KINASE INHIBITORS OF THE FMS RECEPTOR FOR MACROPHAGE COLONY STIMULATING FACTOR

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ABSTRACT

FMS is the receptor for macrophage colony stimulating factor (M-CSF or CSF-1) and is essential for the differentiation and survival of most macrophages, microglia and osteoclasts Infiltration of macrophages and high levels of M-CSF in serum are associated with poor prognosis in human breast, ovarian and endometrial cancers. Consequently, inhibitors of FMS have considerable therapeutic potential for the treatment of cancer, as well as macrophage-mediated inflammatory disease and bone disorders caused by excessive osteoclastogenesis .

Here I report the identification of four clinically advanced small molecule kinase inhibitors which can also target FMS, as shown by various biochemical and cell-based assays. The inhibitors I identified are imatinib, nilotinib, tandutinib and dasatinib (IC50s = 600, 250, 400 and 0.5 nM, respectively), where the IC50 values refer to inhibition of FMS-dependent cell growth.

All of these inhibitors effectively inhibited osteoclastogenesis at concentrations expected to be achieved in patients and nilotinib was a particularly potent inhibitor (IC50 = 50 nM) indicating that nilotinib is a strong candidate for treatment of diseases where osteoclasts are over activated such as osteoporosis and osteolytic bone disease. Dasatinib was found to be a potent inhibitor of tumour associated macrophages derived from ovarian tumour ascites (IC50 = 0.5 nM) which is similar to the concentration required to inhibit FMS and within concentrations considered to be achieved *in vivo*.

Using these FMS inhibitors I have also confirmed that the downregulation of the FMS receptor in response to ligand binding is independent of kinase activity and instead requires a structural change in the receptor. Intriguingly, at least two tyrosine autophosphorylation sites of FMS are still phosphorylated in the presence of some of these inhibitors and therefore in the absence of FMS kinase activity. This implies that other kinases may be involved in FMS activation.

PUBLICATIONS

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ABBREVIATIONS

ABL	Abelson (proto-oncogene)
ALL	Acute lymphoblastic leukaemia
AKT	β-analyl-α-ketagluterate transaminase
ATP	Adenosine Triphosphate
BCR-ABL	Breakpoint Cluster Region – Abelson (Oncogenic fusion protein)
BMM	Bone Marrow Mononuclear cells
CML	Chronic Myeloid Leukaemia
CSF-1	Colony stimulating factor -1 (M-CSF, FMS ligand)
DMSO	Dimethylsulphoxide
EGF	Epidermal Growth Factor
EGFR	EGF receptor
Erk2	Extracellular Signal-Regulated Kinase 2
FCS	Foetal Calf Serum
FGF	Fbroblast growth factor
FLT-3	FMS-like Tyrosine Kinase-3
FMS	McDonough feline Sarcoma (Receptor for M-CSF)
GISTs	Gastrointestinal Stromal Tumours
GM-CSF	Granulocyte macrophage colony stimulating factor
Grb-2	Growth factor Receptor Bound Protein-2
GTP	Guanine triphosphate
IC50	Inhibitory Concentration50 (value at which 50% inhibition is achieved)
Ig	Immunoglobulin
IGF1	Insulin-like growth factor 1
IL-3	Interleukin-3
IL-4	Interleukin-4
JAK	Janus-activated kinase
JMR	Juxtamembrane Region
KDa	Kilodalton
KIT	Stem cell factor receptor
MAPK	Mitogen Activated Protein Kinase
M-CSF	Macrophage-Colony Stimulating Factor (CSF-1, FMS ligand)
NGF	Nerve Growth Factor
OPG	Osteoprotegerin
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
PI-3 K	Phospho-Inositol-3 Kinase
PLCγ	Phospho-Lipase C γ
PTHrP	Parathyroid hormone related protein
Ras	Rat Sarcoma (Signalling molecule in the MAPK pathway)
RANKL	Receptor Activator of Nuclear Factor kappa B Ligand
RTKs	Receptor tyrosine kinases
SCF	Stem Cell Factor (Ligand for the KIT receptor)
SHIP-1/2	Inositol 5'-phosphatase 1/2
siRNA	Silencing RNA
SRC	Member of the SRC (Sarcoma) family kinases
STAT	Signal Transducers and Activators of Transcription
TGF β	Tranforming growth factor beta
TRAP	Tartrate-resistant acid phosphatase
VEGF	Vascular Epithelial Growth Factor
v/v	volume/volume
w/v	weight/volume

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CHAPTER 1. INTRODUCTION

1.1 Receptor Signalling

Signalling by cell surface receptors is fundamental to many cellular processes and disease states. Soluble extracellular signalling molecules including hormones, cytokines, growth factors, neurotransmitters and pheromones bind to their specific receptor, inducing a structural change in the receptor and receptor activation. This activates a cascade of biochemical pathways which can have a great variety of effects on the cell including cell proliferation, differentiation, survival, activation and control of motility. These receptors are classified according to the cellular response they induce and by their primary structure.

Cell surface receptors can be divided into four main groups: G protein coupled receptors, cytokine receptors, ion-channel linked receptors and tyrosine kinase receptors. G protein coupled receptors span the membrane seven times and so are also often called heptahelical receptors. They have a diverse range of ligands including light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters and represent 30 – 45 % of current drug targets (Drews, 2000; Hopkins and Groom, 2002). G protein coupled receptors are activated by binding to their specific ligand and are then able to activate adenylyl cyclase through a linkage to G proteins. Adenylyl cyclase then generates cyclic adenosine monophosphate from ATP which is able to activate many kinases to form a signalling cascade.

Cytokine receptors are receptors for cytokines and usually have associated tyrosine kinase activity. Cytokines are low molecular weight proteins which regulate

immunity, inflammation and haematopoiesis and are usually biologically effective at very low concentrations and often work in synergy with one another. Cytokine receptors are often linked to immunodeficiency diseases and include various receptor families including the interferons, the interleukins and the tumour necrosis factor receptor family.

Ion-channel linked receptors are restricted in their expression to electrically excitable cells including neurons and muscle cells and are responsive to chemical messengers such as Na^+, K^+, Ca^{2+} and Cl^-. They are classified into 3 mains groups: Cys-loop receptors such as the serotonin receptor, ionotropic glutamate receptors such as kainate and ATP-gated channel receptors such as PX2. Ion channel receptors are the main target of anaesthetics and alcohols such as ethanol and are usually located at synapses between cells.

Receptor tyrosine kinases (RTKs) are cell membrane receptors categorised by an intrinsic tyrosine kinase activity, giving the ability to autophosphorylate tyrosine residues within the receptor. Receptor activation occurs when extracellular polypeptide ligands bind to ligand binding domains of the receptor on the surface of the cell, causing dimerization of pairs of receptor monomers. A conformational change in the receptor is induced and the kinase becomes activate and is able to phosphorylate itself and protein substrates. The activated receptors are then able to signal to a complex network of intracellular signalling molecules which can lead to many cellular outcomes including cell proliferation, differentiation and migration (figure 1.1).

