was more severe in the animal injected with KRIB cells. These are comparable with the radiographs taken at time of sacrifice, however, the poorly defined regions of osteolysis in the radiographs are clearly detailed in the μCT, providing much greater insight into the destructive nature of the tumour. From the μCT images it is evident that the tibias with the transplanted K-HOS cells are uneven in appearance as opposed to the smooth contour of the non-transplanted tibia (**Figure 6.4**).

Figure 6.4 Micro-Computer Tomographic analysis of the tibiae removed from a nude mouse. 5 X 10^3 K-HOS cells were inoculated into the left proximal tibia and the animal was sacrificed 6 weeks later. *Top Image*: μCT of both tibiae, *Bottom Image:* Sliced images of the left and right tibiae using μCT software, reveals the extent of osteolytic damage in the left tibia.

Figure 6.5 Micro-Computer Tomographic analysis of the tibiae removed from a nude mouse. 2 X 10^3 KRIB cells were inoculated into the left proximal tibia and the animal was sacrificed 6 weeks later. μCT analysis reveals the extent of osteolytic damage in the tibia.

6.3.3 Histological analysis

Following μCT imaging, the tibias were decalcified and embedded in paraffin for sectioning and staining (refer to section 2.5.6 for further details). Both the K-HOS or KRIB cells reproducibly developed large lesions that invaded the marrow cavity and began to erode the cortical bone (**Figure 6.6**).

Histological analysis of kidney, liver, and spleen sections obtained from animals injected with either the K-HOS or KRIB cells revealed no metastatic lesions to these organs (data not shown). Multiple pulmonary macro- and micro-metastases were identified for both cell types in all animals (**Figure 6.7**).

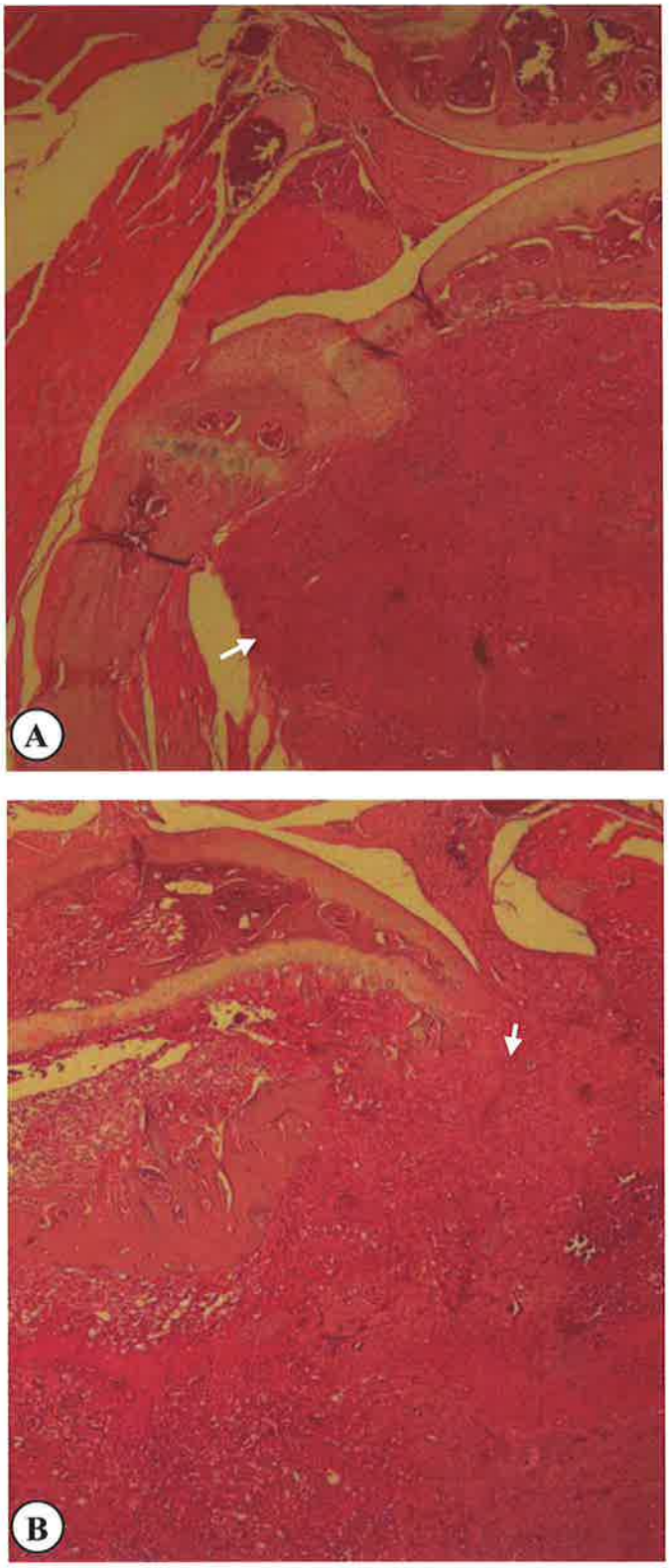


Figure 6.6 Histological examination of intra-tibial OS. Tumours were identified within the bone marrow cavity after injection of either **(A)** KRIB and **(B)** K-HOS into tibiae of nude mice. Regions of tumour are identified with white arrows.

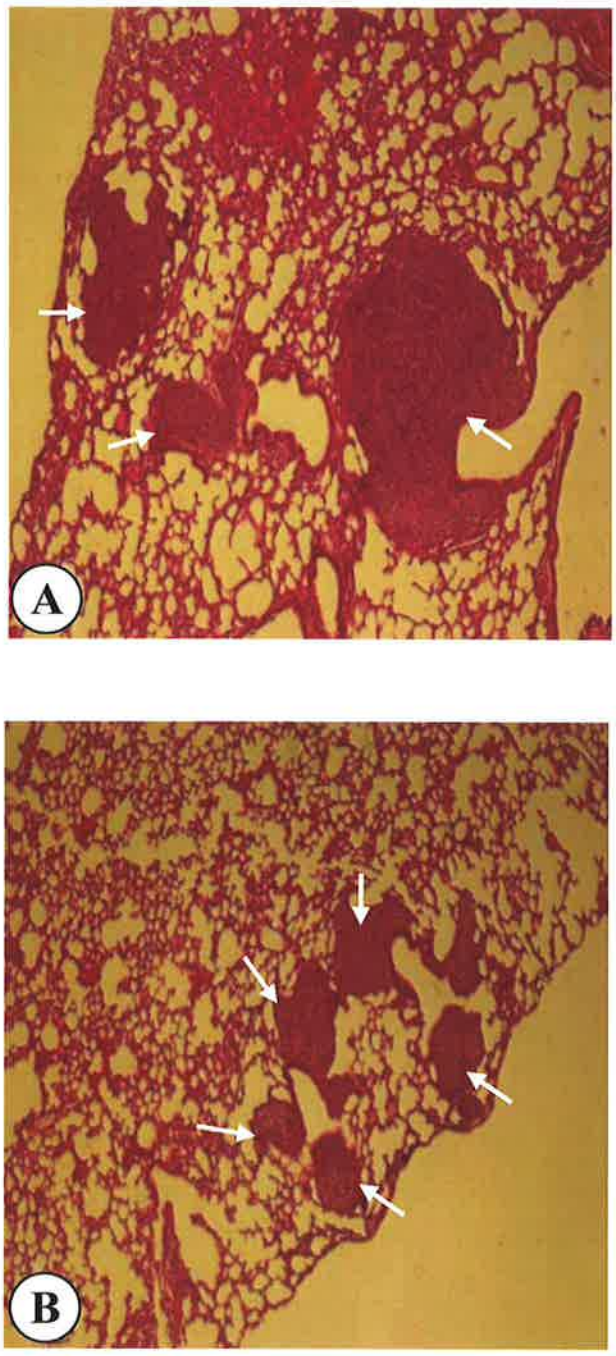


Figure 6.7 Histological examination of lung tissue from nude mice inoculated with human OS cells. Multiple micro-metastatic tumours were identified in the lungs of both **(A)** KRIB and **(B)** K-Hos inoculated mice. Regions of tumour are identified with white arrows.

6.4 DISCUSSION

The establishment of an *in vivo* mouse model of OS allows for the investigation of the efficacy of potential anti-cancer agents. Both K-HOS and KRIB OS cell lines were able to produce locally growing tumours in the immediate area surrounding the tibial site of injection. It is clearly evident that the animals inoculated with KRIB OS cells showed a higher degree of osteolysis when compared to animals inoculated with K-HOS OS cells. Histology also confirmed the presence of tumours within the marrow cavity and eroding into the cortical bone. Pulmonary metastases were identified in animals injected with either cell line, however metastases were not discovered in any other organs. Micro-CT analysis provided far greater image resolution than radiography and supported the radiographs taken at time of sacrifice. However unlike μCT imaging, the use of radiography allowed for the continual monitoring of the tumour area, with the first signs of osteolysis observed at week 3 in the mouse injected with the highest concentration of KRIB cells. One limitation of continual monitoring using radiography is that the tumour has to penetrate through the cortex before it is evident on a radiograph. Thus it is not a clear indication of early tumour development. Both cell lines however required at least 4 weeks to develop macroscopic tumours. A possible reason for the reduced amount of osteolysis in the animal injected with 1 X 10^4 K-HOS cells compared to the animal with 5 X 10^3 K-HOS cells may be due to the delivery of the cells. This needs to be confirmed with a greater number of animals to test the accuracy of the delivery system. It is difficult to ensure that such a high number of cells in such a small volume are delivered accurately, therefore the number of cells delivered may vary. In addition, it is impossible to inject into exactly the same position of the tibia in every mouse, with variations in injection sites potentially providing differing environments for the cells. Finally, it is likely that cells are left in the surrounding soft tissue as the needle withdraws from the tibia, leading to extra-osseous growth of these

cells, which does not represent the normal growth and progression of OS. The tumour burden outside the tibia may be one reason for the uneven appearance of the bone surface observed in the K-HOS tumour-ridden tibiae but not within direct proximity to the injection site, indicating bone remodelling of the periosteal surface. It is possible that these tumours may have a mixed osteolytic and osteoblastic phenotype and the observed response may be due to the extra-osseous tumour stimulating bone remodelling in both the tibia and fibula.

As mentioned in previous chapters, the KRIB cell line does not show a significant augmented apoptotic effect when treated with both ZOL and TRAIL. Therefore the KRIB animal model of OS is unsuitable for the investigation of the efficacy of these agents *in vivo*. For this reason, the K-HOS animal model was chosen for future studies.

CHAPTER SEVEN

INVESTIGATION INTO THE EFFECT OF ZOLEDRONIC ACID AND TRAIL TREATMENT IN NUDE MICE:

Will it work *in vivo*?

7.1 INTRODUCTION

In vivo studies in numerous animal models of metastatic cancer have demonstrated a reduction in tumour-induced osteolysis with ZOL treatment [Dallas *et al.* 1999; Hall and Stoica 1994; Nemoto *et al.* 1990]. Anti-angiogenic properties of ZOL have also been reported, making this class of bisphosphonates attractive agents in the treatment of metastatic cancers [Croucher *et al.* 2003]. The use of ZOL in patients with bone metastases from various types of primary tumours has shown significant clinical benefits, perhaps superior to other BPs. To date however, there have been no reports of ZOL treatment of OS *in vivo.*

The ability of TRAIL to induce apoptotic cell death in a wide variety of tumour cell lines, but not normal cells *in vitro* or *in vivo,* makes it appealing for the treatment of cancer. More importantly, sensitisation of OS cells but not normal human bone cells to TRAIL induced-apoptosis by ZOL, delivers huge potential into anti-cancer therapy.

With the establishment of a mouse model of OS, the aim of the work described in this chapter was to investigate the effect of ZOL and TRAIL as single agents and in combination, in delivering an apoptotic stimulus to OS cells *in vivo*. Such an outcome would bring the hope of achieving substantial apoptotic response in this tumour type without the toxic side effects often seen with conventional and current therapies.

7.2 MATERIALS AND METHODS

7.2.1 Cells and cell culture

The OS cell line K-HOS used in this chapter is listed in section 2.5.2. The methods for cell preparation and propagation are described in section 2.1.3 and 2.5.1. Cell suspensions of K-HOS cells in sterile PBS, at 6 X 10^3 cells/10µl, were implanted into the tibiae of nude mice. Mice were weighed twice weekly and progress of the tumour growth and osteolytic activity was monitored using radiography. At time of sacrifice, organs were collected for histology and tibiae were removed for micro-CT analysis.

7.2.2 Mouse specifications and maintenance

Animal specifications and housing are detailed in section 2.5.3.

7.2.3 *In vivo* treatment: delivery and schedule

For detailed explanation of the intra-tibial implantation method refer to section 2.5.4. Treatment schedules for ZOL and TRAIL are listed in section 2.5.5.

7.2.4 Histology and image analysis

Histological sections were prepared according to the protocol detailed in section 2.5.6. Radiography and micro-CT analysis were performed as described in sections 2.5.8 and 2.5.7, respectively.

7.2.5 Statistical analysis

Data obtained for each treatment group regarding the presence of osteolytic lesions and lung metastases, was assessed using Fisher's exact test. In each of these

cases, the interaction between ZOL and TRAIL was assessed using the Breslow-Day test for homogeneity of odds ratios.

Impact of ZOL and TRAIL and an interaction between the two on bone volume in the left leg was assessed *via* 2-way Analysis of Covariance (ANCOVA), where bone volume in the right leg was the covariate. Impact of ZOL and TRAIL, and an interaction between the two, on tumour area (where tumours were detected) was assessed using the 2-way Analysis of Variance (ANOVA).

7.3 RESULTS

7.3.1 ZOL protects the skeleton from tumour-induced osteolysis

K-HOS cells, at a concentration of 6 X 10^3/10µl (see section 2.5.4), were prepared for intra-tibial injection of 4 to 6 week old Balb/c Nu/Nu female mice. Tibiae of 39 animals were inoculated on Day 0 and animals were assigned randomly into 4 groups: (**1**) the control untreated group (9 mice), (**2**) the TRAIL treated group (9 mice), (**3**) the ZOL treated group (10 mice) and (**4**) the combination of ZOL and TRAIL group (11 mice). To monitor the progression of tumours, mice were weighed twice a week and radiographs were taken fortnightly while under anaesthetic.

Commencement of treatment was at Day 7 post cancer cell transplantation, to allow sufficient establishment of tumour cells within the bone environment. TRAIL was administered *via* intraperitoneal injection at a dose of 30mg/kg/dose, once a day for 5 consecutive days, followed by once weekly injection at the same dose (**Figure 7.1**). ZOL was commenced on Day 7, and was given subcutaneously once weekly at a dose of 100 µg/kg for each injection until sacrifice.

By week 4, several animals had developed large tumours that hindered normal movement of the inoculated leg and the mice were beginning to show signs of discomfort. It was recommended by the animal ethics committee that the experiment be terminated early, to prevent any suffering of the mice. For this reason the experiment was terminated at Day 35.

Radiographs of all the animals were taken on the day of sacrifice, prior to removal of the tibiae for µCT and histological analysis. **Figure 7.2** shows radiographs, with clearly defined osteolytic lesions in 3 of the 9 control untreated animals. The TRAIL treated group resulted in 2 animals with noticeable lesions, based on the radiographs (**Figure 7.3**). **Figure 7.4** and **7.5** show radiographs taken from the ZOL and

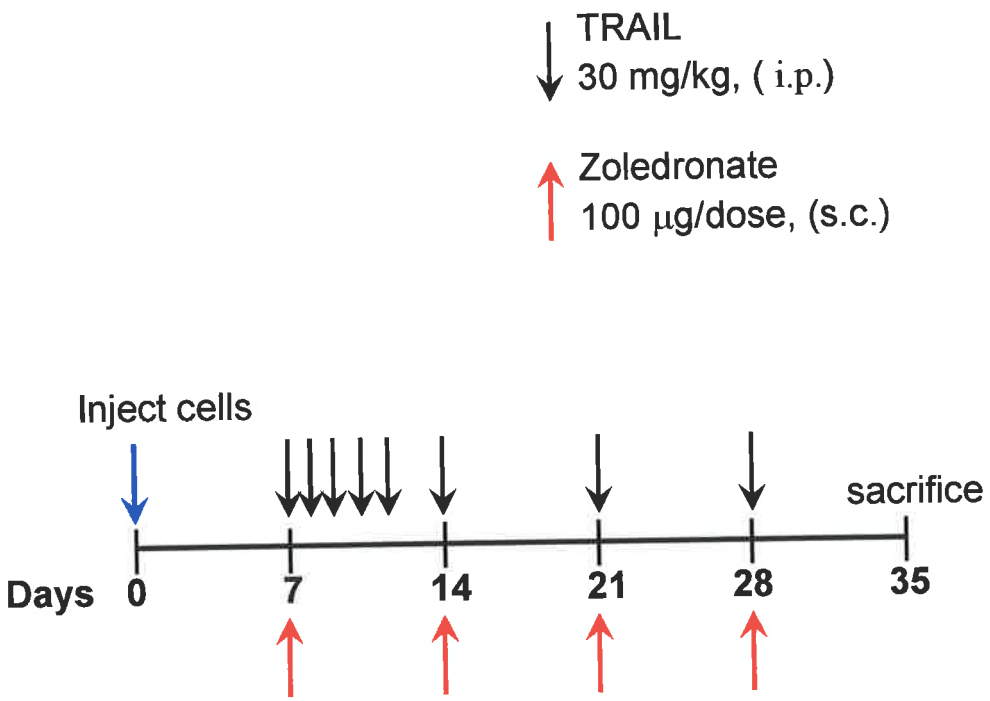


Figure 7.1 Dosing regimen for *in vivo* OS experiment. Mice were inoculated with K-HOS cells into the left proximal tibia at day 0. Treatments were commenced from day 7 onwards with TRAIL and/or ZOL. Mice were sacrificed at day 35 for further analysis. A suitable control group of mice were also included which received the intra-tibial injection but given no treatment.