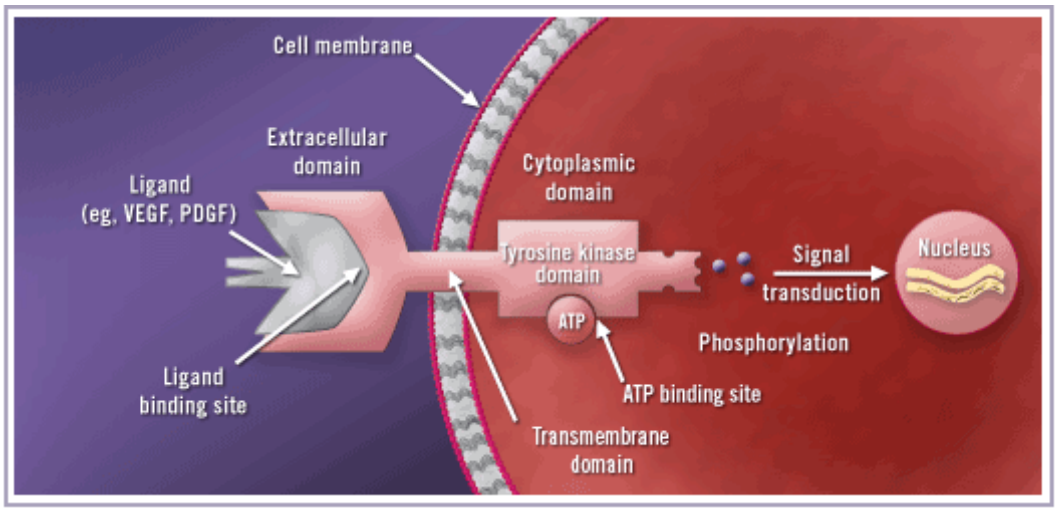
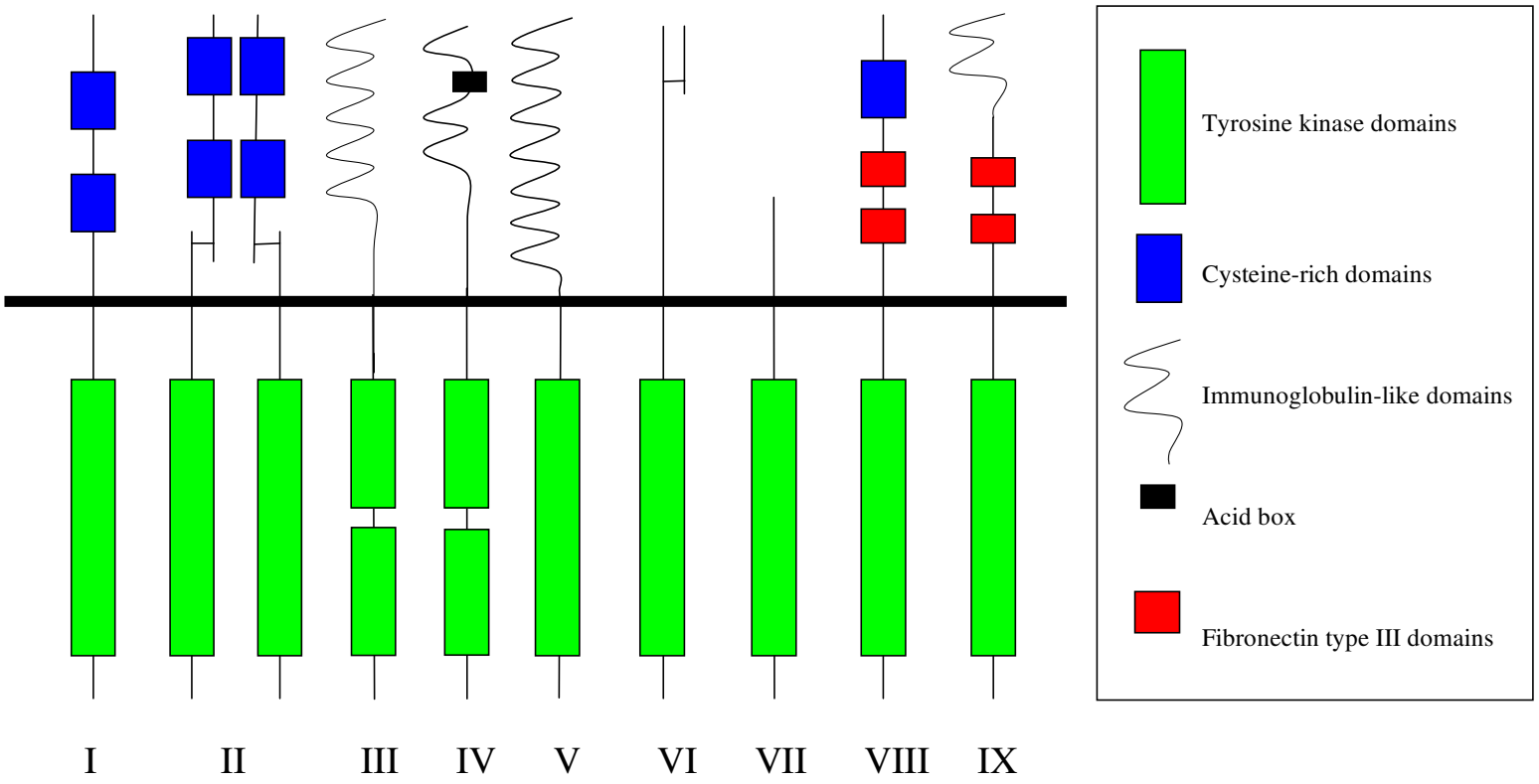


Figure 1.1. Schematic diagram of a receptor tyrosine kinase showing the extracellular ligand binding domain, the cytoplasmic domain, containing the kinase domain and the ATP binding site. Modified from http://www.multi-targetedtherapy.com/rtkCancer.asp

The RTK family includes the receptors for many growth factors and colony stimulating factors including Epidermal Growth Factor (EGF), Vascular Epithelial Growth Factor (VEGF) Nerve Growth Factor (NGF) and the Platelet –Derived Growth Factor family. The receptor tyrosine kinases have been categorised various ways and a popular classifiaction based upon the structures of these receptors is shown in figure 1.2 which was proposed by (Ullrich and Schlessinger, 1990). Figure 1.3 shows an alternative evolutionary tree which includes all of the receptor tyrosine kinases and the cytoplasmic tyrosine kinases.

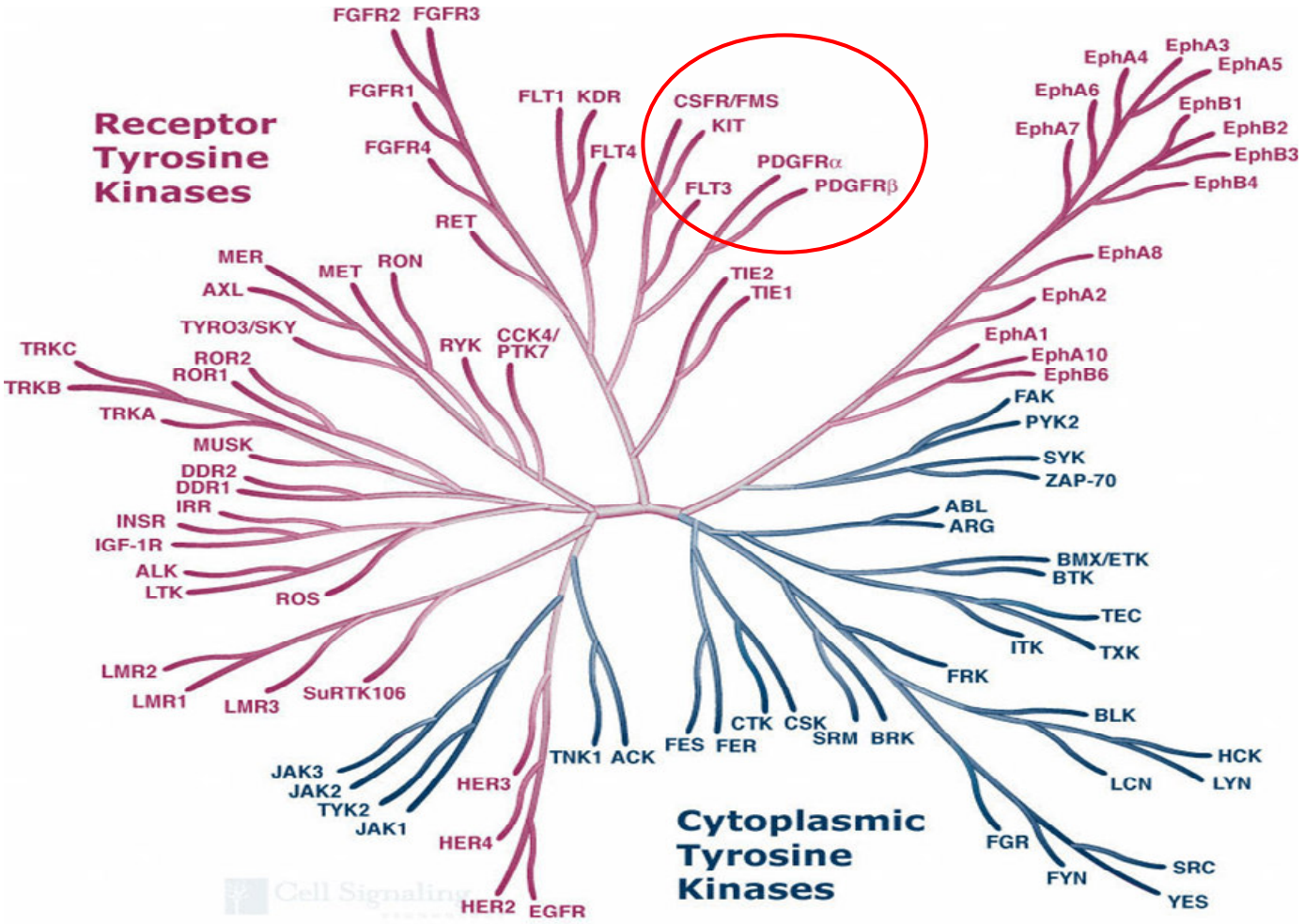
Figure 1.2. Structural classification of receptor tyrosine kinases into nine subfamillies proposed by Ullrich and Schlessinger.



I	EGF Receptor, HER2, HER3, HER4
II	Insulin Receptor
III	PDGF, M-CSF, KIT and FLT-3 receptors
IV	FGF receptors
V	VEGF receptor, FLK receptors
VI	Met/HGF receptor
VII	TrkA, TrkB, TrkC
VIII	Ephrin receptors
IX	AXL receptor

(Ullrich and Schlessinger, 1990)

Figure 1.3. The evolutionary tree of receptor tyrosine kinases and cytplasmic tyrosine kinases with the PDGF receptor family highlighted.



1.1.2 Signalling by the PDGF Receptor Family

Receptor tyrosine kinases are divided into subgroups with respect to their sequence and structure (figure 1.2). The Platelet Derived Growth Factor Receptors (PDGFR) or Type III RTK family are a closely related family of tyrosine kinase receptors which are activated by the binding of their specific ligands. Figure 1.3 shows an evolutionary tree consisting of the receptor tyrosine kinases and the cytoplasmic tyrosine kinases with the PDGF receptor family circled in red. This family consists of KIT, FLT3, PDGF α and β and FMS, which bind to Stem Cell Factor (SCF) (Lev et al., 1994) FLT3 (Stirewalt and Radich, 2003), PDGF ligands (Heldin and Westermark, 1999; Bergsten et al., 2001; LaRochelle et al., 2001; Li et al., 2000) and Macrophage Colony Stimulating Factor (M-CSF) (Yeung and Stanley, 2003) respectively. Ligand binding induces activation and autophosphorylation of the receptor and this allows recruitment and binding of other proteins with phosphotyrosine recognition domains. This results in a signalling cascade which induces changes in conformation and phosphorylation status of many downstream molecules and leads to activation of specific transcription factors. An example of a common, well documented signalling cascade downstream of the PDGF receptor family is the ERK/MAPK pathway. Generally, upon ligand binding, adapter complexes which have SH2 and SH3 domains such as the GRB1/SOS complex translocate to the membrane and bind to the receptor. These complexes are able to convert Ras-GDP to Ras-GTP by guanine nucleotide exchange. Ras is then able to activate the MAP kinase kinase kinase (MAP3K) family member Raf which in turn activates the MAP kinase kinase (MAPKK) family members MEK1 or MEK2. These in turn activate the MAP kinase members ERK 1 and ERK2 which are then able to translocate through the nuclear membrane and activate transcription factors such as

TCF and Elk1. This is a simplified view and signalling cascades in fact involve interplay between many kinases and phosphatases and crosstalk between a number of signalling pathways, including three further MAP kinase signalling pathways, the PI3 kinase and JAK/STAT signalling pathways. These pathways regulate many cellular effects and therefore deregulation of signal transduction is implicated in many diseases.

All members of the PDGF receptor family have the same basic structure including five extracellular immunoglobulin (Ig) domains, a single transmembrane spanning helix, an autoinhibitory juxtamembrane region (JMR) and a cytoplasmic kinase domain, consisting of an N terminal and C terminal lobe and a characteristically large loop region called the kinase insert region (figure 1.4 shows a schematic diagram of the FMS receptor). The PDGF receptor family is most associated with haematopoiesis. The FLT-3 and KIT receptors are important receptors for early haematopoietic progenitor cells and can promote their survival and proliferation (Simmons et al., 1994; Drexler, 1996). KIT is also expressed on mast cells and is widely expressed during embryogenesis and in melanocytes and germ cells and is known to have multiple roles in development (Keshet et al., 1991; Lyman and Jacobsen, 1998). The PDGF receptor has many signalling roles in the mesenchymal cell lineage in both adults and during development but the haematopoietic role of PDGF is less clear although it is an important factor for megakaryocyte differentiation and proliferation (Yang et al., 1997). The FMS receptor is an important receptor for the development and differentiation of cells of the monocyte/macrophage cell lineage and is also expressed by some cells of the female reproductive tract including

trophoblasts and some decidual cells where it has a regulatory role (Wiktor-Jedrzejczak et al., 1990; Cecchini et al., 1994; Regenstreif and Rossant, 1989).