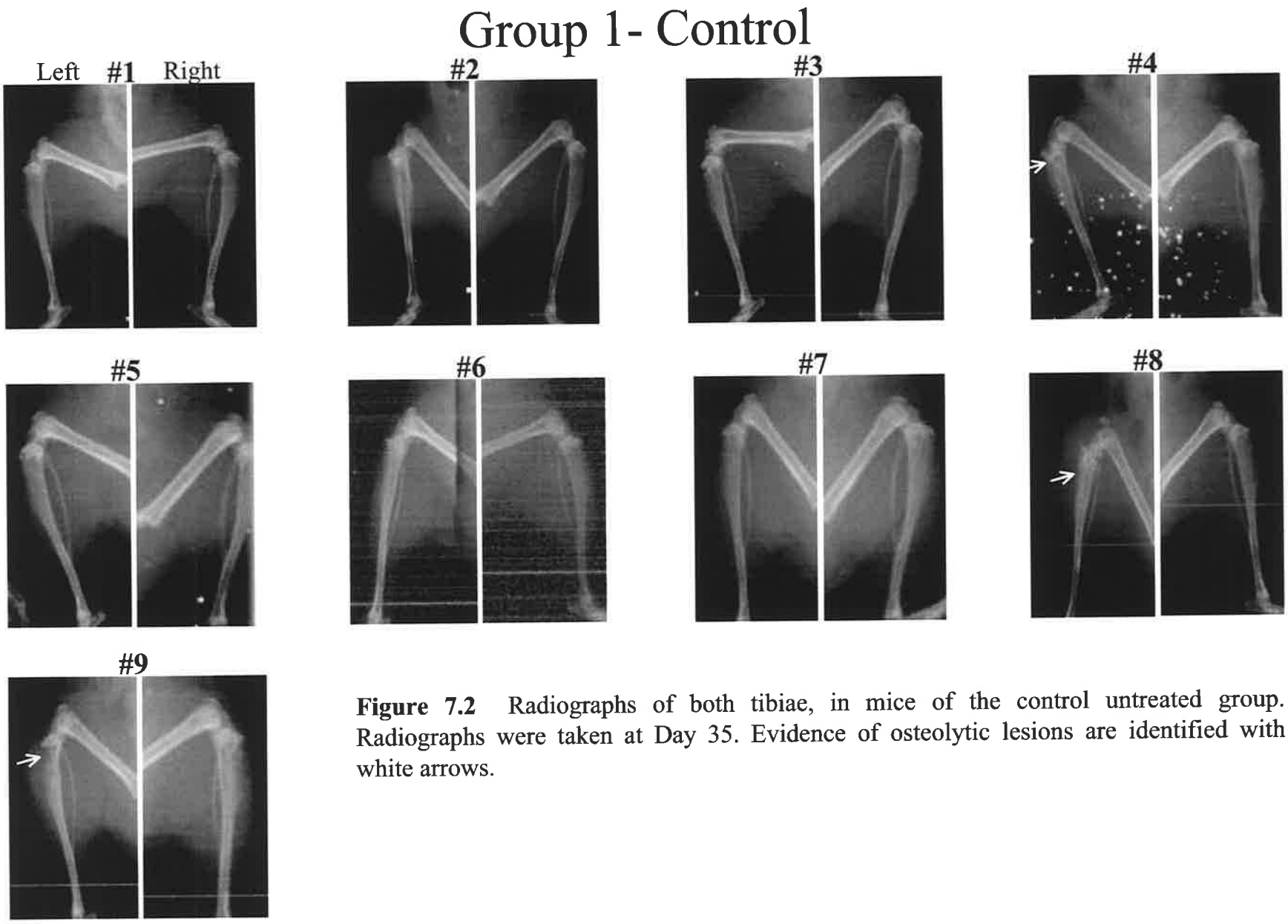


Figure 7.2 Radiographs of both tibiae, in mice of the control untreated group. Radiographs were taken at Day 35. Evidence of osteolytic lesions are identified with white arrows.

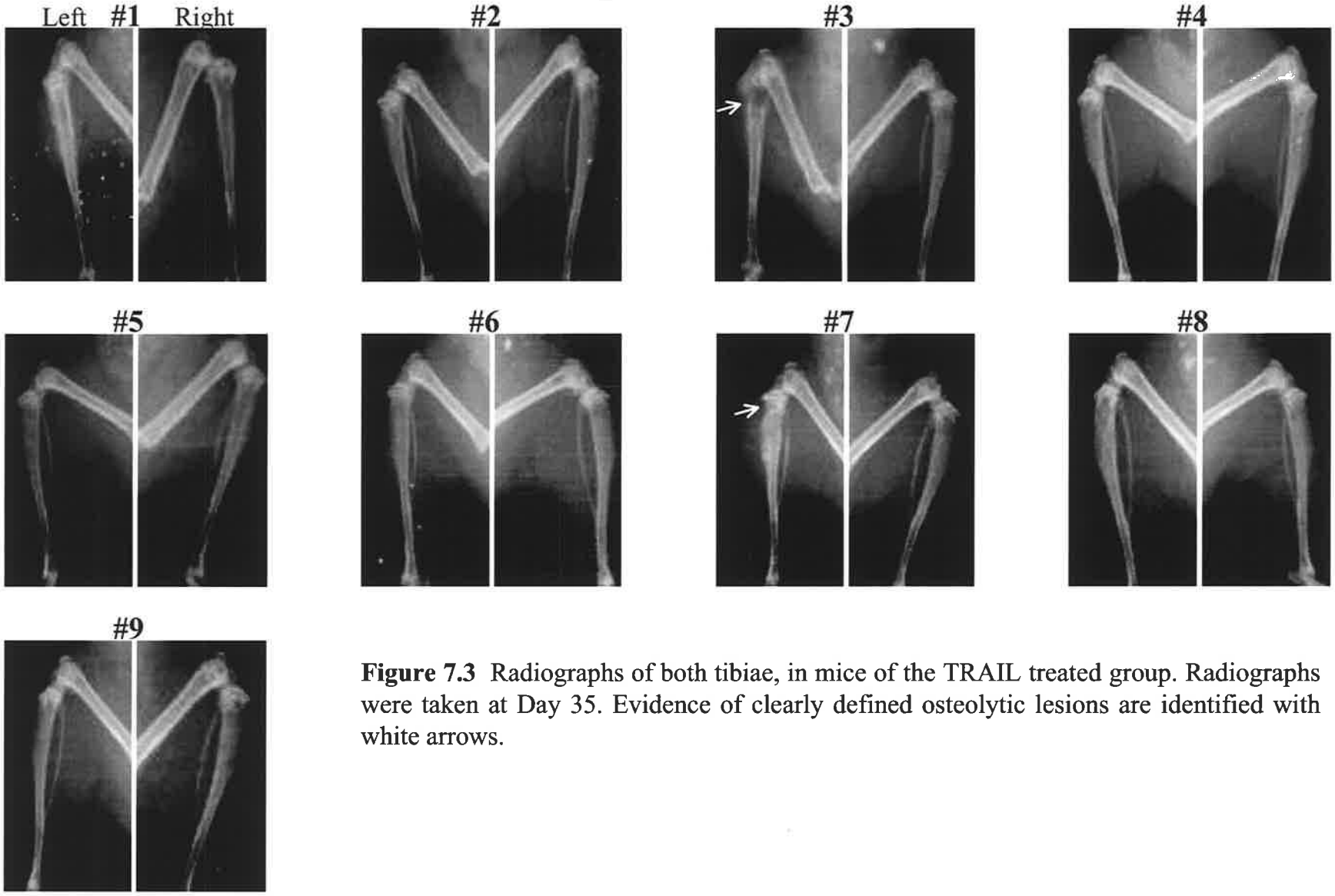


Figure 7.3 Radiographs of both tibiae, in mice of the TRAIL treated group. Radiographs were taken at Day 35. Evidence of clearly defined osteolytic lesions are identified with white arrows.

Group 3- ZOL

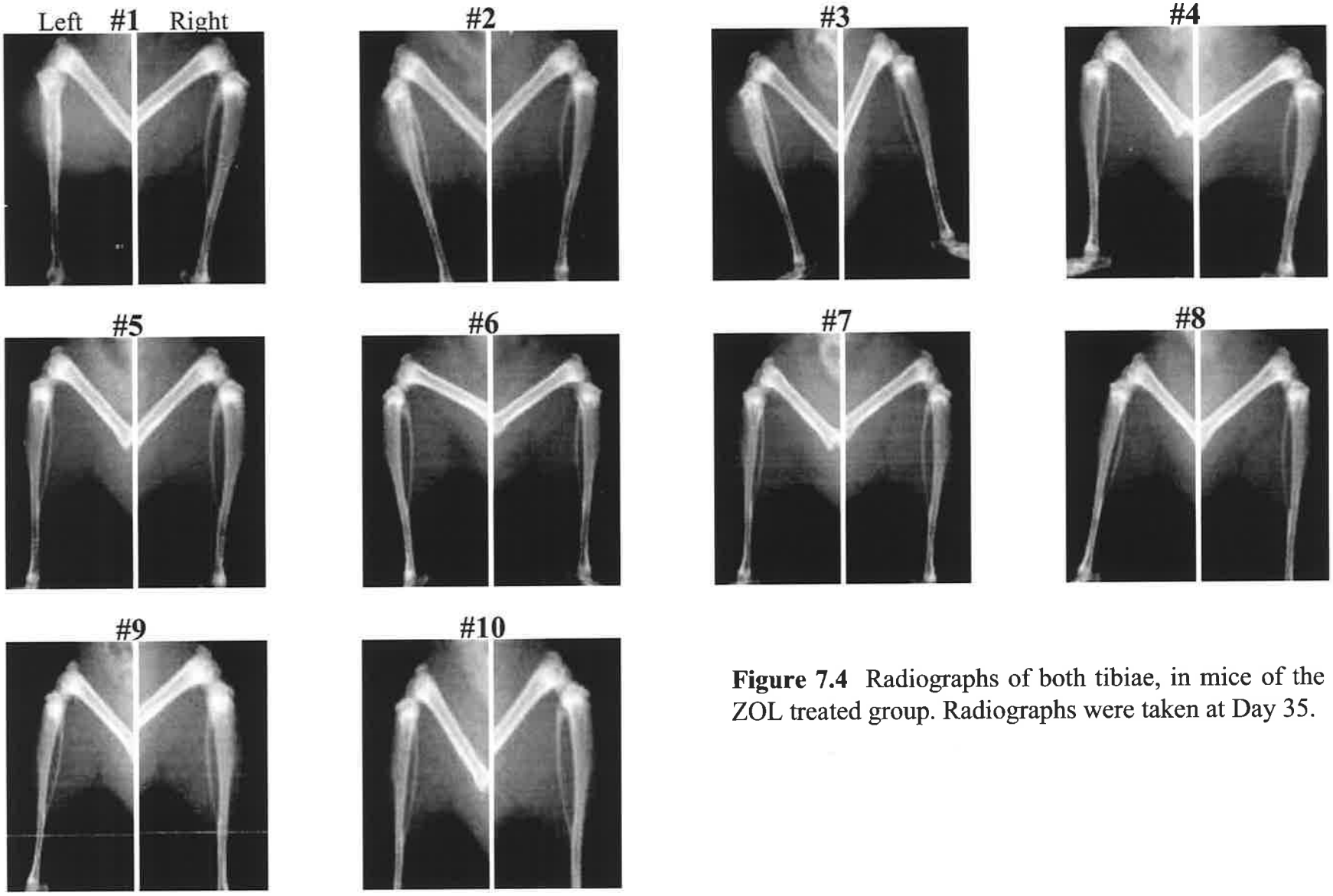


Figure 7.4 Radiographs of both tibiae, in mice of the ZOL treated group. Radiographs were taken at Day 35.

Group 5- ZOL + TRAIL

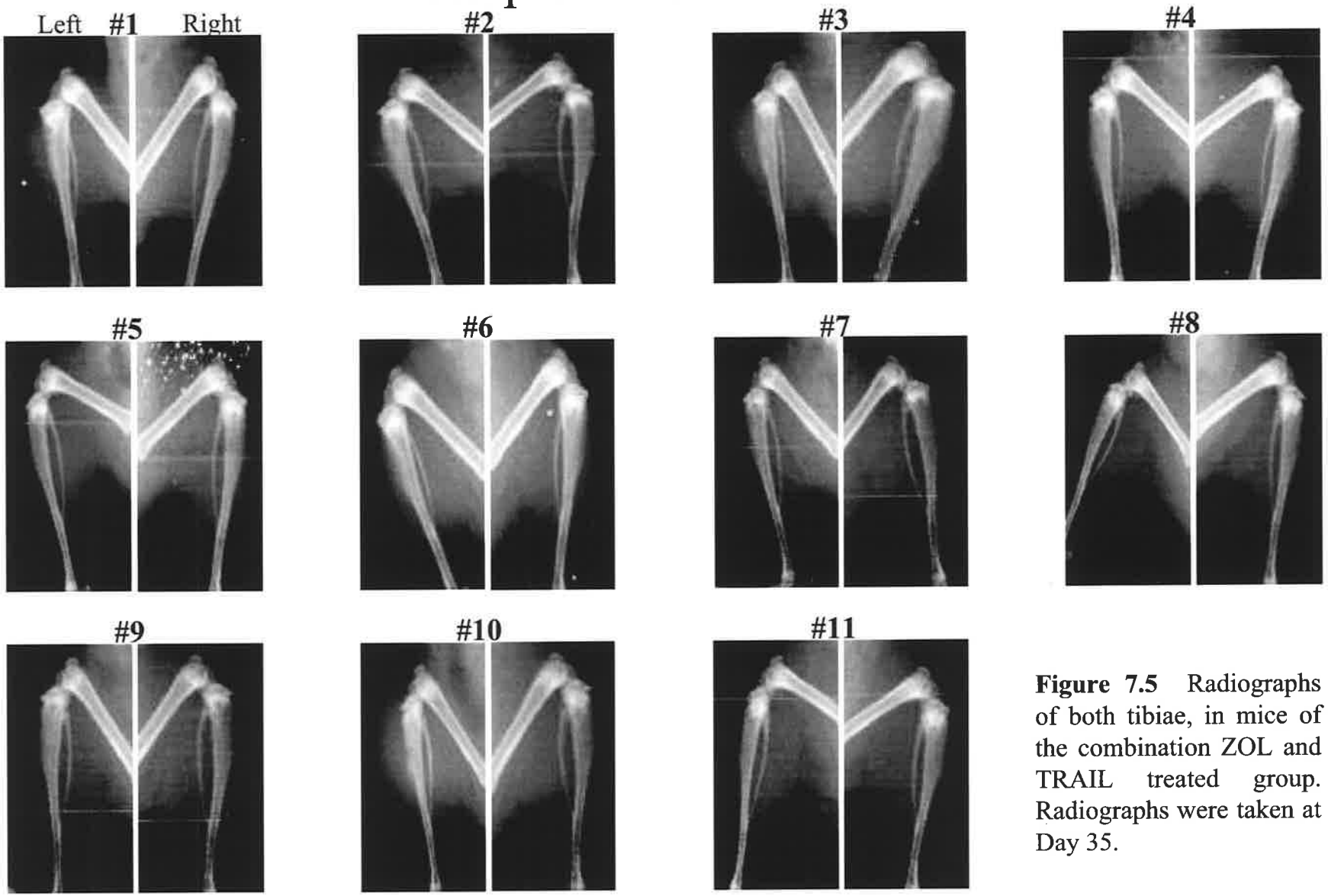


Figure 7.5 Radiographs of both tibiae, in mice of the combination ZOL and TRAIL treated group. Radiographs were taken at Day 35.

combination of ZOL and TRAIL treated groups, respectively. Both groups show no signs of bone lysis from the radiographs. The level of resolution of these images makes it difficult to identify small lesions with any certainty. Micro-CT analysis proved far more useful in identifying the extent of damage from the OS tumours, as well as the efficacy of the chosen treatments.

Using the SkyScan-1072 micro-CT, one pair of tibiae per run was scanned, with a total of 4 runs per day. Following scanning, the resulting TIFF files from one run (which totalled approximately 1 gigabyte of computer memory), were converted into BMP files, a process requiring between 6-8 hours. The files were then imported into the μCT 3-D software and 3-D images generated. The 3-D software enables editing and rotation of the image, allowing modifications to be made and further investigation of the tibiae, such as slicing through the middle to reveal the inner bone cavity. Although the process of generating 3-D images using μCT is a lengthy and laborious task, the quality of the images is considerably more informative than radiographs alone, which justifies the investment of time in this procedure.

The μCT images generated for each group of animals were collated into treatment groups for comparative analyses. **Figure 7.6** shows a more comprehensive view of the effect of K-HOS cells in the tibia of untreated control mice when compared to the corresponding radiograph (**Figure 7.2**). Five out of 9 animals in this group exhibited obvious differences between the inoculated tibia and the control tibia. It is worth noting that, although the same 3 animals (#4, #8, #9) identified as having osteolysis in the radiographs show lytic lesions in the μCT images, animals #1 and #5 also had small lytic lesions, which were not apparent on the radiographs. Animals #4 and #8 had both osteolytic and presumed 'osteoblastic' lesions, as discussed below. Animal #2 exhibited only the 'osteoblastic' phenotype. This feature would not have otherwise been identified, if μCT analysis had not been conducted. The images from the untreated

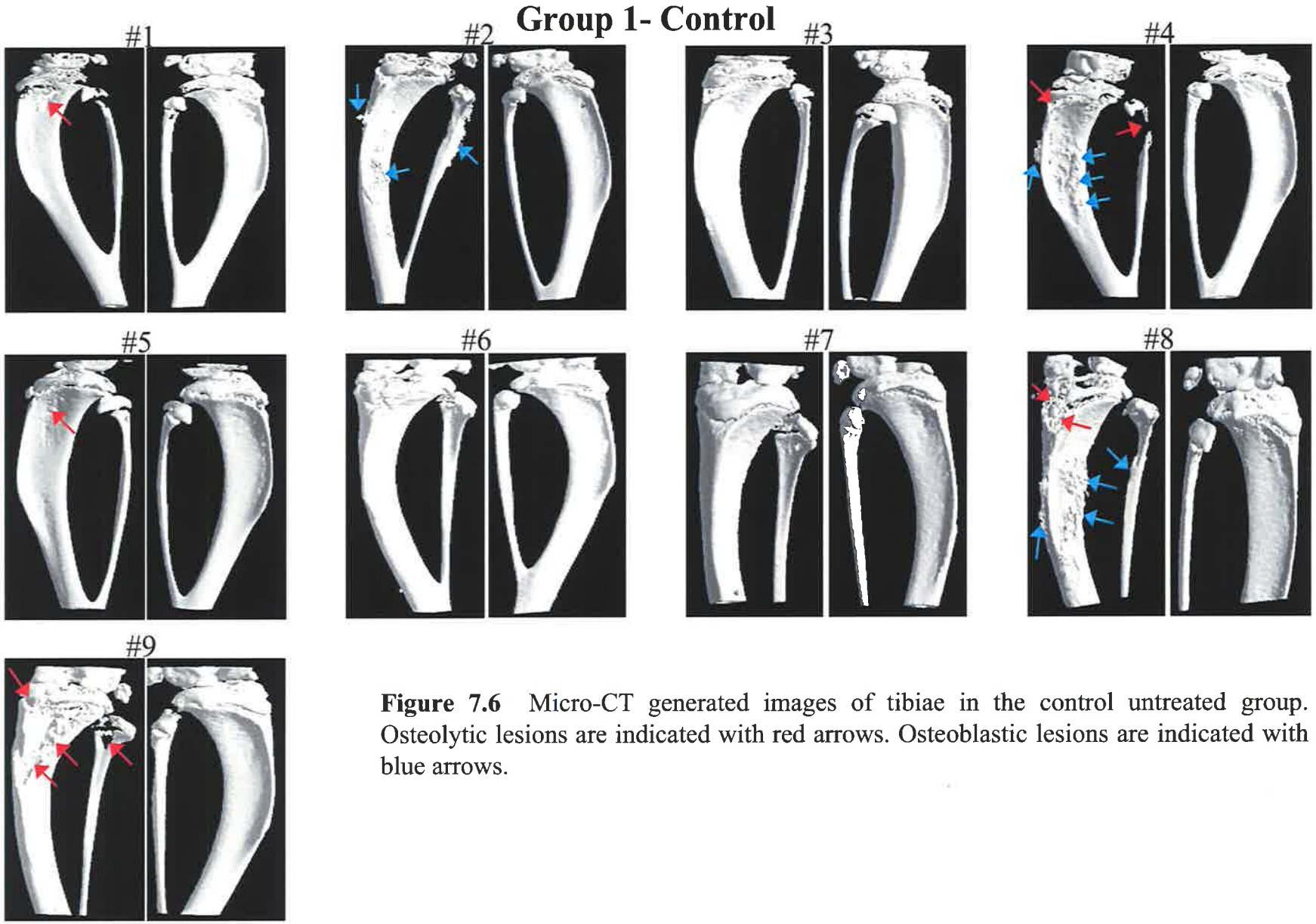


Figure 7.6 Micro-CT generated images of tibiae in the control untreated group. Osteolytic lesions are indicated with red arrows. Osteoblastic lesions are indicated with blue arrows.

control group were sliced through the middle (**Figure 7.7**) and showed significant damage through the inner bone cavity, due to osteolytic lesions, in animals #1, #4, #5, #8 and # 9. Animal #9 also showed thickening of the bone in the areas of increased bone formation. For the TRAIL treated group, radiographs revealed osteolytic lesions in animal #3 and #7 (**Figure 7.3**). The lesions were confirmed using μCT analysis, however another 3 animals (#1, #2 and #8) had the 'osteoblastic' phenotype in the inoculated tibia, as shown in **Figure 7.8**. Longitudinal tibial sections of μCT images also confirmed this finding, however increased bone thickness was not evident in animal #8 as the outer bone surface is not visible in this photo (**Figure 7.9**).

None of the ZOL treated animals showed any signs of osteolysis, as judged from the radiographic images (**Figure 7.4**). This was reflected in the μCT images, with 3 of the 10 animals showing the 'osteoblastic' phenotype, as depicted in **Figure 7.10** and **7.11**. Similarly, the radiographs from the combination of the ZOL and TRAIL treated animals showed no osteolytic lesions (**Figure 7.5**) and this was supported by the μCT images; however, 7 of the 11 animals did show areas of increased bone formation, as seen in **Figure 7.12**, which was not always apparent in the sliced images (**Figure 7.13**).

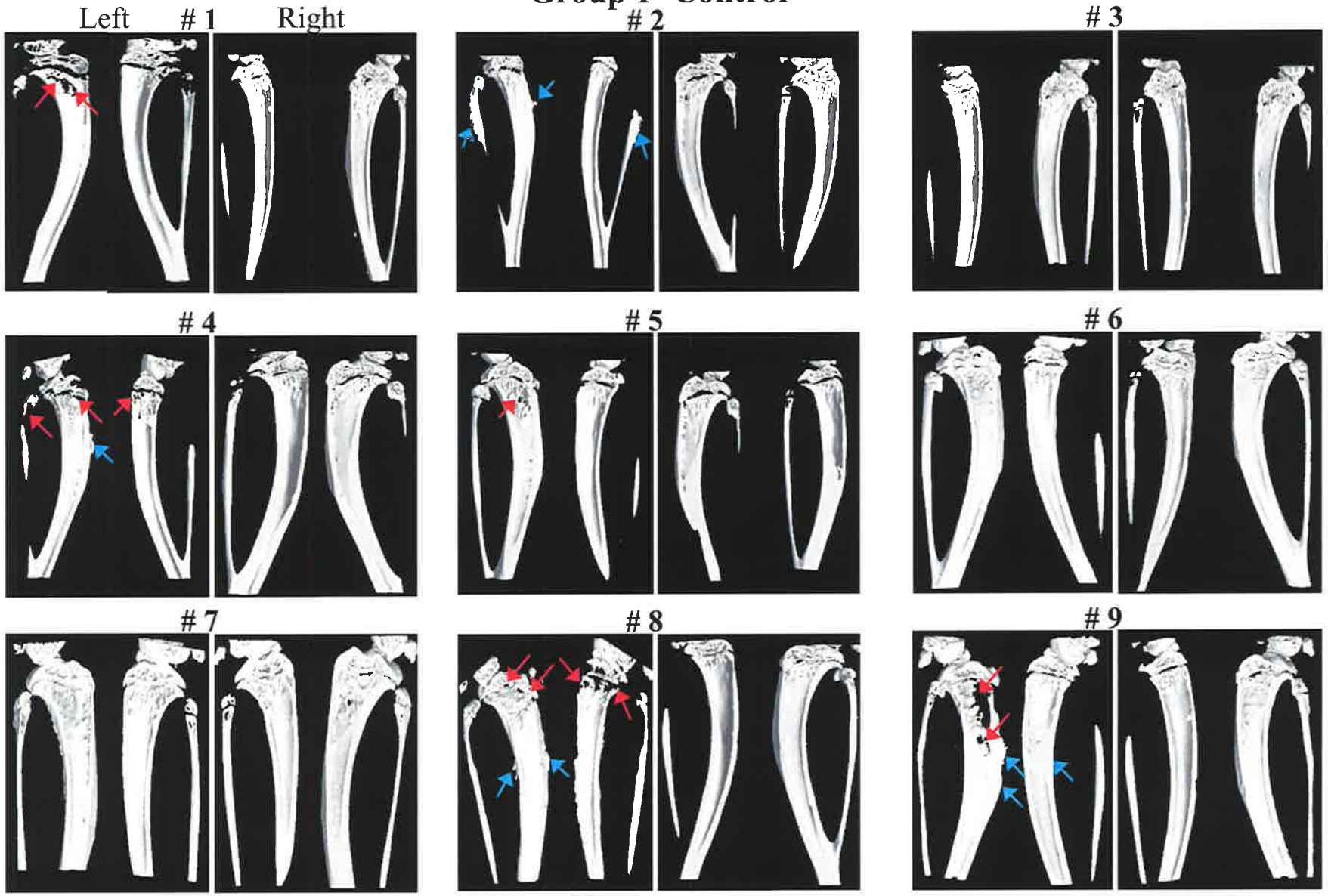
Due to the absence of osteolytic lesions in both the ZOL and combination ZOL and TRAIL treatment groups, it is not possible to assess the homogeneity of odds ratios between ZOL and TRAIL, therefore it could not be tested whether the combined effect is statistically different from ZOL or TRAIL alone. However, the ZOL treated group is statistically significantly different to the control untreated group ($p=0.01$) in terms of the number of osteolytic lesions.

Of particular interest was the development of the 'osteoblastic' phenotype observed in 18 of the 39 tumour-injected animals. **Figure 7.14** follows the process of μCT analysis beginning with scanning of the tibia, generation of the 2-D images and then creation of a 3 dimensional image. Increased areas of bone density were evident in

Figure 7.7 Micro-CT generated images of tibiae sliced in half from all animals in the control untreated group.

Osteolytic lesions are indicated with red arrows. Osteoblastic lesions are indicated with blue arrows.

Group 1- Control



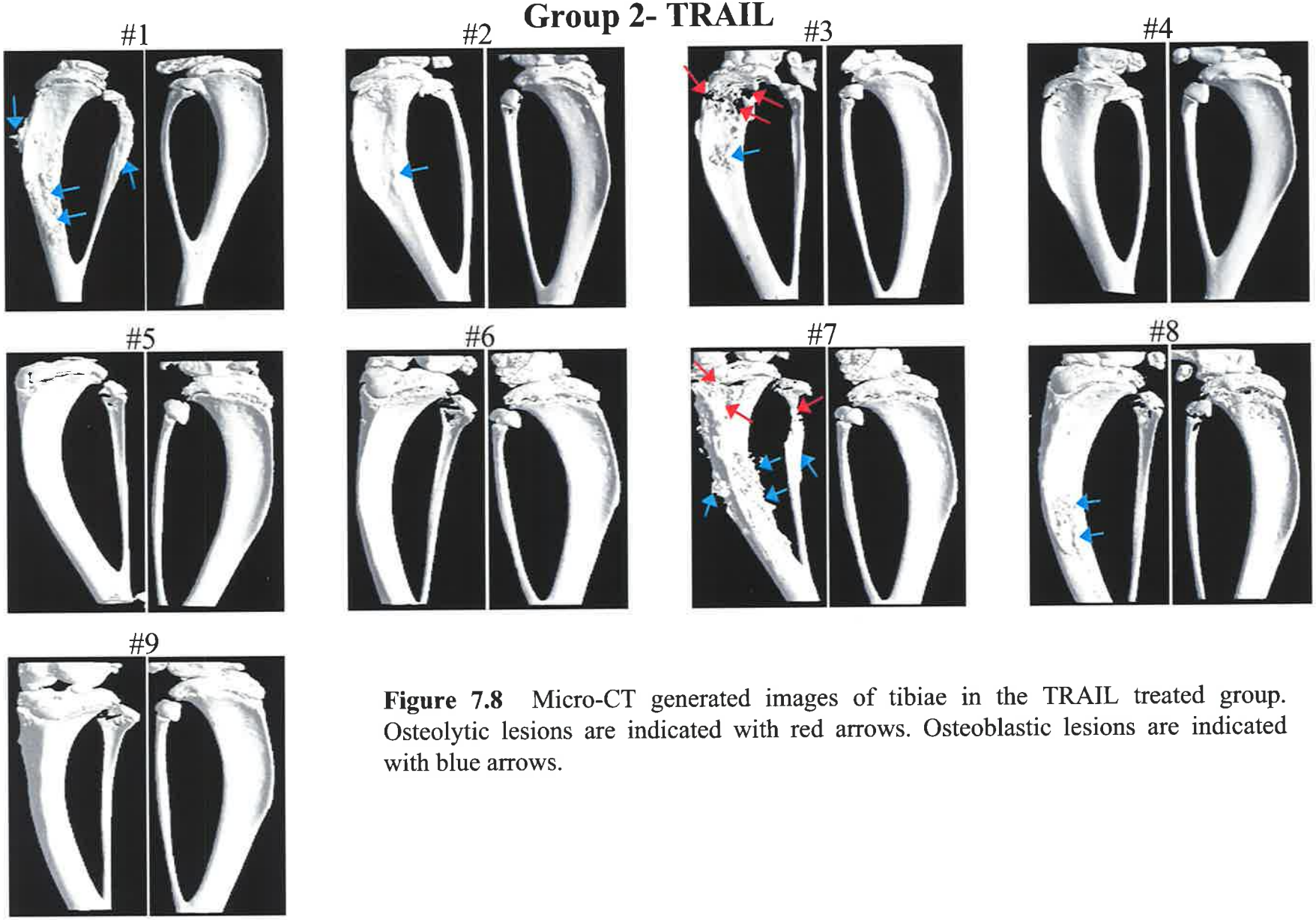
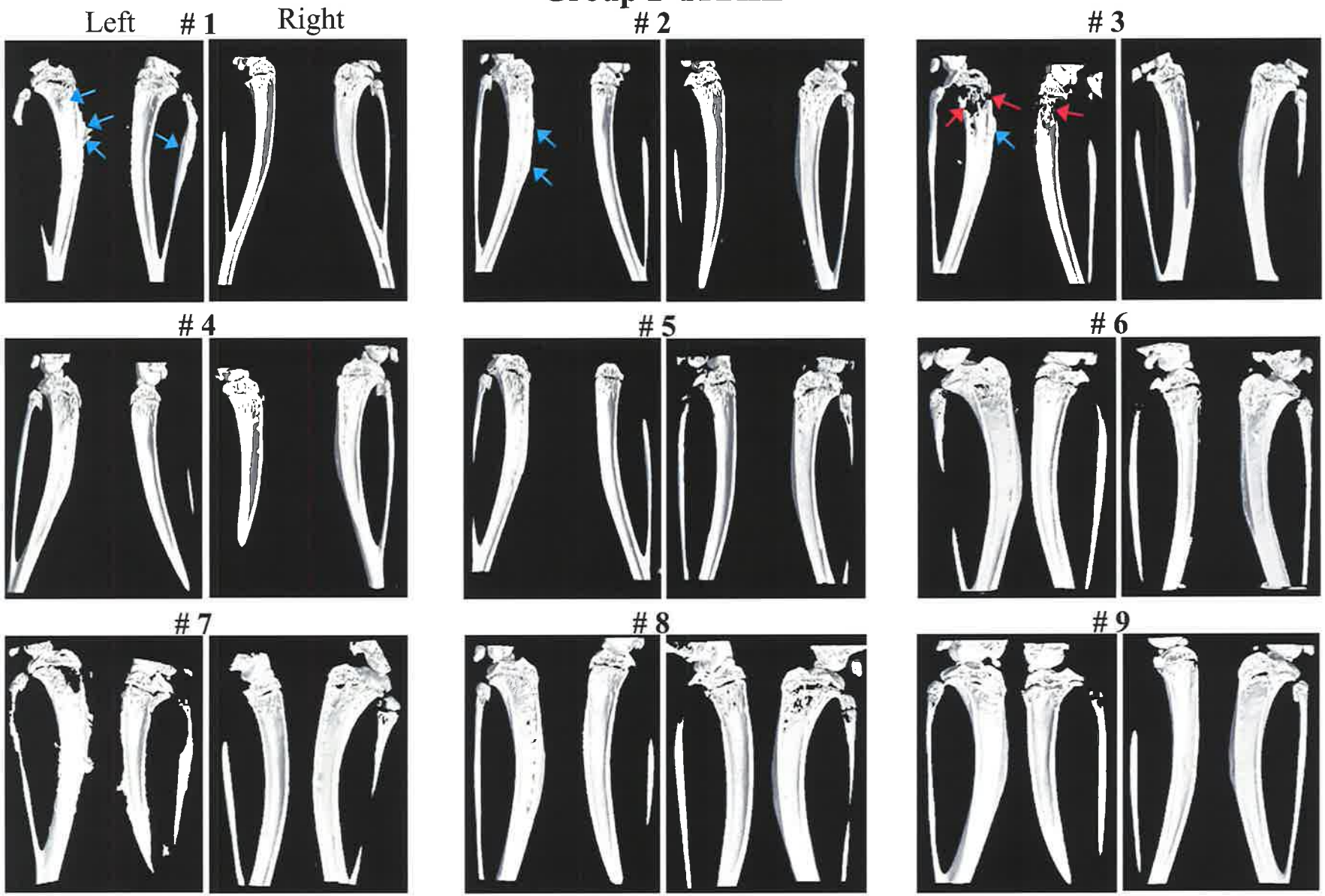


Figure 7.8 Micro-CT generated images of tibiae in the TRAIL treated group. Osteolytic lesions are indicated with red arrows. Osteoblastic lesions are indicated with blue arrows.