1.1.3. Activation of the PDGF receptor family

The structures of FLT-3 (Griffith et al., 2004), PDGF (Oefner et al., 1992) and FMS (Walter et al., 2007) have been determined but the mechanisms of activation of the KIT receptor are the most extensively characterised, the crystal structure of KIT is shown in figure 1.4 (Mol et al., 2003). The kinase domain of this family of receptors is able to switch between an inactive and active confirmation upon ligand-binding. Kinase activation requires a short 20 to 30 amino acid region called the activation segment to rotate around a single aspartate residue (Asp 810) within a highly conserved region Asp810-Phe811-Gly812 (DFG) sequence. This allows access to the substrate and correct orientation of amino acids for catalysis (Mol et al., 2003). Stabilisation of this structure requires that part of the activation loop (amino acids LARDI , 813-817) , moves position by some 20 Angstroms and converts from a helical or loop structure to a beta strand (known as β9, shown in green in figure 1.4) that pairs with the invariant β6 strand of the kinase domain that is found outside the activation segment (shown in yellow in figure 1.4). The formation of the β6/ β9 pair is important for stabilisation of the active structure of all kinases (Dibb et al., 2004).

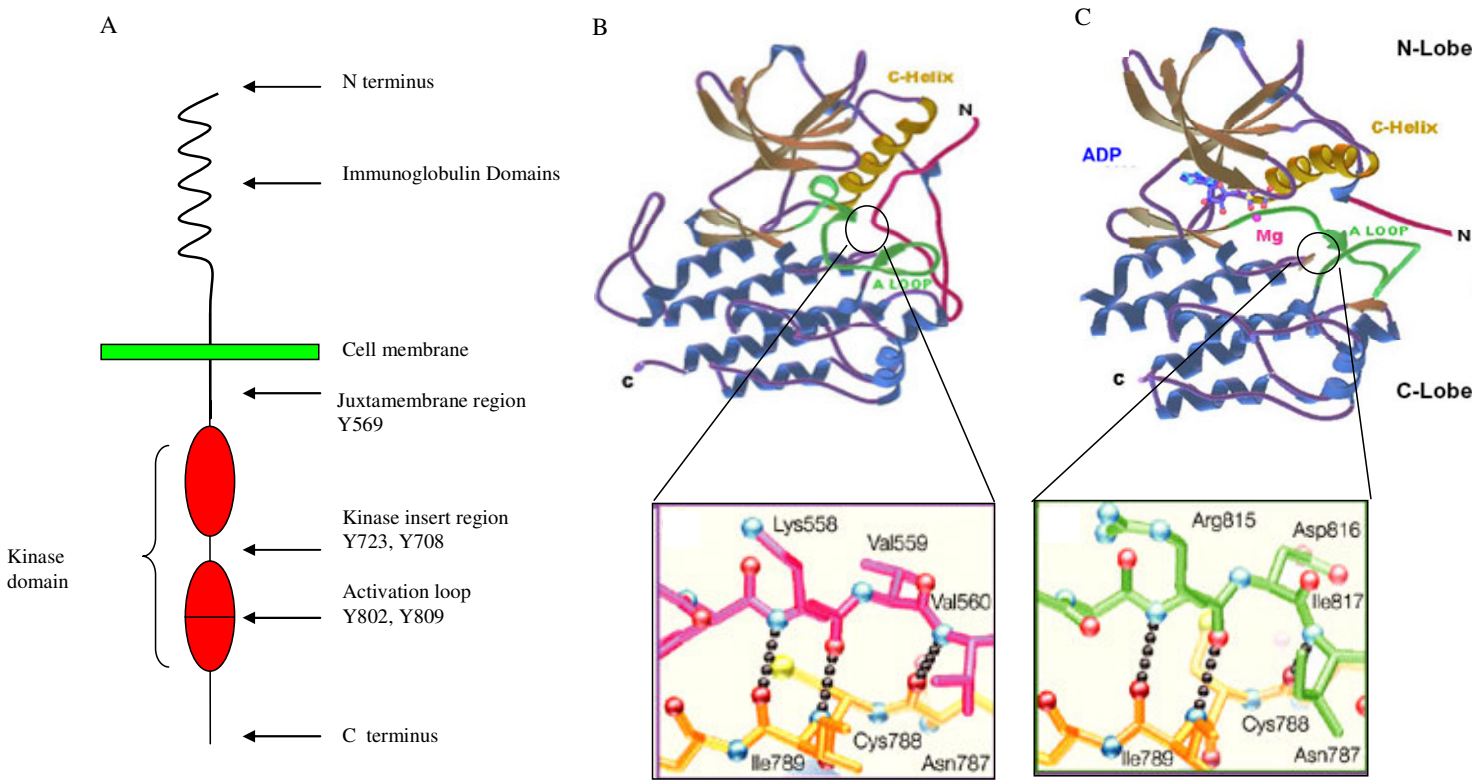


Figure 1.4. The structure of the FMS and KIT receptors. (A) Schematic diagram of the FMS receptor showing key tyrosine phosphorylation sites (Y = tyrosine). (B,C) Inactive and active KIT kinase crystal structures. (B) In the inactive conformation the JMR inserts into the interface between the N and C-lobes and forms hydrophobic interactions with a ß-sheet from the kinase domain (ß6). The magnified area shows hydrophobic bonds between the amino acids 556-560 from the JMR (pink) and the kinase domain ß6 strand (yellow). The JMR competes with the activation loop for this position, preventing the kinase domain from moving into an active conformation. (C) In the active conformation, the activation loop binds with ß6 from the kinase domain (yellow) and forms ß6/ ß9 which is required for kinase activity. JMR is shown in pink, ß6 strand from the kinase domain in yellow and, b9 from the activation loop in green. Modified from (Mol et al., 2004, Dibb et al., 2004).

When KIT is in its inactive conformation the juxtamembrane region (JMR) has an autoinhibitory role and is inserted into the cleft that the activation loop occupies in the active conformation and thereby competes with the activation loop for binding with the β6 strand (shown in pink in figure 1.4). This blocks the activation segment from moving into its active position and this autoinhibitory structure is maintained whilst the JMR is not phosphorylated (Mol et al., 2003; Mol et al., 2004a). Amino acids 556-560 of the juxtamembrane region are particularly important for this autoinhibitory role as they bind directly with the β6 strand.

There are many oncogenic mutations of KIT which affect the interaction of the β6 strand and JMR. Amino acids QWKVV (556-560) of the JMR are an oncogenic hotspot and are frequently mutated in cancers, particularly in Gastrointestinal Stromal Tumours (GISTs) (Russell et al., 1987) Small deletions of this region or single amino acid mutations, usually of Val559 to Asp, prevent the inhibitory interaction with β6 and shift the equilibrium of the receptor towards the active state. FLT-3 mutations are also common in acute myeloid leukaemia (AML) and internal tandem repeat sequences in the juxtamembrane region are the most common mutation found. These mutations also disrupt the structure of the JMR and therefore its autoinhibitory function, allowing the activation loop access to the β6 strand.