Figure 7.9 Micro-CT generated images of tibiae sliced in half, from all animals in the TRAIL group.

Osteolytic lesions are indicated with red arrows. Osteoblastic lesions are indicated with blue arrows.

Group 2-TRAIL



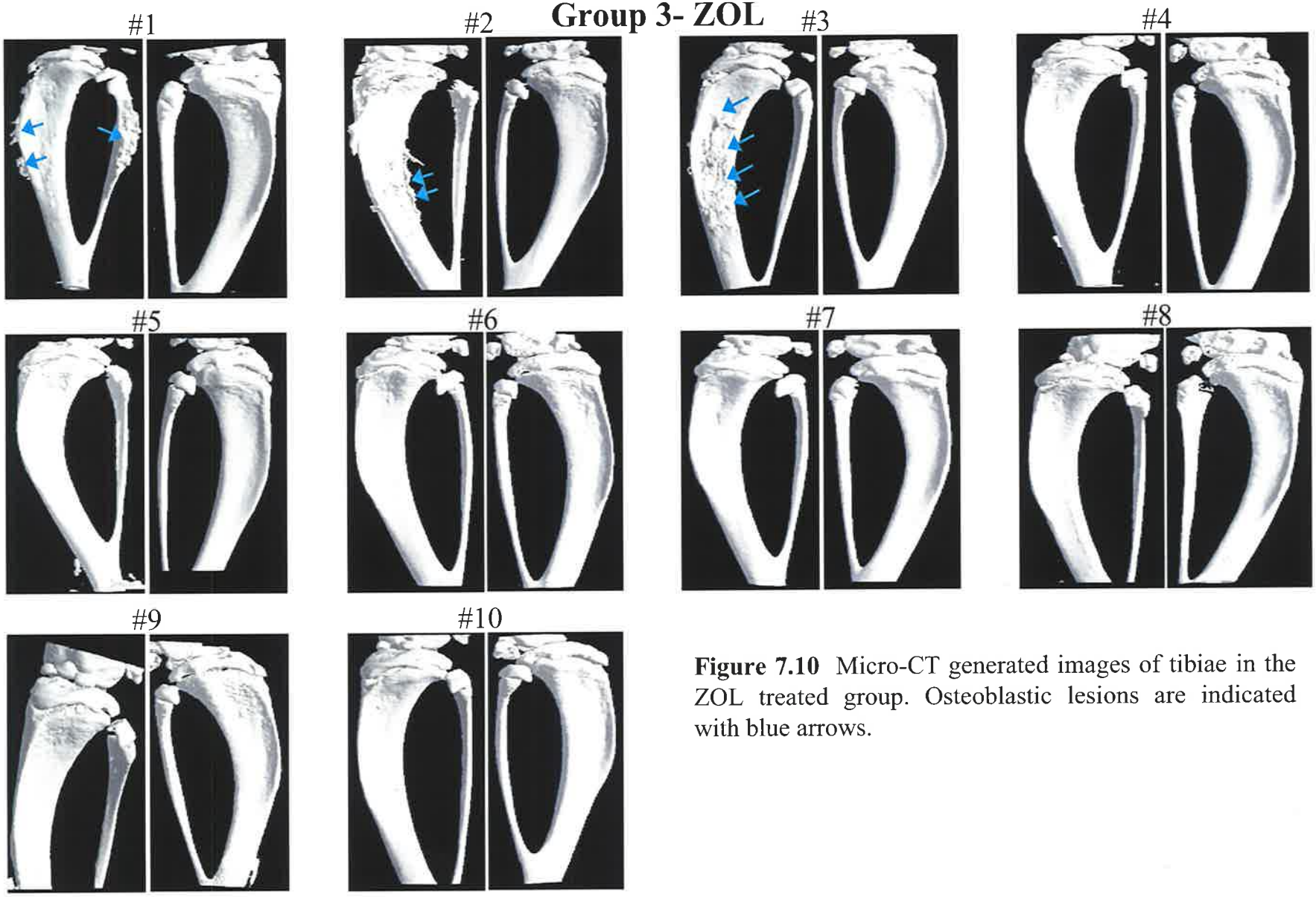
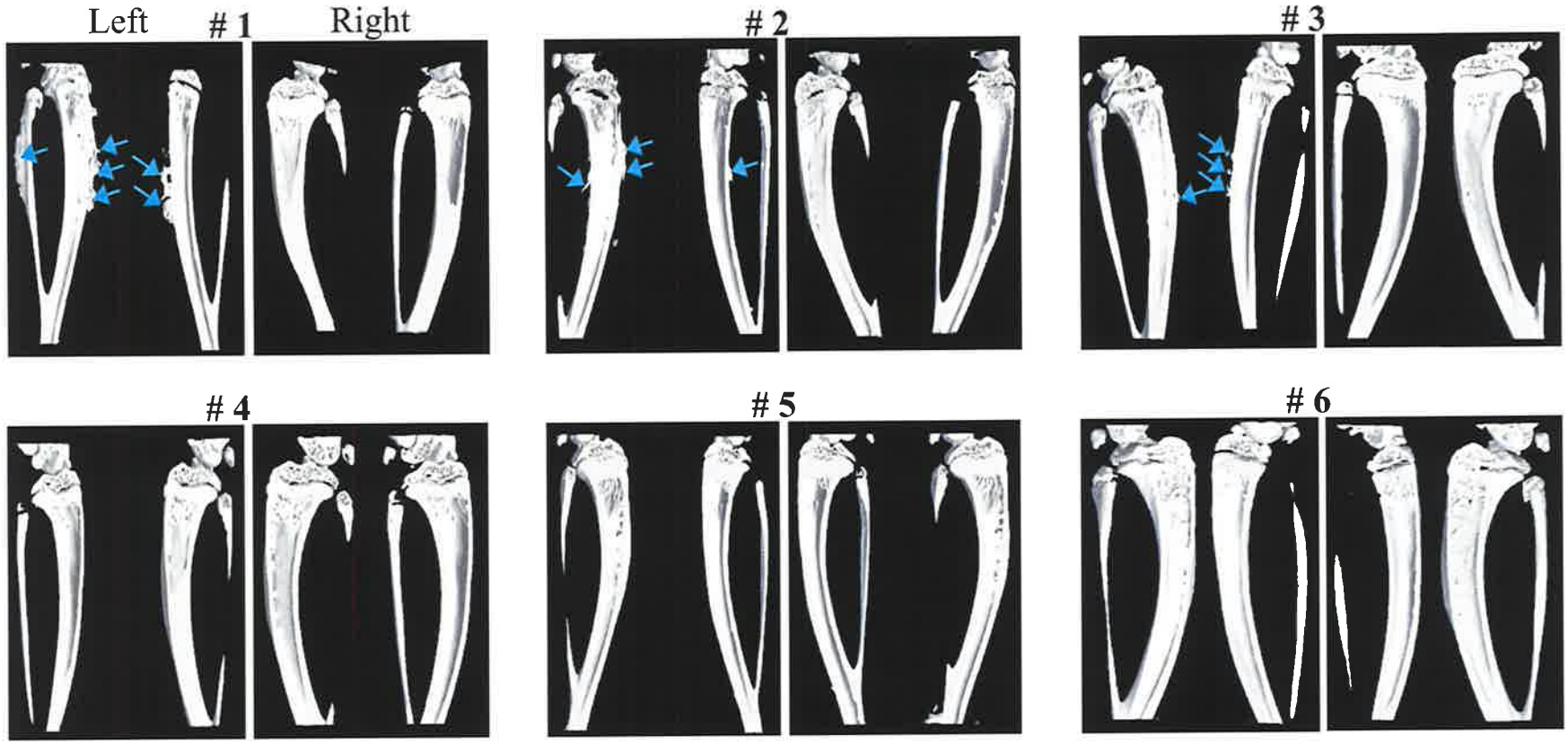


Figure 7.10 Micro-CT generated images of tibiae in the ZOL treated group. Osteoblastic lesions are indicated with blue arrows.

Group 3- ZOL



Group 3- ZOL

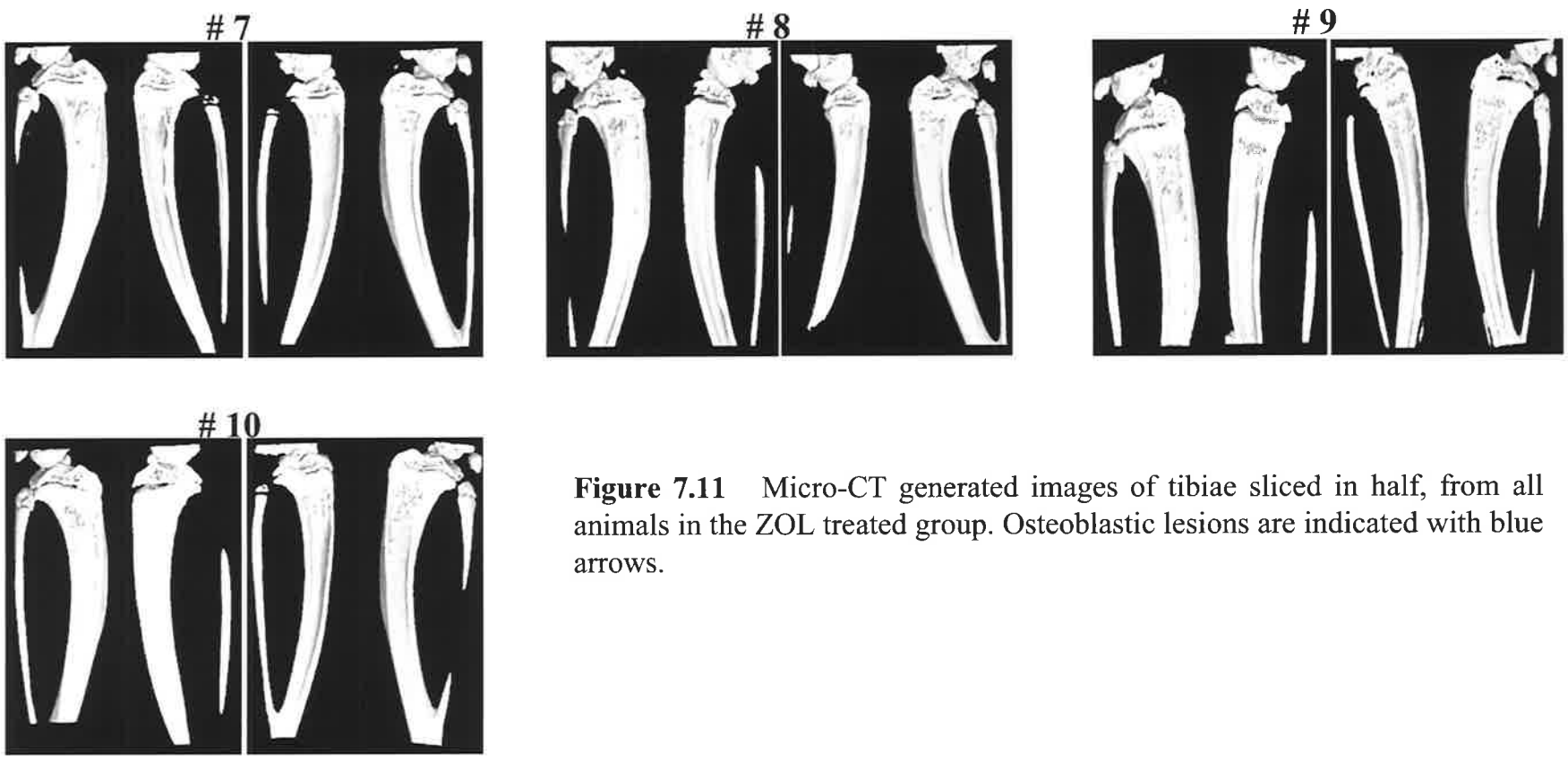


Figure 7.11 Micro-CT generated images of tibiae sliced in half, from all animals in the ZOL treated group. Osteoblastic lesions are indicated with blue arrows.