Oncogenic mutations of the activation segment are also common, particularly those that stabilise the active conformation of the receptor. Aspartate 816, which is part of the β9 strand on the activation loop is an oncogenic hotspot and is often substituted to valine (D816V) in mast cell leukaemia (Longley et al., 1999). Asp β9 mutations are usually constitutively activating mutations and may give the Asp β9 sheet a higher

propensity to form the β6β9 motif, pushing the equilibrium towards the active state (Dibb et al., 2004). Mutations of Asp β9 are resistant to the tyrosine kinase inhibitor imatinib, which is able to bind to the inactive, but not to the active kinase conformation implying that Asp β9 mutations lock the kinase domain into an active structure (Ma et al., 2002).

1.1.4. The FMS Receptor

The FMS receptor is encoded by the *c-fms* proto-oncogene and is involved in regulation of cell proliferation, differentiation and survival in a variety of cells types of the monocyte/macrophage cell lineage and some cells of the female reproductive tract. FMS was first discovered due to its similarity to the v-fms oncogene which is found in the Susan McDonough strain of the feline sarcoma virus (Sherr et al., 1985). In the haematopoietic system, FMS is expressed early in differentiation of the monocyte/macrophage cell lineage and is continuously expressed in a non-dividing macrophages and osteoclasts (Rohrschneider et al., 1997). Valuable insights into the function of the FMS receptor came from the nullizygous mouse $Csf1^{op}/Csf1^{op}$ which has an inactivating mutation in the M-CSF coding gene mapping to chromosome 3 and as a result has no endogenous M-CSF (Wiktor-Jedrzejczak et al., 1990). $Csf1^{op}/Csf1^{op}$ mice are osteopetrotic, toothless due to bone thickening, have a deficiency in tissue macrophages and osteoclasts and have fertility defects and brain abnormalities including a reduced number of microglia (Cecchini et al., 1994; Michaelson et al., 1996; Wiktor-Jedrzejczak et al., 1982; Wegiel et al., 1998). This phenotype can be partially corrected when recombinant human M-CSF is administered to neonatal animals, giving an almost complete recovery of macrophage and their progenitor cells and partial recovery of osteoclasts (Ryan et al., 2001).

M-CSF deficient mice also have a diminished ability to support tumour growth. The growth of subcutaneous injections of Lewis Lung cancer cells is impaired (Nowicki et al., 1996) as is the metastatic potential of mammary tumours when $Csf1^{op}/Csf1^{op}$ mice are crossed with polyoma middle T mice which are susceptible to breast cancer and metastasis (Lin et al., 2001). Both of these phenotypes are reversed when M-CSF is administered.

Along with all other members of the PDGF receptor family, the FMS receptor has the capacity to autophosphorylate on specific tyrosine residues. Four tyrosine residues have so far been identified as major autophosphorylation sites by *in vitro* assays and these are Y699, Y708, Y723 and Y809 (Tapley et al., 1990; van der Geer and Hunter, 1990; Reedijk et al., 1992) (figure 1.4). In addition to these, at least three other minor sites are known to become phosphorylated upon stimulation of FMS with M-CSF and all of these tyrosine phosphorylation sites are known to interact with and provide docking sites for at least one other protein once they become phosphorylated (Pixley and Stanley, 2004). The FMS receptor activates a complex network of intracellular signalling pathways and in Bac1.2F5 macrophages, around 0.02% of total cellular protein becomes phosphorylated upon M-CSF stimulation. A summary of known downstream signalling molecules of FMS is shown in figure 1.5 and table 1 summarises some of the reported downstream effectors of FMS signal transduction (Yeung and Stanley, 2003).

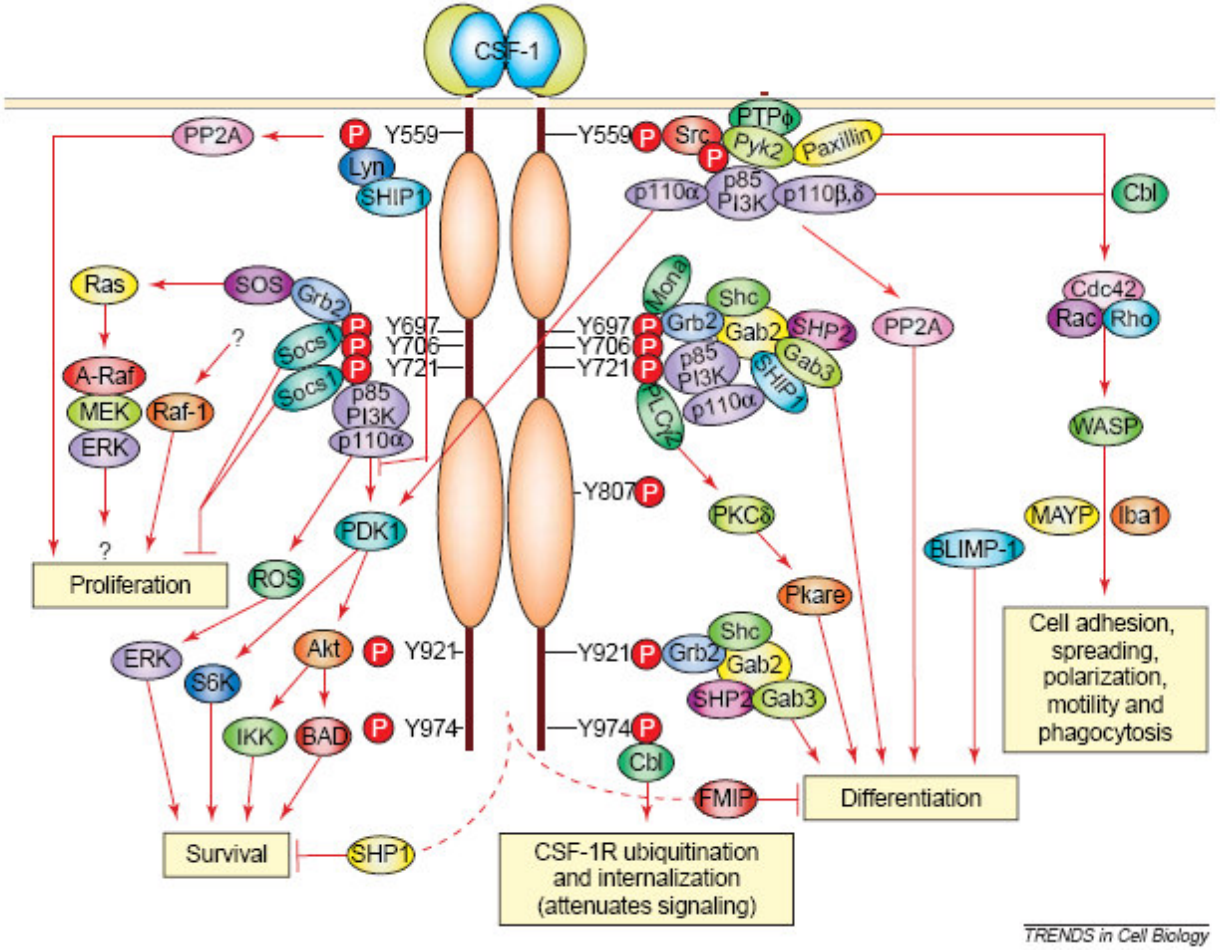


Figure 1.5. Signal transduction from the FMS receptor is complex and involves activation of various signalling pathways and an initial direct association of FMS substrates to the receptor at specific tyrosine residues. These signalling molecules are able to activate their respective pathways and induce various cellular effects including proliferation, survival, differentiation and cell adhesion and motility. Diagram taken from Pixley, F.J & Stanley, E.R. (2004) CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol, 14, 628-38.