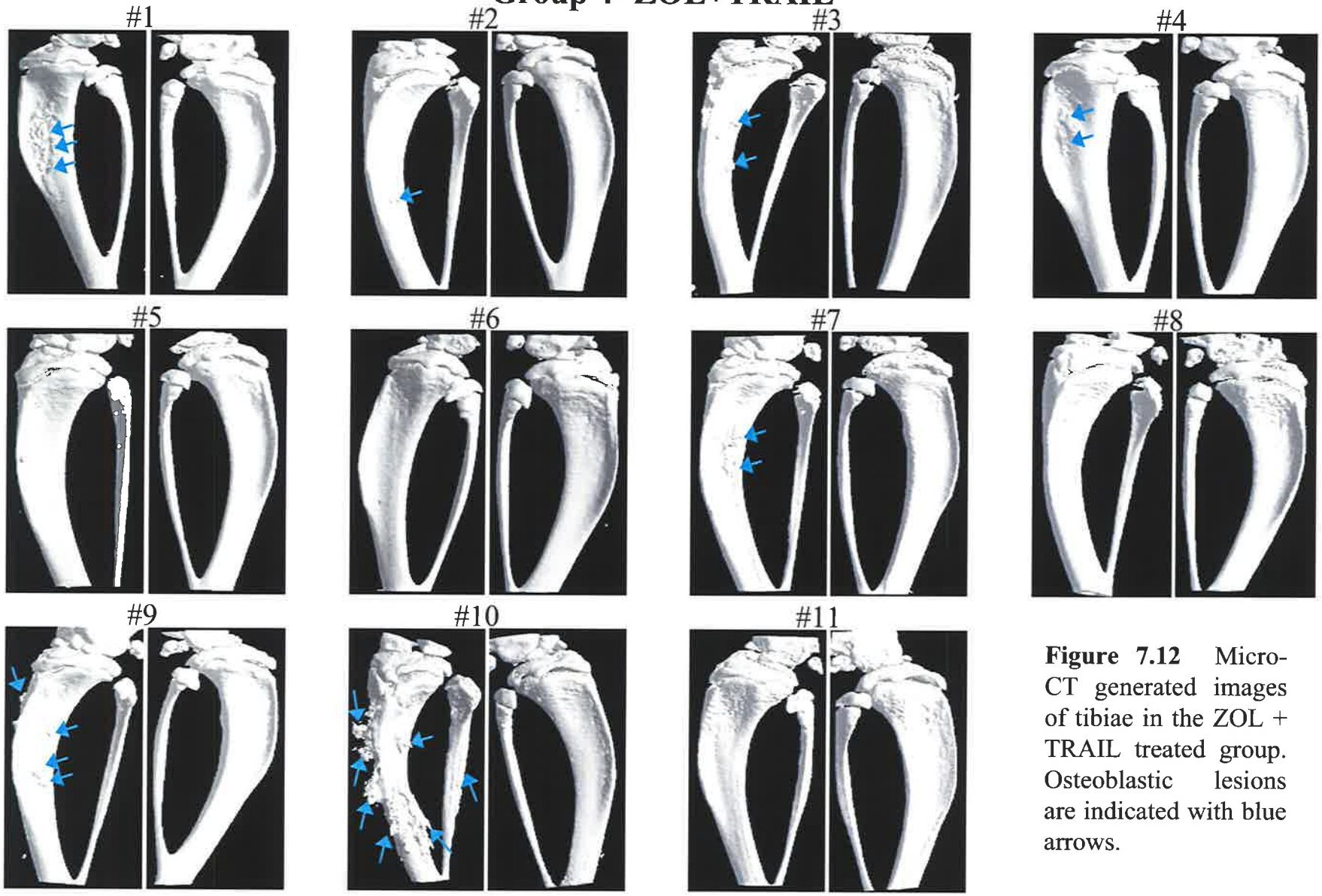
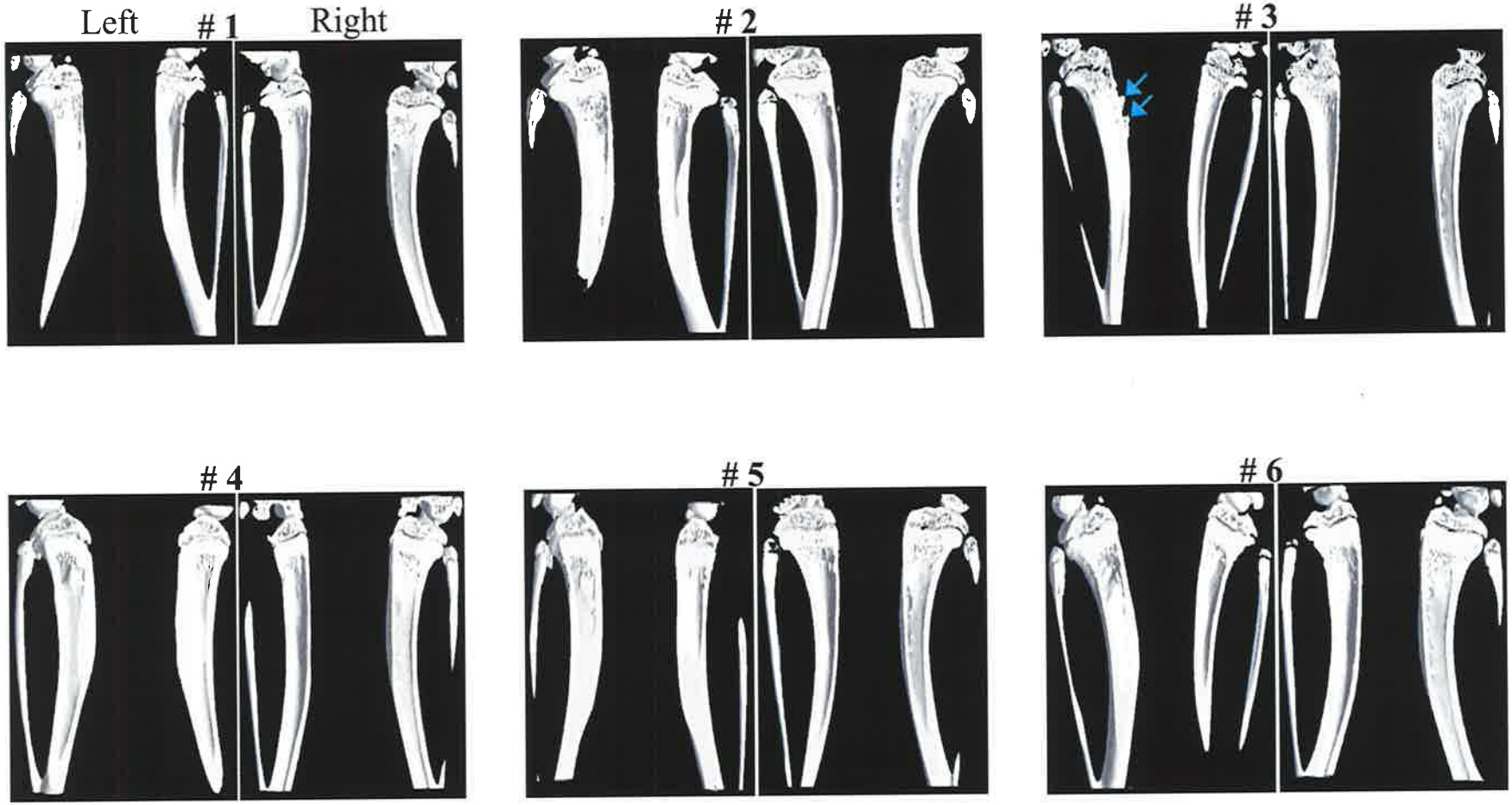


Figure 7.12 Micro-CT generated images of tibiae in the ZOL + TRAIL treated group. Osteoblastic lesions are indicated with blue arrows.

Group 5- ZOL+TRAIL



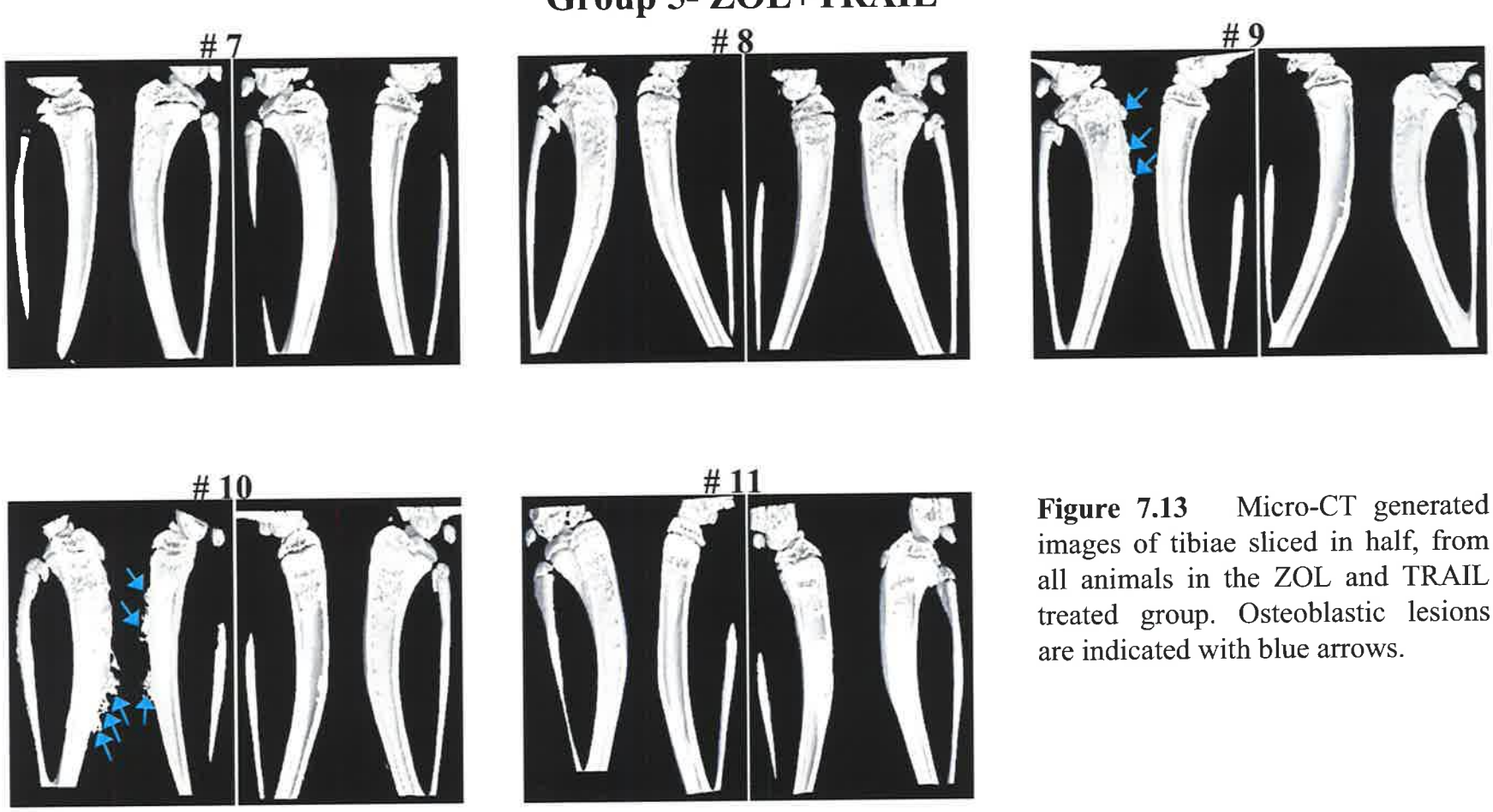


Figure 7.13 Micro-CT generated images of tibiae sliced in half, from all animals in the ZOL and TRAIL treated group. Osteoblastic lesions are indicated with blue arrows.

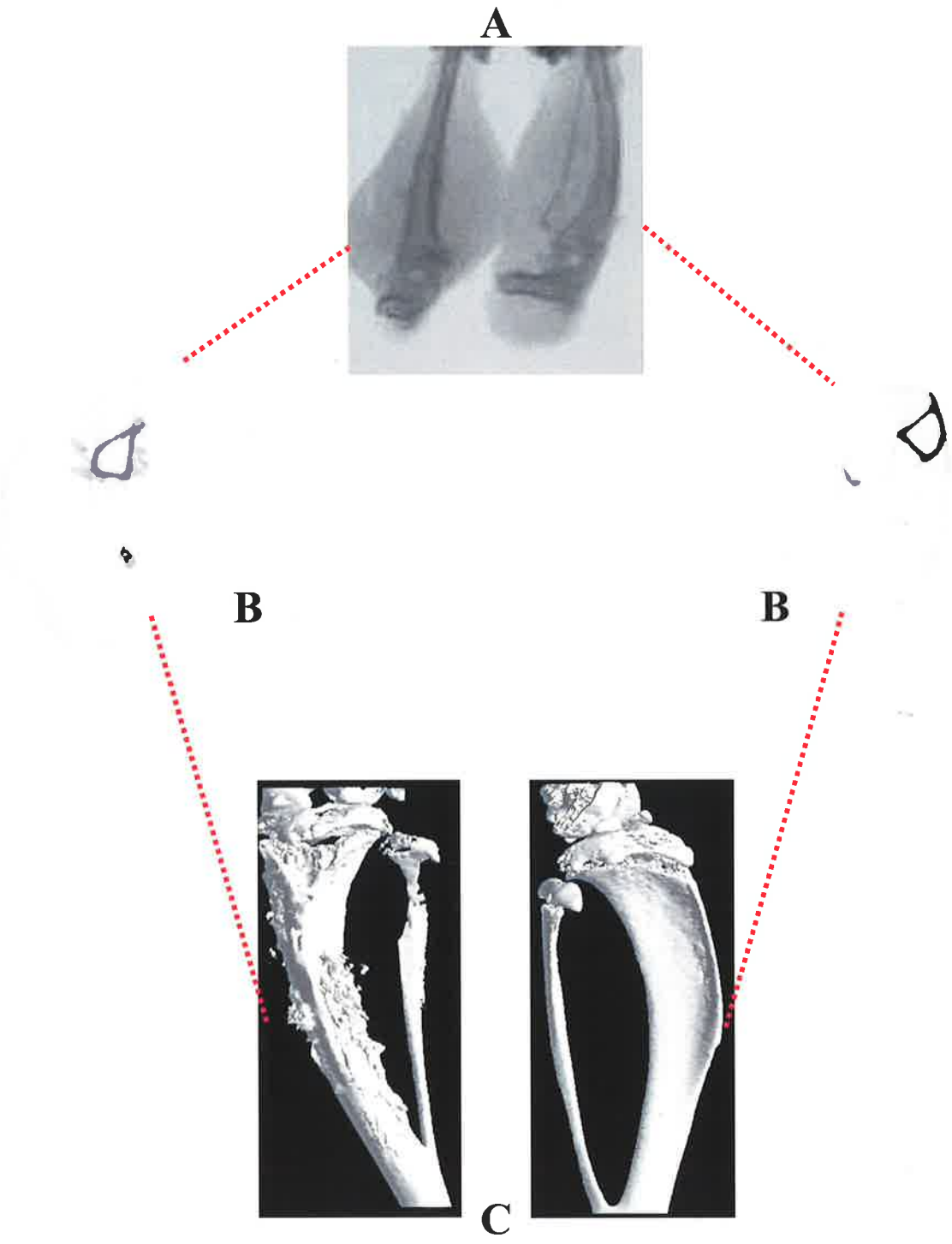


Figure 7.14 Comparison images showing the characteristic 'osteoblastic' phenotype observed in the K-HOS treated tibia (left) but not the contralateral tibia (right). ***A.*** Image of the tibia as observed during μCT analysis. ***B.*** 2D image of the tibia obtained during μCT analysis. The image on the left shows areas of increased density surrounding the tibia, represented by darker flame-like protrusions which are absent in the non-injected right tibia ***C.*** 3D images generated from 2D data reveal a high degree of 'osteoblastic' activity in the tibia injected with K-HOS cells.

the 2-D images, with flame-like protrusions typical in all tibiae exhibiting the 'osteoblastic' phenotype. These protrusions only extend from the tibiae outwards and, rather than pockets of mineral deposits as a result of the tumour itself, appear to be regions of accelerated bone formation. Possible reasons for this are discussed later in the chapter. Due to the decalcification process prior to histology, the 'osteoblastic' phenotype was not clearly visible in many sections, however some sections of the inoculated tibiae show clear evidence of these mineral deposits, as shown in **Figure 7.15**.

7.3.2 ZOL increases bone volume

Closer examination of the radiographs demonstrated that in all ZOL treated animals, and not in the untreated animals, the bones were highly radio-dense, suggesting increased bone density. This effect of ZOL was more pronounced in areas of increased bone turnover such as the distal femurs and proximal tibiae and was not restricted to the tumour site since the contralateral tibiae also showed this effect (**Figure 7.16**). Longitudinal sections of μCT images were compared between treatment groups. These images revealed that both groups treated with ZOL showed significant increases in trabecular density and cortical bone thickness, as shown in **Figure 7.17**. This supports the increase in radio-density observed in the radiographs (**Figure 7.16**).

The computer program CTan was used to analyse bone volume of the scanned tibiae. Using the 2-D images obtained from the μCT scan, the growth plate was identified and 150 sections, starting from the growth plate/tibial interface and moving toward the distal tibia, were selected, with each section representing 17.36 microns. Graphs representing bone volume were generated and compared to the control tibia. Differences between the left and right tibia were noted across all treatment groups, as

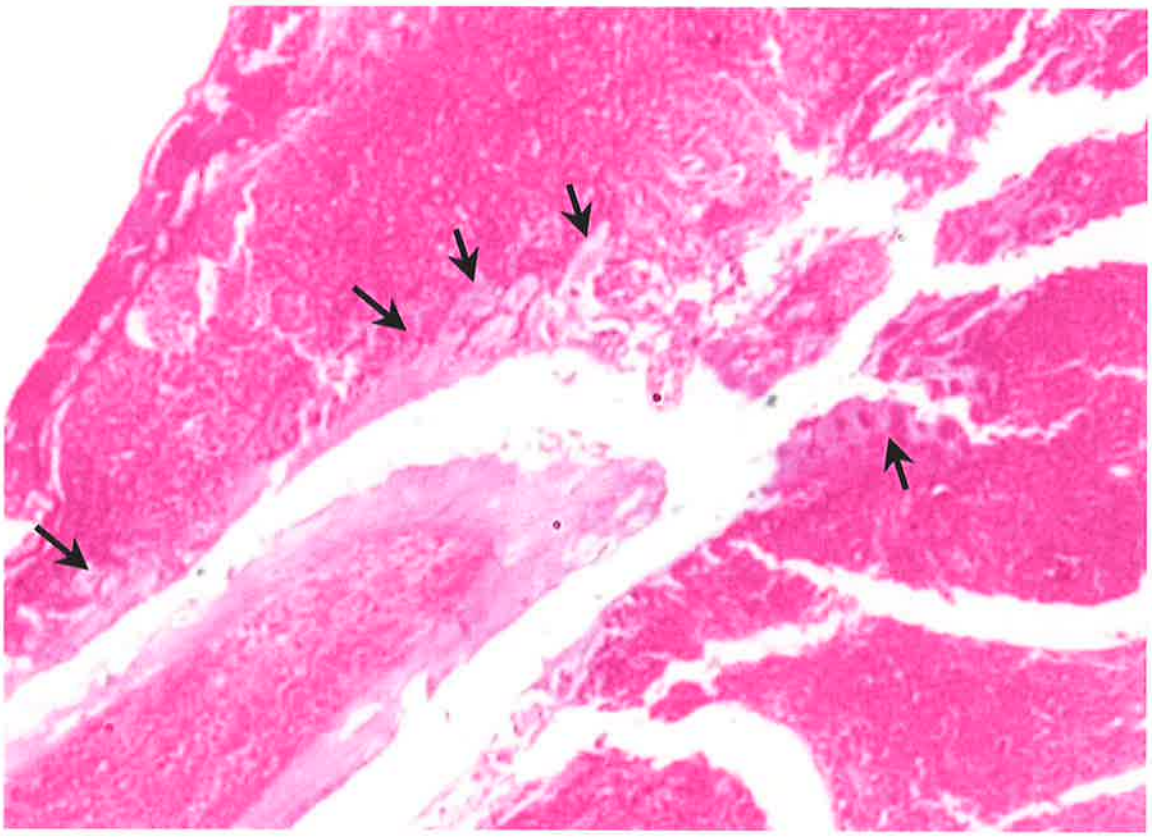


Figure 7.15 The photograph shown is a haematoxylin and eosin stained section of a mouse tibiae at 35 days post inoculation with K-HOS cells. Black arrows indicate areas of uneven bone growth which are shown as protrusions in the μCT images.