Table 1.1. Summary of signalling molecules identified downstream of the FMS receptor.

Signalling molecule	General function	Reference
Shc	adapter protein	(Lioubin et al., 1994)
AKT	PI3K/AKT pathway activator	(Kelley et al., 1999)
Grb2	adaptor protein	(Lioubin et al., 1994)
Sos1	activator of Ras	(Lioubin et al., 1994)
Cbl	ubiquitin ligase	(Wang et al., 1996)
P85 PI3K	regulatory unit of PI3K	(Bourette and Rohrschneider, 2000)
Fyn	Src family kinase	(Courtneidge et al., 1993)
Yes	Src family kinase	(Courtneidge et al., 1993)
Src	Src family kinase	(Courtneidge et al., 1993)
Jak1	Activator of JAK/STAT pathway	(Novak et al., 1995)
STAT1, STAT3	Part of JAK/STAT pathway	(Novak et al., 1995)
Socs1	Adaptor protein	(Bourette et al., 2001)
Paxillin	Focal complex adapter	(Pixley et al., 2001)
Protein kinase C	Protein kinase	(Imamura et al., 1990)
Ras	MAP3K signalling molecule	(Heidaran et al., 1992)
Vav	Rho family GTP-ase	(Yeung and Stanley, 2003)
SHIP1	SH2 domain containing protein	(Bourette and Rohrschneider, 2000)
SHP1 and SHP2	SH2 domain containing phosphatase	(Berg et al., 1998)

Two major FMS signalling pathways are thought to be the phosphoinositide 3-kinase (PI3K) pathway and the MAPK/ERK pathways. PI3K is activated by growth factor receptors at the cell surface and subsequently generates inositol 3-phosphorylated second messengers which activate AKT (also known as PKB) and stimulate its serine-threonine kinase activity (Franke et al., 1997). AKT can then leave the cell membrane and inhibit apoptotic elements such as the caspase family. PI3K activation is thought to be important for macrophage survival and inhibition of AKT activity causes macrophage apoptosis (Kelley et al., 1999). In macrophages, AKT is known to block caspase-9 activation, so inhibiting apoptosis and promoting cell survival (Kelley et al., 1999). PI3K is recruited both directly to the kinase insert region of FMS (Y723) or in a src dependent manner through phosphorylation of tyrosine 559 (Lee and States, 2000). PI3K signalling is also negatively regulated by FMS signalling through a feedback system which involves recruitment of the phosphatase SH2-domain-containing polyinositol phosphatase-1 (SHIP-1) to the membrane. SHIP-1 dephosphorylates important second messengers of PI3K such as phosphatidylinositol 3,4, 5-bisphosphate and this downregulates AKT activity (Baran et al., 2003).

The MAPK/ERK pathway is thought to have a central role in controlling cellular proliferation and in macrophages is involved in cell activation, differentiation and proliferation. Upon FMS activation, Raf is recruited to the receptor via adapter proteins and is able to phosphorylate MEK-1 on serine/threonine residues (Howe et al., 1992). MEK-1 is a threonine/tyrosine kinase and once activated can phosphorylate and activate ERK-1 and ERK-2 (also known as p42/p44 MAPK) (Crews et al., 1992). The activated ERKs are able to then translocate though the

nuclear membrane and regulate transcription factors and therefore cellular outcome. In macrophages, the cellular outcome of ERK signalling is determined by many complex factors including the duration of signalling and by the cell surface receptor from which the signal originates. For example, various authors have proposed models in which the duration of FMS signalling to ERK is important for cellular outcome. When ERK is stimulated through the FMS receptor, which has an ERK phosphorylation peak of around 5 minutes, cell proliferation is stimulated and differentiation is supressed. In contrast to this, chronic signalling though lipopolysaccharide or through the phorbol ester tetradecanoyl-phorbol-13-acetate has an ERK phosphorylation peak of around 20 minutes and inhibits cell proliferation and induces differentiation (Valledor et al., 2000; Suzu et al., 2007).

1.2. FMS function in haematopoetic cells

M-CSF is an important growth factor for cells of the monocyte macrophage cell lineage and plays an important role in regulating the production of various specialised cell types which originate from the monocyte precursor. Monocytes are derived from CD34+ hematopoetic stem cells and are relatively primitive haematopoietic cells that can differentiate into many cells types including macrophages (Dewar et al., 2003), dendritic cells (Thomas and Lipsky, 1996), microglia (Asheuer et al., 2004) and osteoclasts (LaRochelle et al., 2001).

1.2.1 The role of FMS in osteoclast production and function

Osteoclasts are capable of absorbing bone matrix and interact with osteoblasts to constantly remodel skeletal structures of the body. Monocytes differentiate into osteoclasts in response to M-CSF and Receptor Activator of Nuclear Factor kappa B

ligand (RANKL) stimulation and adhere to the bone matrix (Yasuda et al., 1998). Once adhered to the bone, osteoclasts secrete acid and lytic enzymes which resorb and remodel the bone and are the sole bone absorbing cell of the body (figure 1.6) (Baron et al., 1985). Once differentiated from their precursor cells, osteoclasts acquire novel characteristics such as calcitonin receptor and vitronectin receptor expression, tartrate-resistant acid phosphatase (TRAP), cathepsin K and the ß3 integrin (Fuller *et al* 1993). The process of differentiation is negatively regulated by osteoprotegerin (OPG) and downregulation of OPG leads to osteoporosis and excessive bone remodelling (Simonet et al., 1997). FMS signalling through M-CSF stimulation is not only essential for their differentiation but also promotes osteoclast motility, spreading, cell polarization and promotes cell survival by preventing apoptosis (Fuller *et al* 1993).

There are many skeletal diseases that can be attributed to excess osteoclast activity causing an imbalance of bone resorption and bone synthesis. These diseases include osteoporosis, periodontal disease, rheumatoid osteoarthritis, multiple myeloma and metastatic bone cancers (Boyle et al., 2003; Teitelbaum, 2006).

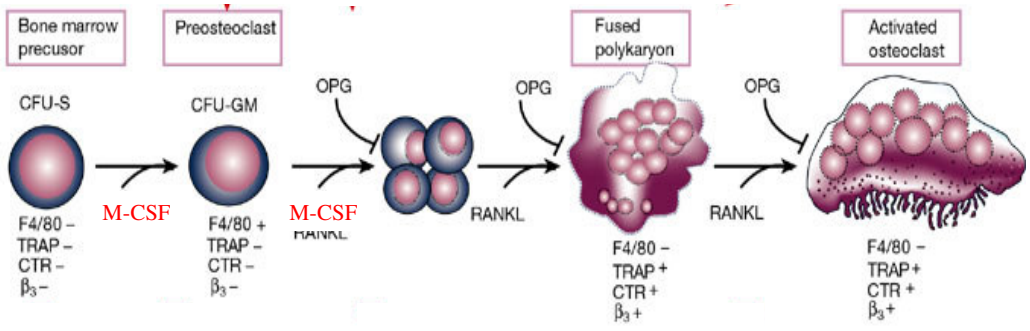


Figure 1.6. Osteoclastogenesis. Osteoclasts originate from bone marrow precursor CD34+ cells which require M-CSF for survival and differentiation into pre-osteclasts. At this stage, both M-CSF and RANKL are required to stimulate the preosteoclasts to fuse to produce a polykaryon and with further RANKL stimulation a mature

multinucleated osteoclasts. This process is inhibited by OPG. Modified from (Boyle et al., 2003)