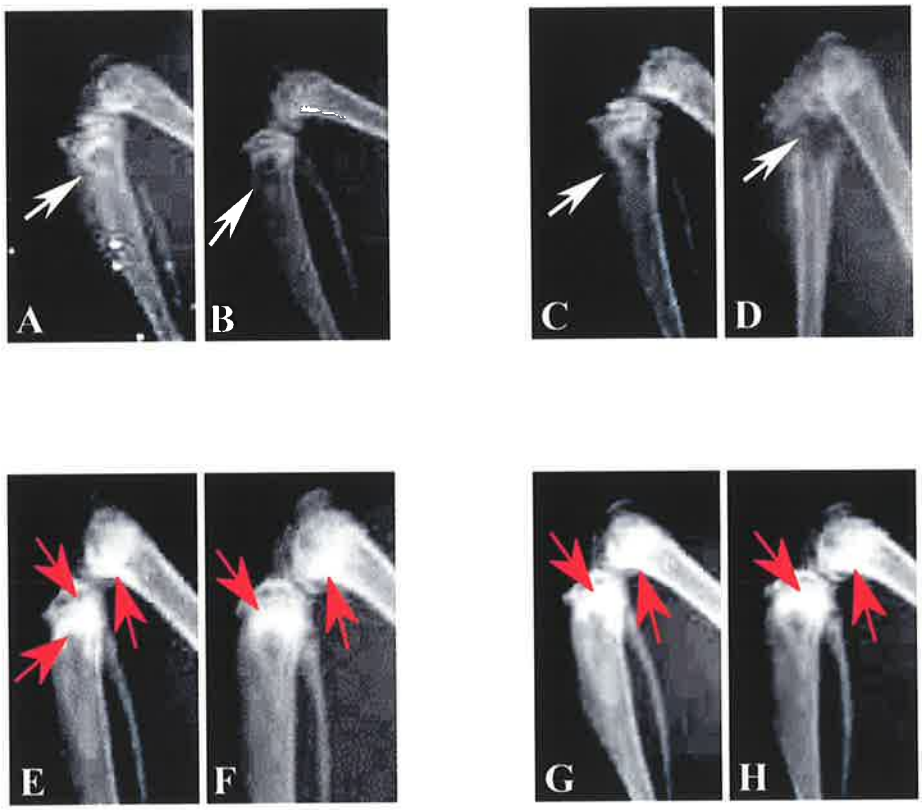


Figure 7.16 Radiographs of the inoculated tibiae at two week intervals. Photo **A** and **B** represent a control untreated animal; **C** and **D** represent a TRAIL treated animal; **E** and **F** represent a ZOL treated animal; and **G** and **H** represent a combination ZOL and TRAIL treated animal. White arrows indicate areas of osteolytic lesions; Red arrows indicated areas of increased radiodensity.

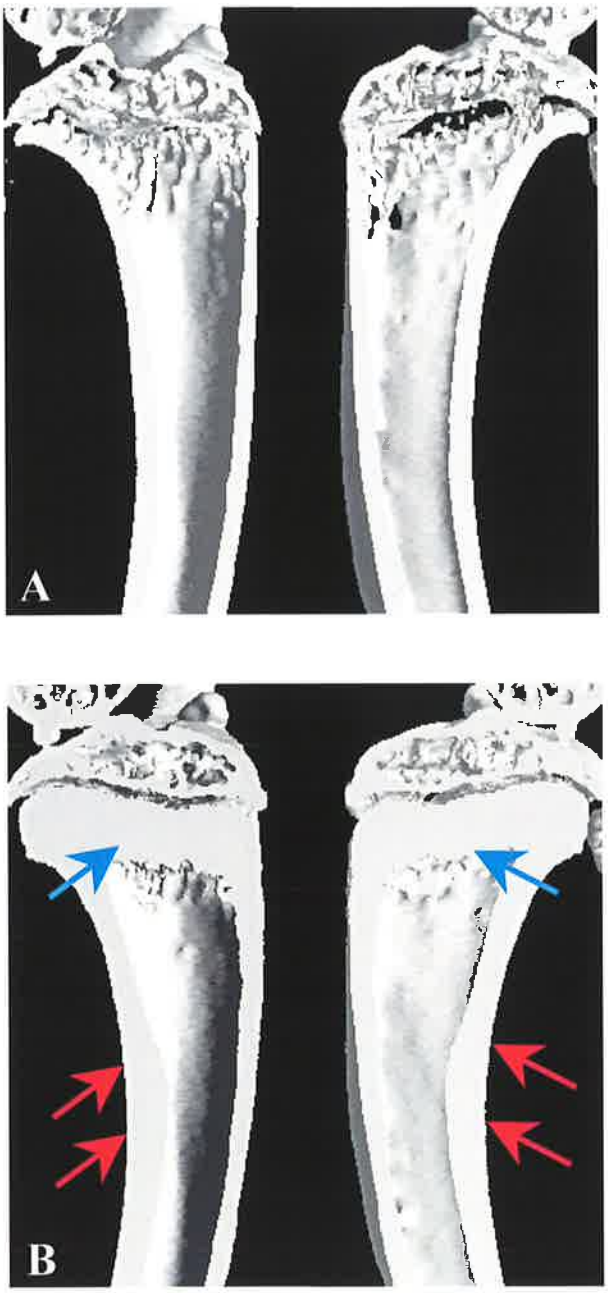


Figure 7.17 Micro-CT images of tibiae sliced through the middle from; ***A.*** control untreated animal and ***B.*** ZOL treated animal. Blue arrows indicate increases in trabecular density; Red arrows indicate increases in cortical bone thickness.

shown in **Figure 7.18**, with a slightly decreased average bone volume in the inoculated tibia of the control group and a slightly increased average bone volume in the inoculated tibia of the TRAIL treated group. However, it is important to note that there was a significant increase in bone volume between the ZOL treated groups and the non-ZOL treated groups. Bone volume increased by up to 2 mm^3 with ZOL treatment. This correlates with the increased radio-density of the radiographs and the increased thickness of the trabecular and cortical bone observed with μCT analysis. Marked differences in histology, between the ZOL treated and untreated animals were also apparent (**Figure 7.19**). The histological findings correlate well with the radiographs and the μCT images.

There was a statistically significant increase in bone volume due to ZOL treatment ($p=0.0414$) compared to the non-ZOL treatment groups. There was no interaction between ZOL and TRAIL nor was there any significant effect of TRAIL on bone volume in tumour-bearing legs.

7.3.3 ZOL had no effect on tibial tumour burden but TRAIL reduced tumour burden

The tibiae were fixed in 10% buffered formalin, followed by acid decalcification at room temperature. Tibiae were paraffin embedded, sectioned longitudinally and stained with haemotoxylin and eosin prior to analysis on an inverted microscope. Analysis of the sections revealed that there were several animals that had no evidence of any tumour burden. A photograph was taken of the best representative section of the tibia for each animal and imported into the Scion program. The area of the tumour in mm^2 was determined for each animal and tabulated, as shown in **Table 7.1**. Animals #2, #4, #8 and #9 of the control untreated group, all had macroscopic tumours which is

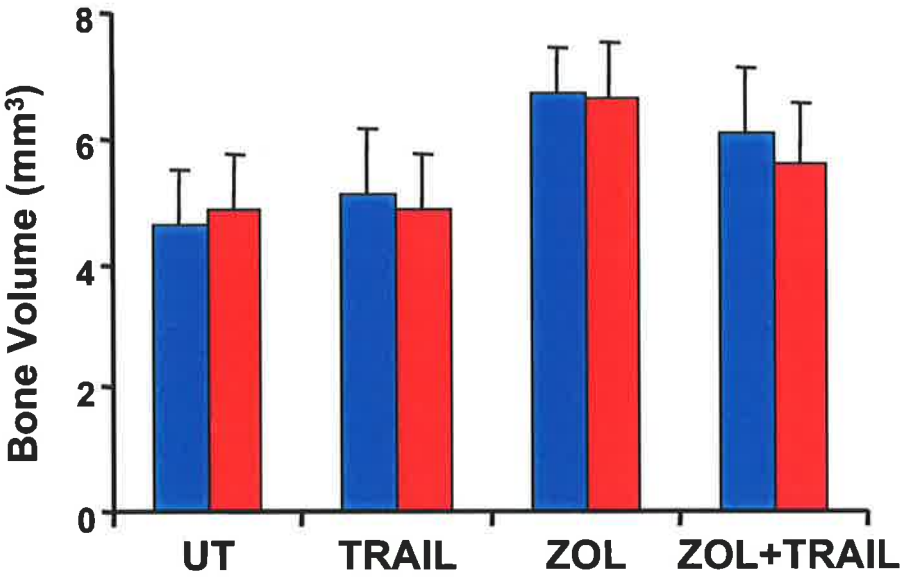


Figure 7.18 Bone volume comparisons between the control untreated group, the TRAIL treated group, the ZOL treated group and the combination ZOL and TRAIL treatment group. The above graph shows the bone volume in the ■ left tibiae and ■ the right tibiae of each group based on analyses using the program CTan. Bone volume was measured from 150 sections of the proximal tibia starting from the growth plate. Data shown in each case is the average bone volume from all animals in that group: points are means ±SD.

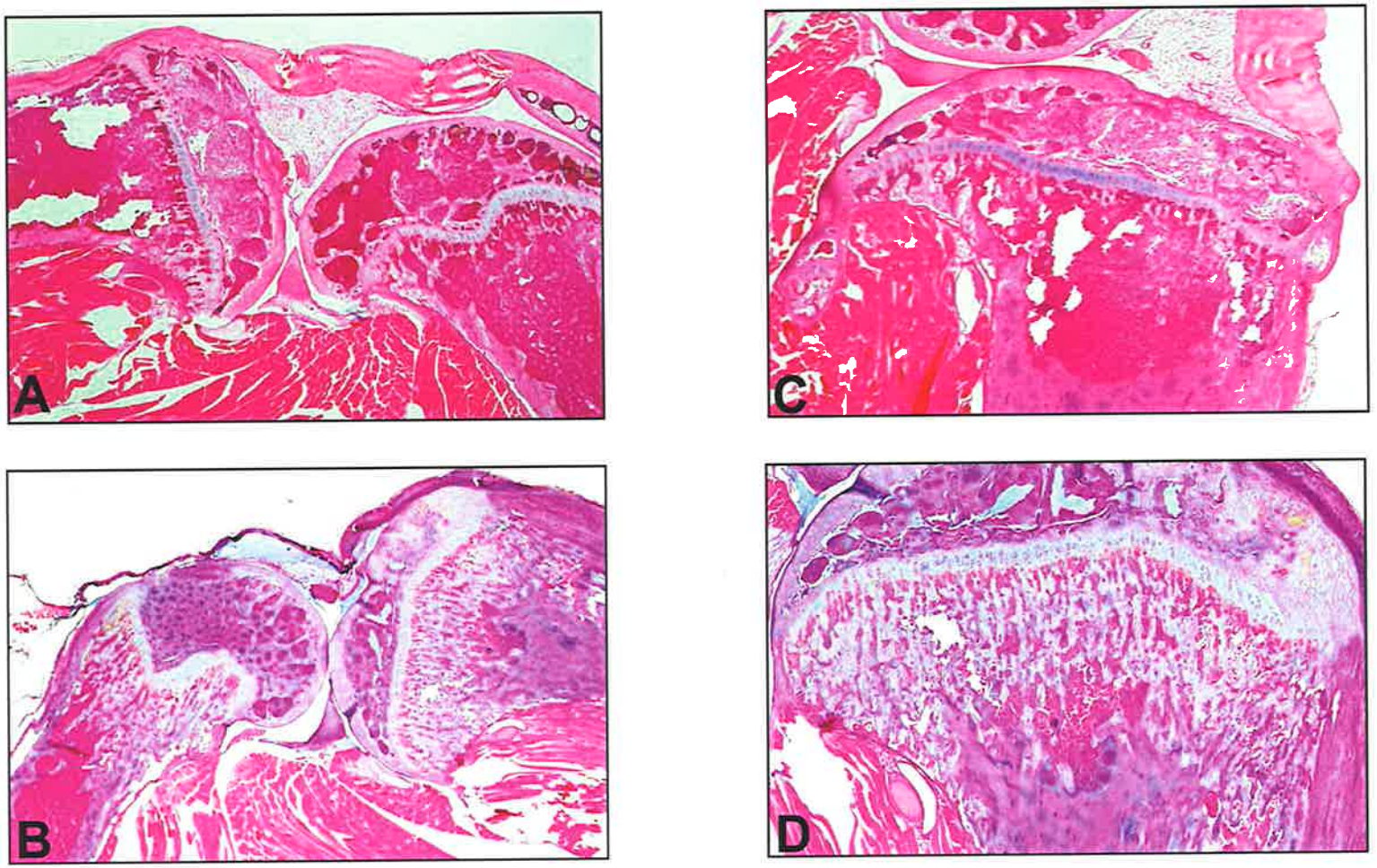


Figure 7.19 Photographs shown are representative of haematoxylin and eosin stained sections of mouse tibiae at 35 days post inoculation with K-HOS cells. Panel (**A**) and (**B**) represents an untreated control animal with normal trabecular density and cortical bone thickness. Panel (**C**) and (**D**) represents a ZOL treated animal with increased trabecular density and cortical bone in both the tibia and the femur.

Animal	UT	T	Z	Z+T
#1	0	22.21	29.04	8.16
#2	26.34	4.58	28.96	9.26
#3	0	5.58	21.2	16.77
#4	11.58	0	0	3.65
#5	0	0	0	0
#6	0.1	0	0	0
#7	0	18.97	0	7.94
#8	19.71	3.76	0	0
#9	14.81	0	0	8.33
#10	-	-	0	31.21
#11	-	-	-	0

Table 7.1 Area of tumour in the inoculated tibia. Histological photographs were taken and tumour area measured in mm^2. Animals showing no visible tumour were assigned a value of zero.

evident in the histology, resulting in large areas of tumour burden. Animal #6 had a very small area of tumour mass within the marrow cavity but showed no visible signs of tumour from either the radiographs or the μCT images. The average area of tumour in the affected animals of the control untreated group was 14.5 mm^2. In the TRAIL treated group, 5 out of 9 animals had tumours, based on histological analysis. Of these, only 3 out of the five were evident from the radiographs but all were identified from the μCT images. The average area of tumour in the affected animals of the TRAIL treated group was 11.02 mm^2. It is clearly evident from the radiograph (**Figure 7.4**) that animals #1, #2 and #3 of the ZOL treated group had visible tumours and this is reflected in the tumour area calculated from the histological sections. In the combination of ZOL and TRAIL treated group, 7 out of 11 animals developed quantifiable tumours, with an average area of 12.2 mm^2. However, tumour growth was solely confined to regions outside the bone for all animals treated with ZOL. The average tumour area for each group was calculated, excluding animals with no tumour cells present, and is shown in **Figure 7.20**. From the graph, it can be seen that ZOL treated animals had the greatest tumour area, with TRAIL treated animals showing the least tumour area. There was a statistically significant reduction in tumour area in the treatment groups given TRAIL (P=0.0481), when compared to non-TRAIL treated groups.