Metastasis to the bone is a common secondary cancer and various cancers predispose to bone metastasis including renal cancer, lung cancer, thyroid cancer, prostate cancer, breast cancer and multiple myeloma (Mundy, 2002). Bone metastasis can be either osteolytic, and associated with excess osteoclast formation or osteoblastic and associated with excess bone formation. Osteoblastic bone metastasis is most commonly associated with prostate cancer whereas osteolytic bone metastasis is most associated with breast cancer and together bone metastasis is present in 70 % of cases of mortality from these two cancers (Coleman and Rubens, 1987; Charhon et al., 1983). The distinction between osteolytic and osteoblastic lesions is not entirely straightforward as osteolytic cancer is commonly associated with local bone formation and osteoblastic cancer is commonly associated with local areas of osteolysis and osteoclast formation. Bone metastasis could therefore be considered to alter the balance of normal bone formation and absorption (Stewart et al., 1982). Breast cancer metastasis to the bone is well characterised and is thought to be mainly due to the release of a peptide from tumour cells called parathyroid-hormone-like-protein (Guise et al., 1996). This stimulates osteoblasts to produce high levels of pro-osteoclastogenic growth factors such as RANKL and in turn promotes excess osteoclast formation and therefore bone absorption. In an animal model of osteolytic bone metastasis, inhibition of the parathyroid-hormone-like-protein with neutralising antibodies significantly decreased the size and number of osteolytic lesions (Guise et al., 1996).

Osteoclasts and tumour cells are thought to have a mutually beneficial relationship and osteoclasts produce many growth factors which promote tumour growth such as Transforming growth factor beta and Insulin like growth factor-1. This results in a continuous cycle of tumour production, osteoclastogenesis and bone destruction and this is summarised in figure 1.7 (Yin et al., 1999). It may therefore be possible that inhibiting osteoclastogenesis may also decrease tumour burden. Consistently, in an animal model of breast cancer metastasis to the bone, treatment with the bisphosphonate osteoclast inhibitor Risedronate effectively inhibited osteoclastogenesis and also inhibited tumour cell metastasis to the bone but had no effect on metastasis to other soft tissues (Sasaki et al., 1995). This association is also extended to other animal models, and various explanations have been suggested for this effect including inhibition of tumour invasion, angiogenesis, tumour cell adhesion to the bone and general alterations in the bone microenvironment (Clezardin et al., 2003; Green, 2003; Green and Clezardin, 2002; Denoyelle et al., 2003). More investigation into this area is needed, but it is clear that osteoclasts are an important therapeutic target.

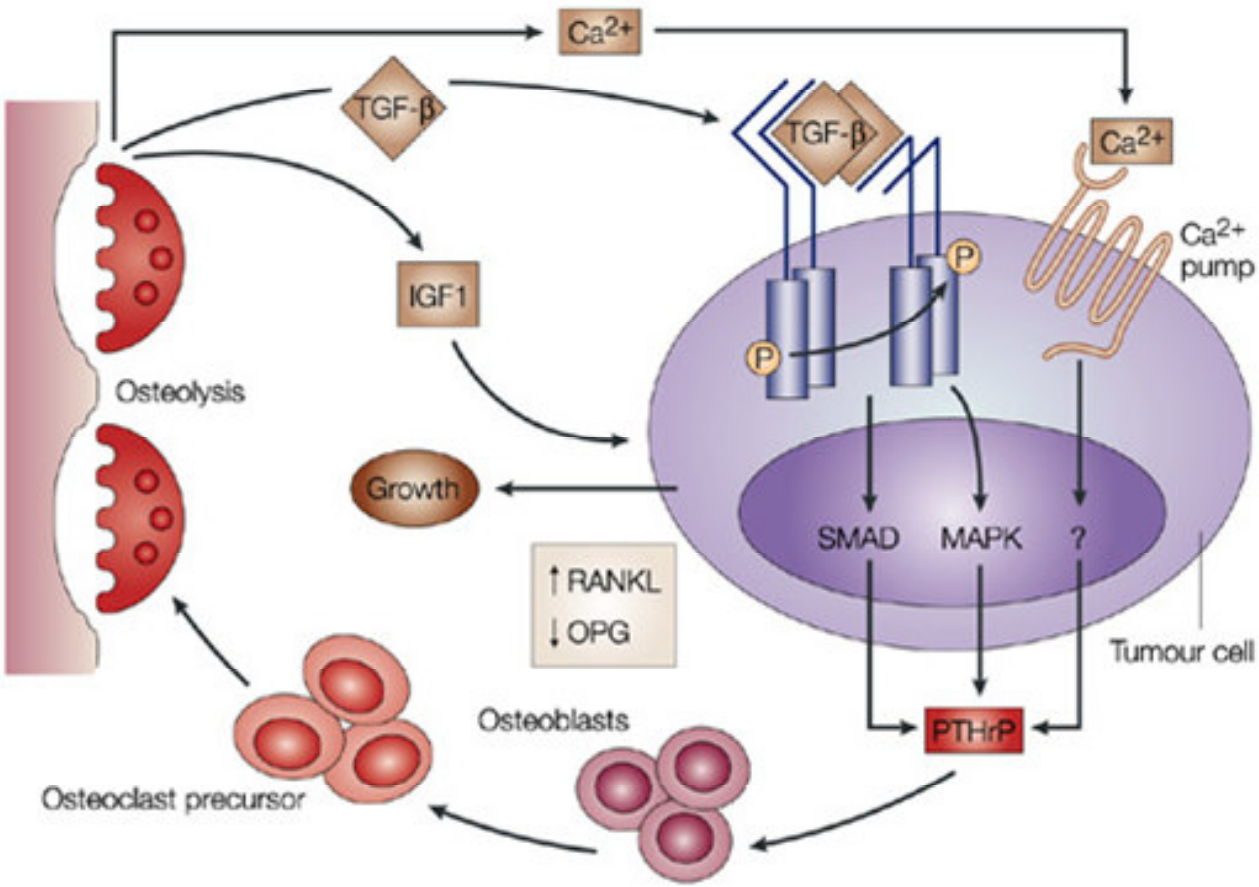


Figure 1.7. The interplay between osteoclasts and tumour cells in osteolytic bone metastasis from breast cancer. Osteoclasts and tumour cells create a mutually supportive bone environment which is conducive to osteoclastogenesis and tumour proliferation. Tumour cells produce parathyroid hormone related protein (PTHrP) which stimulates osteoblasts to upregulate production of RANKL. This stimulates osteoclastogenesis and results in bone destruction. Mature osteoclasts produce growth factors such as TGF beta and IGF1 which promote tumour cell survival and proliferation.
Diagram taken from Mundy, G. R. (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. ***Nat Rev Cancer,*** **2, 584-93**