7.3.4 ZOL and TRAIL have no effect on lung metastases

Upon sacrifice of the animals, the lungs were removed and prepared for histology. Approximately 3 sections at 3 levels were obtained for each animal and viewed under the microscope for the detection of lung metastases. The number of animals identified with lung metastases were, 2, 3, 3, and 3 for the untreated control group, TRAIL treated group, ZOL treated group and combination ZOL and TRAIL treated group,

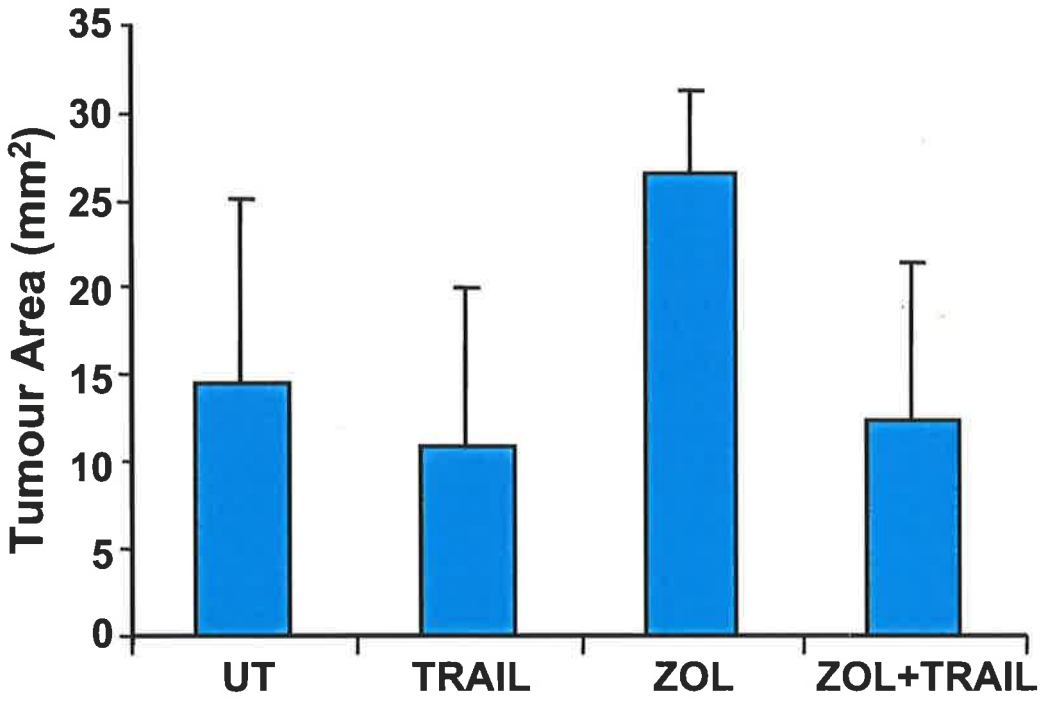


Figure 7.20 Average tumour area per group. Histological sections were photographed and used to measure tumour area in mm^2. Animals with no tumour cells present were eliminated from the calculation. Data shown in each case are from a representative section of each animal: points are means ±SD.

respectively. All mice showing lung metastases also exhibited osteoblastic lesions but only 1 mouse showed both osteolytic and osteoblastic lesions (**Table 7.2**). Mice presenting with only osteolytic lesions did not possess lung metastases.

There was no statistically significant interaction between ZOL and TRAIL, and neither ZOL nor TRAIL significantly impacted independently on the presence of lung metastases.

	UT			T			Z			Z+T		
Animal	Osteolytic Lesions	Osteoblastic Lesions	Lung Metastases	Osteolytic Lesions	Osteoblastic Lesions	Lung Metastases	Osteolytic Lesions	Osteoblastic Lesions	Lung Metastases	Osteolytic Lesions	Osteoblastic Lesions	Lung Metastases
#1	+	-	-	-	+	+	-	+	+	-	+	+
#2	-	+	+	-	+	+	-	+	+	-	+	+
#3	-	-	-	+	+	-	-	+	+	-	+	+
#4	+	+	+	-	-	-	-	-	-	-	+	-
#5	+	-	-	-	-	-	-	-	-	-	-	-
#6	-	-	-	-	-	-	-	-	-	-	-	-
#7	-	-	-	+	+	+	-	-	-	-	+	-
#8	+	+	-	-	+	-	-	-	-	-	+	-
#9	+	-	-	-	-	-	-	-	-	-	+	-
#10							-	-	-	-	-	-
#11										-	-	-

Table 7.2 Frequency of osteolytic, osteoblastic lesions and lung metastases. Animals left untreated (UT) and treated with T (TRAIL) and/or Z (ZOL) are assigned + for the presence of, and - for absence of lesions or lung metastases. Osteolytic and osteoblastic lesions were identified using micro-CT analysis and lung metastases were identified using histological sections.

7.4 DISCUSSION

This is the first study to investigate the effect of ZOL in an animal model of human OS. K-HOS cells were inoculated into the left proximal tibia of nude mice. When left untreated, these developed large lesions that invaded the marrow cavity and began to erode the cortical bone. Treatment with 30mg/kg/dose of TRAIL for 5 consecutive days followed by once weekly injections, had little effect, and large osteolytic lesions similar to control animals were seen. In mouse models, TRAIL has demonstrated remarkable efficacy against tumour xenografts of colon carcinoma [LeBlanc *et al.* 2002; Ashkenazi *et al.* 1999] breast carcinoma [Walczak *et al.* 1999] and glioma [Pollack *et al.* 2001; Fulda *et al.* 2002]. In this current study, it was not surprising that osteolytic lesions developed despite TRAIL treatment, as TRAIL had no effect on K-HOS cells *in vitro*. In contrast, all animals treated with 100 μg/kg/dose of ZOL, once weekly for four weeks, showed remarkable conservation of the tibiae with no radiographic or μCT evidence of bone destruction. In addition, no evidence of tumour growth within the bone was identified upon histological examination. This data supports previously published reports of the protection of bone by ZOL from cancer-induced osteolysis [Alvarez *et al.* 2003; Hiraga *et al.* 2004; Lee *et al.* 2002; Croucher *et al.* 2003; Green 2003; Green 2002; Peyruchaud *et al.* 2001]. As both the ZOL treated and combination of ZOL and TRAIL treated animals showed no osteolysis, it could not be determined whether the combination treatment was any more beneficial than ZOL alone. A dose response study with differing ZOL concentrations would be required to investigate this matter further.

It has been reported that at an active bone resorption site the concentration of BPs is highest and equates to the concentration required to induce apoptosis of cancer cells *in vitro* [Sato *et al.* 1991]. This concentration of BPs is not achievable in visceral organs *in vivo* [Fleisch 2000]. As OS-induced lesions are areas of high osteoclastic activity, it

may be possible that ZOL is released in the direct vicinity of the surrounding cancer cells. It is not known whether ZOL directly affects cancer cells *in vivo* or whether it causes changes to the bone environment that indirectly impact these cells. Studies demonstrating increased apoptosis of tumour cells in osteolytic lesions in mice treated with ZOL and other BPs have been demonstrated but this is not conclusive proof of a direct anti-tumour effect [Hiraga *et al.* 2001; Yaccoby *et al.* 2002]. To reproduce the augmented apoptotic effects observed with the combination treatment of ZOL and TRAIL *in vitro*, it is therefore essential that the tumour cells be directly exposed to ZOL *in vivo*.

Comparisons of radiographs between treatment groups revealed highly radio-dense areas within the bone of the ZOL treated animals, suggesting increased bone density. The 100 μg/kg/dose of ZOL used is approximately equivalent to the human 4 mg dose, which is given once a month to tumour patients and not every week, as was administered to the mice [Berenson *et al.* 2001; Berenson *et al.* 2002]. This corresponds to a 4-fold higher dose and this was perhaps manifested in the development of dense bone, especially at the ends of the growing long bones. The use of μCT 3-D reconstructions and histological sections confirmed that ZOL treated animals showed significant increases in cortical bone thickness and trabecular density, when compared to the untreated animals. This observation is commonly found in BP treated animals [Sohara *et al.* 2003; Corey *et al.* 2003]. The bone volume, as measured by μCT, significantly increased in ZOL treated animals and was more pronounced in areas of increased bone turnover, such as the distal femurs and proximal tibiae, but was not restricted to the tumour site since the contralateral tibiae also showed this effect. This correlated with the radio-dense radiographs and the histology. Statistical analyses confirmed a significant increase of bone volume in ZOL treated animals compared to untreated. Clinically, patients are given BPs primarily to prevent further bone loss,

rather than increasing bone density [Fleisch 2000], although some increase in bone mass appears to occur with this class of anti-resorptive [Reid *et al.* 2002; Orwoll *et al.* 2000; Schnitzer *et al.* 2000]. However, it is difficult to compare events in young growing animals with those in older and osteoporotic human individuals.

The average area of tumour growth was lowest in the TRAIL treated and the combination of ZOL and TRAIL treated groups than the untreated and ZOL treated groups, suggesting that TRAIL may have an effect on the extra-osseous tumour. Although TRAIL alone had no effect on K-HOS cells *in vitro*, it is plausible that there may be an effect *in vivo* due to other factors. Statistical analysis based only on the animals with detectable tumour cells, showed a significant decrease in tumour area in TRAIL treated animals. The average tumour area per group was also calculated using these animals. The rationale for this was to exclude animals, in which there may have been technical errors upon intra-tibial injection, resulting in the absence of a lesion. However, the statistical significance did not hold true upon inclusion of all animals per group. Further experiments are required to support or reject the current data.

Histological examination of lung tissue revealed no difference between the treatment groups with regard to metastatic lesions. The only correlation made between the mice with metastases was that they all had 'osteoblastic' lesions. It is possible that the large non-osseous tumours often associated with these lesions had a larger, more complex vasculature and were therefore more likely to allow transport of cancer cells to the lungs, where they were able to establish and grow. Although ZOL protects the skeleton from cancer-induced cell death, it does not prevent lung metastases, which are often associated with increased mortality. This may indicate that there are no direct effects of ZOL *in vivo* under these conditions and that the observed effects on tumours in bone are related to inhibition of osteolysis. Alternatively, the lack of inhibitory effects on lung metastases may be due to poor bioavailability of ZOL, because it

accumulates in bone with limited access to soft tissues. Reports of the effect of N-BPs on visceral metastases have been conflicting, with trends towards either a decrease [Alvarez *et al.* 2003; Nobuyuki *et al.* 2001; Sasaki *et al.* 1998] or an increase in visceral metastases [Stearns and Wang 1996; Sasaki *et al.* 1998; Sasaki *et al.* 1995; Cruz *et al.* 2001]. The use of ZOL in conjunction with current chemotherapy would provide treatment for both OS-induced osteolysis and lung metastases. The efficacy of ZOL combined with anti-cancer agents *in vitro* has been well documented [Matsumoto *et al.* 2005; Vogt *et al.* 2004; Hiraga *et al.* 2003; Tassone *et al.* 2000; Jagdev *et al.* 2001]. Most clinical studies have been performed using BPs in combination with conventional anti-cancer agents [Body 2003; Body *et al.* 1998; Diel *et al.* 1998; Coleman and Seaman 2001; Berenson *et al.* 2001; Rogers *et al.* 2000; Rosen *et al.* 2003; Rosen *et al.* 2004; Rosen *et al.* 2003]. These clinical studies have all focused on metastases to the bone rather than OS, so there is a need to investigate the protective effect of BPs, specifically ZOL, in combination with agents capable of targeting both osseous and non-osseous tumours. Alternatively, ZOL could be used in combination with other IMP such as the statins as another way of targeting both osseous and non-osseous tumours.

Micro-CT analysis proved to be the most informative tool in assessing the effect of ZOL on human OS cells *in vivo*. It provided an extremely detailed analysis of the inoculated tibia, which could be further manipulated for other purposes, such as dissecting, rotating and bone volume analyses. Radiographs clearly showed relatively large osteolytic lesions in 3 animals of the control untreated group, however, the improved sensitivity of μCT allowed smaller lesions to be recognized, identifying a total of 5 animals with lesions. Micro-CT confirmed the absence of osteolysis in the tibiae of all animals treated with ZOL. In addition, μCT identified the presence of an 'osteoblastic' phenotype, in the form of small protrusions extending from the bone surface in over half the animals, which could not be discerned in the radiographs, or in

most of the histological sections due to the decalcification process. One possibility is that K-HOS cells may possess a mixed osteolytic and osteoblastic phenotype. ZOL has been shown to exhibit inhibitory effects on both osteoblastic and osteolytic lesions and is the only BPs to show a direct reduction in skeletal related events in the treatment of osteolytic and osteoblastic metastatic bone disease [Body 2003; Corey *et al.* 2003; Lipton *et al.* 2002]. A contradictory report has suggested that ZOL is effective in limiting osteolytic lesions *in vivo* but not osteoblastic lesions associated with prostate tumour cells, suggesting that osteoclast activity may not be critical for the development of osteoblastic lesions [Lee *et al.* 2002]. Another possibility for the observed 'osteoblastic' phenotype is that OS produce bone morphogenetic proteins (BMPs), which are potent inducers of osteoblasts and are able to induce ectopic bone formation *in vivo* [Yoshikawa *et al.* 2004; Raval *et al.* 1996; Anderson *et al.* 1992]. One report described this as "sunburst" spiculation of the periosteum as observed by radiography [Yoshikawa *et al.* 1985]. Almost all animals exhibiting the 'osteoblastic' phenotype had large macroscopic tumours. It is possible that cells, which were left in the surrounding soft tissue as the needle withdrew from the tibia, led to extra-osseous tumour growth, and does not represent typical development *in vivo*. As the areas of increased bone formation include the fibula and regions well below the inoculation site, it is likely that the extra osseous tumour growth is responsible for this phenomenon and is not representative of normal tumour progression. The observed phenotype may partly be a consequence of the method of delivery of cells to the tibia and this may need to be revised. Regardless of this observation, the lack of any osteolytic lesions in the ZOL treated animals is very exciting and may represent changes in the bone environment that make it less amenable to tumour growth. This may be the reason for the increased tumour area observed in ZOL treated animals, forcing the cells to establish outside the bone.

One limitation of this study is the lack of osteolysis observed in some of the control untreated animals. There could be numerous reasons for this observation and the premature termination of this experiment due to animal ethics issues also remains a possible reason for the incomplete collection of adequate data. This was the first time that the animal ethics committee were confronted with this procedure and it proved to be problematic for them. If the experiment had been taken to full term, it is possible that more lesions may have developed and that greater variations may have been observed between the treatment groups. This however remains to be seen and further experiments are required with the addition of analgesics, to reduce pain of the animals and extend the length of the experiment. Other reasons for the lack of osteolysis include, variations between tibial injections including inadequate delivery of cells and variations in injection sites potentially providing differing environments for the cells. The volume of inoculum was reduced from 20µl in the pilot study to 10µl in the current study. This was to improve the likelihood of complete delivery of entire volume into the tibia and to reduce variability between injections. Experience and practice should help reduce the large variations between animals. The use of mice at 4 weeks of age may improve the delivery technique, as the bones are much softer and easier to inject into.

Although there is extensive *in vivo* preclinical evidence that BPs, and in particular ZOL, have anti-tumour activity and can reduce skeletal and perhaps also extraskeletal tumour burden in a variety of tumour types, no such data exist for OS. These preliminary animal studies on human OS supplement the current literature by showing that ZOL treatment protects the skeleton from OS-induced osteolysis.

CHAPTER EIGHT

GENERAL DISCUSSION

8.1 GENERAL DISCUSSION

The data reported in this thesis are part of an ongoing investigation into new and effective agents for the treatment of OS, which is an aggressive and destructive malignancy. Individuals with OS, and particularly older patients continue to have a poor prognosis despite considerable improvement in the treatment regimes. New alternative approaches for the treatment of OS are essential for improvement in patient survival. Such agents include TRAIL and inhibitors of the mevalonate pathway including BPs, statins and PTIs.

Bisphosphonates have the ability to inhibit osteoclast-mediated resorption, and have also been identified as having direct anti-tumour activity. Reports of the more potent N-BPs to dose-dependently inhibit the proliferation and survival of tumour cells have been made in numerous tumour cell types [Senaratne and Colston 2002; Stearns and Wang 1996; Fromigue *et al.* 2000; Sato *et al.* 1991; Lee *et al.* 2001]. The most potent of the currently available N-BPs, ZOL, was investigated for effects on human OS cells. ZOL treatment induced a dose- and time-dependent decrease in proliferation and survival in all of the OS cell lines tested, although it was variably effective in reducing cell number in the different cell lines. Variations in the cytostatic and apoptotic effects of N-BPs *in vitro* have been reported and most probably depend on the potency of the N-BP and the cell line used [Fromigue *et al.* 2000]. ZOL acts by inhibiting a key enzyme of the mevalonate pathway leading to the cell's inability to post-translationally modify (or prenylate) proteins, such as the GTPases, which are required for essential processes of survival. [Coxon *et al.* 2000; Dunford *et al.* 2001; Luckman *et al.* 1998; Fisher *et al.* 2000; Rodan 1998; Rogers *et al.* 2000]. Treatment of OS cells with ZOL *in vitro* led to loss of attachment followed by apoptosis and was concomitant with an increase in caspase activity, most likely due to a secondary effect since caspase inhibition did not protect these cells from the ZOL-mediated effects. Alternatively,

addition of geranylgeraniol (GGO), was able to protect these cells by acting as an intermediate of the mevalonate pathway, allowing geranylgeranylation to proceed and thus cell survival.

Other effects of ZOL on human OS cells include S-phase arrest of the cell cycle and an increased expression of cell surface TRAIL death receptors. The increase of death receptors but not the decoy receptors at the cell surface may explain the observed augmented apoptotic effect of ZOL and TRAIL in most cell lines, however previous reports have not found a correlation between receptor expression and TRAIL sensitivity [Hersey and Zhang 2001; Ozoren and El-Deiry 2003]. Treatment of OS cells with ZOL and TRAIL together led to an augmented apoptotic effect, which was observed in all cell lines to some extent, correlating to the sensitivity of the cell lines to ZOL alone. More importantly, the cell lines that were resistant to TRAIL treatment alone were strongly sensitised to TRAIL-induced apoptosis by the co-addition of ZOL. As was noted with ZOL treatment, OS cells given the combination treatment showed higher levels of caspase activity. The co-addition of caspase inhibitor to ZOL and TRAIL combination treatments led to an apoptotic effect equal to that seen with ZOL alone. On the other hand, addition of GGO to the combination treatment prevented sensitisation to TRAIL-induced apoptosis.

Normal human osteoblast-like (NHB) cells did not exhibit such an augmented apoptotic effect with co-treatment of ZOL and TRAIL. Similarly, there was no upregulation of the TRAIL death receptors at the cell surface as was observed in the OS cells, which may explain the lack of augmentation with combination treatment. This is very encouraging as the combination of ZOL and TRAIL is much more effective at inducing apoptosis in cancer cells than normal cells, which could reduce toxicity of *in vivo* treatment, compared with that associated with currently used chemotherapeutic regimes.

Other inhibitors of the mevalonate pathway (IMP), such as statins, FTIs and GGTIs were also employed to investigate their effectiveness as alternative agents for the treatment of OS. As was observed with ZOL, treatment of OS cells with statins, FTI and GGTI also led to dose-dependent apoptosis that was augmented with the co-addition of TRAIL. Resembling the ZOL effects on NHB cells, these other IMP all failed to exhibit an augmented apoptotic effect in NHB cells using combination treatment with TRAIL. This highlights the importance of the mevalonate pathway inhibitors as anti-cancer agents.

Although *in vitro* experiments are necessary for the initial investigation of potential therapeutic agents, these experiments are invalid if the agents are not able to reach their destination at the concentration required to generate the desired effect without associated toxicity. Fortunately, BPs and statins have been used for many years for the treatment of various diseases and FTIs are currently being assessed in clinical trials [Goldstein and Brown 1990; Body 2000; Brunner *et al.* 2003]. Although GGTIs have not reached clinical trials yet, there is encouraging pre-clinical data and further investigation is warranted. This provides a sound basis for the use of these agents for *in vivo* analysis. It is not known whether tumour cells are directly exposed to ZOL *in vivo*, due to its high affinity for the bone. As the *in vitro* data described in this thesis suggests, ZOL availability to cancer cells is vital for sensitisation to TRAIL-induced apoptosis. Therefore, the use of statins, FTIs and GGTIs as alternatives to ZOL, with potentially improved bioavailability, may offer advantages. In addition, these agents may also be used for the treatment of other non-osseous cancers.

An OS mouse model involving the injection of human cells into the tibia was established in a pilot study and the most suitable conditions were identified for further investigations. The K-HOS cell line was chosen for the *in vivo* approach due to the ability of these cells to establish in the marrow cavity and cause osteolytic lesions in

mice. Also the synergistic apoptotic activity of ZOL and TRAIL co-treatment *in vitro* has been demonstrated, an effect not seen in KRIB cells. The animals were inoculated into the left proximal tibia and treatments were commenced one week post cancer cell transplantation.

Using radiography, micro computed tomography (μCT) and histology, inoculated tibiae were assessed from animals of the control untreated, TRAIL-treated, ZOL-treated and combination ZOL and TRAIL-treated groups. All 21 animals given ZOL, showed remarkable conservation of the tibiae with no radiographic evidence of bone destruction. The radiographs demonstrated that in all ZOL treated animals, and not in the untreated animals, the bones were highly radio-dense, suggesting increased bone density. Longitudinal sections of μCT 3-D reconstructions and histological sections of the corresponding tibiae clearly showed significant increases in cortical thickness and trabecular density in the ZOL treated animals when compared to the untreated animals. This was supported with significant increases in bone volume using the 2-D images obtained by μCT analysis.

Using histological sections, tumour area was calculated and comparisons between affected animals were made. A significant reduction in tumour area was observed in animals treated with TRAIL, but no effect was seen with ZOL treatment. As the number of animals per group was small, the experiments need to be repeated to place any confidence in the results. The number of animals identified with lung metastases revealed no differences between groups.

From the μCT data it was noted that many animals exhibited 'osteoblastic'-like lesions, which were not apparent from the radiographs. There are many possible reasons for the observed effect, including the release of factors by the surrounding soft-tissue tumour, almost always associated with this phenomena, leading to the stimulation of bone growth.

The development of very large macroscopic tumours which hindered movement and caused pain in a few animals, led to the premature termination of the experiment. However at this time point some animals did not exhibit any signs of osteolysis, possibly due to insufficient time for tumour development to occur. Alternatively, technical difficulties may also be the reason for the variations observed.

The *in vivo* results support current reports that ZOL protects the skeleton from OS-induced osteolysis but fail to exhibit any positive interaction between ZOL and TRAIL combination treatment *in vivo*. The direct effect of ZOL on cancer cells *in vivo* is not known, but may not be adequate to sensitise the cells to TRAIL-induced apoptosis. Further investigation is required to determine whether ZOL exposure to OS cells is adequate to exhibit an augmented apoptotic effect when combined with TRAIL.

The use of TRAIL in combination with inhibitors of the mevalonate pathway offers an exciting therapeutic potential. However, in order to realise this potential, it is important to proceed systematically through cell biology studies, preclinical studies before embarking on clinical trials. Ongoing cell studies are required to further understand the complex biology of TRAIL and its interaction with molecules such as ZOL, statins and PTIs that will enable more informed treatment, particularly of TRAIL-resistant tumours. Further *in vivo* experiments will prove extremely useful for the study of human OS growth and metastasis and will enable a thorough *in vivo* evaluation of these agents, which have been shown to have anti-proliferative and cytotoxic effects *in vitro*. This information will have important implications for future therapy of OS.

8.2 FUTURE DIRECTIONS

This thesis has provided data to support the anti-cancer activity of ZOL, statins, FTIs and GGTIs alone, and in combination with TRAIL, in human OS cells. Some of the mechanisms of action have been explored but many questions remain unanswered. Why are there differences in the sensitivity of OS cells to IMP-induced apoptosis? The reasons for the observed differences in activity and potency of IMP in the different cell lines are unclear but may reflect differences in bioavailability, cellular uptake or intracellular effects of IMP in different cell types. It is important to identify the reasons for these differences so that their effectiveness in inducing apoptosis can be improved. Due to the high degree in variability of OS cells with regards to sensitivity to these treatments, it would be beneficial to the patients to use biopsy sample to test the responsiveness of the tumour cells to these agents *in vitro*. Thus identifying the best course of treatment for each individual patient and improving prognosis.

Further investigation into the molecular mechanisms of action of TRAIL and IMP will assist in their exploitation and possibly enable identification of alternative agents to work in combination with the IMP. Which Bcl-2 family members are involved? Which GTPase proteins are critical for cell attachment? How exactly do IMP sensitise the cell to TRAIL-induced apoptosis? Which caspase independent pathways are involved? Despite the many questions, one important thing remains, that IMP in combination with TRAIL are very effective in inducing apoptosis in some human OS cells but not normal cells, providing the possibility of a new cancer treatment without the associated toxicity. This combination of agents must first be assessed in pre-clinical models.

Although the *in vivo* experiments conducted were able to show no tumour growth or bone destruction in all the animals treated with ZOL, it could not be determined whether the combination treatment was any more beneficial than ZOL alone. Direct investigation of the degree of exposure of ZOL to OS cells *in vivo* may help determine

whether this combination could cause a similar effect *in vivo* to that observed *in vitro*. Detection of unprenylated protein in OS cells in treated animals might provide some insight. If this proves difficult, a dose response study with differing ZOL concentrations and TRAIL would be required to investigate this matter further. Prior to any further *in vivo* experiments, several factors need to be addressed. The technique of intra-tibial injections needs to be refined to improve delivery and reduce variation between animals. The early onset of macroscopic tumours in some individuals must be considered. The use of analgesics will need to be administered to allow the experiment to be taken to full term. Alternatively, the use of bioluminescence equipment will allow better evaluation of the effects of these agents over time. More importantly, it will help identify and monitor the effect of these agents on well-established tumours, so that termination of the experiment will give the most meaningful results. If bioavailability remains a problem with ZOL treatment, the use of other agents, such as statins and PTIs, are alternative possibilities. These agents may act to target OS cells and other non-osseous cancer cells *in vivo*. The use of IMP in combination with other clinically relevant anticancer drugs may also prove extremely useful for the study of human OS growth and metastasis.

Taken together the use of IMP and TRAIL as alternative anti-tumour agents for the treatment of human OS has considerable potential and may lead to a more effective therapeutic regime that has the added advantage of being non-toxic and safe.

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Amendments

Introduction

- Pg 33: For more details of last paragraph refer to Hofbauer LC *et al.*, The OPG/RANKL/RANK system in metabolic bone diseases. *J Musculoskelet Neuronal Interact* 2004 4(3):268-75.
- Pg 47: For more details of paragraph 1 refer to Mundy GR. Mechanisms of bone metastasis. *Cancer* 1997 80(8S):1546-56, and Chirgwin JM. & Guise TA. Molecular mechanisms of tumor-bone interactions in osteolytic metastases. *Crit Rev Eukaryot Gene Expr* 2000 10(2):159-78.
- Pg 51: First paragraph should read 'It has been proposed that these concentrations of BPs may be achievable in bone at an active resorption site although there is no conclusive evidence to prove this hypothesis [Sato *et al.*, 1991].'

Chapter 2

- Section 2.4.12: The 2nd sentence should read ' This program was used to determine standard deviation (SD) and *p* values, based on experiments that were performed in triplicate and repeated at least three times'.

Chapter 3

- Pg 95: The 2nd to last sentence of paragraph 1 should read 'the efficacy of ZOL to inhibit increases in cell number varied considerably between cell lines. The decrease in cell number could be due to inhibition of cell proliferation or to the induction of apoptosis'.
- Fig 3.4: See revised figure (next page).
- Fig 3.14: BTK-143 should be replaced with MG-63.
- Section 3.3.7: The 3rd should read 'However, by 72 hours, ZOL caused an increase in the number of cells arrested in S-phase (38% v 26%), concomitant with a reduction of cells in G_0/G_1 and G_2/M phases.' The 5th sentence should include the following amendment; '....S-phase increasing from 33%.....'
- Pg 99: Our results show a correlation between ZOL dose on cell viability and inhibition of protein prenylation in the more sensitive HOS cell line. A ZOL dose of 10μM, reduced cell viability by approx 60% (see dose curves, Figure 3.1) and also inhibited Rap 1A prenylation. By contrast, the same concentration of ZOL, which was less potent at reducing cell viability of MG-63 cells, still showed inhibition of prenylated Rap 1A. The presence of unprenylated protein may therefore not be a clear representation of cell viability.

Chapter 4

- Pg 112: Although there is some variability in the effect of ZOL on BTK-143 cells when compared to previous data, there is clear augmentation of apoptosis when TRAIL and ZOL are given in combination (as seen from multiple experiments).
- Fig 4.7: The columns representing Z+T for both cell lines had *p* values < 0.005 compared to both Z and T single treatments.

Fig 4.8: The columns GGO+Z for both cell lines had *p* values < 0.001 compared to Z treatment alone.

Fig 4.11: All references to BTK-143 should be replaced with the cell line MG-63.

Section 4.3.6: All references to BTK-143 should be replaced with the cell line MG-63. The last line should read, ' These trends were also identified in the G-292, Saos2 and SJS-1 cell lines (data not shown).'

Chapter 5

Fig 5.9: All t tests performed on data sets represented in this figure generated values of $p > 0.05$.

Fig 5.11: t tests performed on (A) and (B) revealed $p<0.001$ for L+ GGO+/-T and M+GGO+T and $p<0.003$ for M+GGO. (C) and (D) had values of $p>0.05$ for PTI+GGO+/-T.

Table 5.1: Column should read 'increase in cell death as a % of control untreated cells'.

Chapter 7

Section 7.3.2: Add to the end of paragraph 1: 'It has been reported that an increase in bone mineral density adjacent to growth plates after bisphosphonate administration to growing animals is due to the inhibition of bone resorption and the retention of the primary spongiosa formed during growth (Schenk R. *et al.*, *Calc Tissue Res* 1973 11:196-214).'

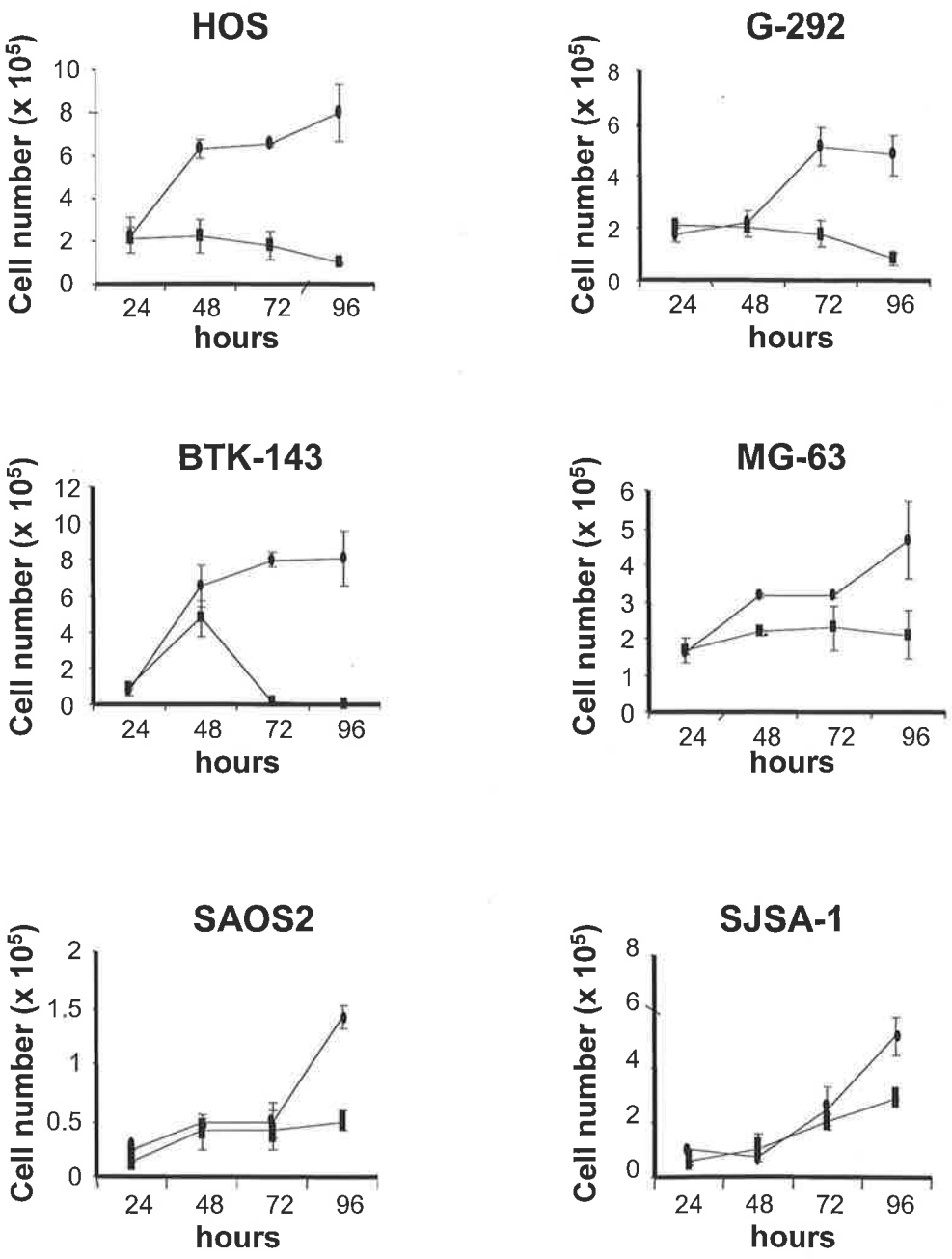

Figure 3.4 The effect of ZOL on OS cell number. Cells were incubated for the times indicated in the absence (-•-) or presence (-■-) of 25 μM ZOL. The number of viable cells was quantitated, based on trypan blue exclusion, using a heamocytometer and expressed as number of cells. Data shown in each case are from representative experiments repeated at least three times: bars, ±SD.