1.2.2 The role of FMS signalling in microglia activation

Microglia are important phagocytes of the central nervous system and constitute approximately 20 % of the non-neuronal cells. Microglia exist in the central nervous system (CNS) as 'resting microglia', which are characterised by low surface antigen expression and are small cells with fine processes (Garden and Moller, 2006). Microglia can become 'activated' when they are stimulated with inflammatory cytokines due to CNS injury and stimulation from other inflammatory cells. The main function of microglia is to protect the nervous system by producing neuro-protective agents and phagocytising cellular debris resulting from injury or disease (Imai and Kohsaka 2002). M-CSF is thought to be important in microglia activation through an M-CSF/FMS autocrine loop which is stimulated by an inflammatory environment. This results in the production of various inflammatory cytokines including Interleukin-1, macrophage inflammatory protein-1-β, Interleukin-6 and M-CSF and also the release of reactive oxygen species such as nitric oxide (Hao et al., 2002). Activated microglia can also contribute to neurodegenerative diseases by producing neurotoxic agents which exacerbate the chronic inflammatory environment and contribute to neuronal damage (McDonald et al., 1997; Liu and Hong, 2003; McGeer et al., 1988). As a result, inappropriate microglia activation is implicated in many diseases of the nervous system such as Parkinson's disease, Alzheimer's disease and Prion disease (Eikelenboom et al., 2002; Liu and Hong, 2003). There is strong evidence that FMS signalling is responsible for maintaining microglia in their active state and also for modulating cell migration and actin cytoskeleton remodelling and microglia proliferation in these diseases (Imai and Kohsaka, 2002; Rogove et al., 2002). M-CSF production is often found to strongly correspond to areas of amyloid plaque production in Alzheimer's disease and in mouse models of neurodegeneration,

overexpression of FMS leads to an increase in microglia activation and an exacerbation of the neurodegenerative phenotype (Mitrasinovic et al., 2001; Murphy et al., 2000).

1.2.3 The role of FMS in macrophages and chronic inflammatory diseases

Macrophages are the most abundant of the cells to be derived from the monocyte lineage and play a large part in the innate immune response. Monocytes in the circulating blood migrate through the endothelial wall and enter the tissue in response to chemotactic signals from other immune cells at the site of tissue damage. Once in the tissue the monocytes differentiate into macrophages which are able to engulf pathogens and debris and secrete a variety of factors including enzymes, complement proteins, cytokines and growth factors. Macrophages destroy invading micro-organisms by phagocytosis and by releasing cytokines and cytotoxic molecules such as reactive oxygen species. This response is pathogenic and causes debilitating tissue damage when inflammation is not resolved appropriately as in various chronic inflammatory diseases such as rheumatoid arthritis, multiple sclerosis and atherosclerosis (Linton and Fazio, 2003). M-CSF is a major chemoattractant, survival and differentiation factor for macrophages and stimulation of macrophages with M-CSF therefore contributes to many inflammatory diseases (Akagawa, 2002; Chitu and Stanley, 2006).

Arthritis is an inflammatory disease which is associated with chronic inflammation in the joints and a massive infiltration of immune cells (Kawanaka et al., 2002). Macrophages and M-CSF levels are thought to be particularly important in disease pathogenesis and this is illustrated in an animal model of collagen-inducted arthritis.

This disease can be induced in wild type mice or in M-CSF knockout mice by subcutaneous injection of collagen. This causes joint inflammation and local tissue destruction and is considered a good model of disease progression of human rheumatoid arthritis (Williams, 2004). It was shown that M-CSF knockout mice were significantly more resistant to monocyte infiltration and joint destruction than their wild type littermates in this model (Campbell et al., 2000). Consistent with this, high concentrations of M-CSF in patient joints in human rheumatoid arthritis is associated with a more aggressive disease and a poor quality of life (Yang et al., 2006; Kawanaka et al., 2002). In addition to the damage caused by infiltrating macrophages, there is commonly a local production of RANKL from stromal cells in the joint which acts with M-CSF to promote increased osteoclastogenesis, leading to osteoarthritis and localised bone destruction (Danks et al., 2002).

1.2.4 The inflammatory tumour microenvironment

In 1863 Rudulf Virchow made a connection between sites of chronic inflammation and tumourigenesis and since then this connection has become well established (Balkwill and Mantovani, 2001). We now know that infections that cause chronic inflammation are known to be responsible for more than 15% of cancers worldwide (Kuper et al., 2000). These infections lead to local areas of chronic inflammation which contain large numbers of infiltrating immune cells. These immune cells produce high levels of mutagenic and cytotoxic substances such as reactive nitrogen and oxygen species which cause neoplastic changes in the surrounding tissue (Maeda and Akaike, 1998). Classical examples of this include the Hepatitis virus which leads to hepatocellular carcinoma due to chronic inflammation in the liver, the HIV virus, which is associated with Kaposi's sarcoma and local chronic inflammation in the skin

and the bacteria helicobacter pylori which is associated with stomach cancer (Coussens and Werb, 2002). In fact the inflammatory tumour microenvironment is important for most tumours regardless of their origin and it is widely thought that non-malignant cells, in particular inflammatory cells, can modulate tumour properties and play an important role in allowing malignant cells to express their full neoplastic phenotype (Pollard, 2004).

These observations have lead to popular a 'seed and soil' hypothesis in which malignant changes in a cell forms the 'seed' but this requires a fertile 'soil' which consists of a supportive environment of non-malignant cells. This supportive environment is often termed the 'tumour microenvironment' and commonly includes resident stromal cells, adipocytes and fibroblasts and a large population of haematopoietic cells that include mostly macrophages and T cells but also lower numbers of mast and dendritic cells (Albini and Sporn, 2007; Hagemann et al., 2006; Pollard, 2004). The interactions between these cell types are complex but it is thought that many of the non-cancerous cell types contribute to tumour survival, growth or metastasis through the production of cytokines, chemokines, proteases and immunosuppressive molecules that together create a tumour supportive environment (see figure 1.8) (Albini and Sporn, 2007; Bissell and Radisky, 2001; Karnoub et al., 2007; Pollard, 2004).

1.3 Macrophages in the tumour microenvironment

Macrophages play a major part in the tumour microenvironment and make up most of the non-malignant cell mass (Pollard, 2004). Macrophages are highly plastic cells and can be polarised to become either tumour supportive or tumour destructive by cytokines and growth factors in their environment. Normally, macrophages work as part of the innate immune system and can display two distinct phenotypes which are involved in both initial inflammatory responses and in wound healing once the inflammation has resolved. These two activated macrophage phenotypes are termed M1 and M2. Normally, macrophages become activated towards an M1 phenotype in response to bacterial invasion and lipopolysaccharide stimulation or contact with cytokines and chemokines released from T cells or NK cells such as interferon γ. This leads to production of immunostimulatory cytokines such as tumour necrosis factor α and interleukin-6 and results in the production of cytotoxic compounds, phagocytosis, antigen presentation and tissue destruction. In contrast to this, macrophages become activated towards an M2 phenotype when they are involved in wound healing and resolution of inflammation and are stimulated by anti-inflammatory molecules such as glucocorticoid hormones, interleukin-4 and interleukin-13 and chemokines which support angiogenesis and matrix remodelling such as VEGF.

Macrophages that are present in the tumour environment are usually termed tumour associated macrophages (TAMs). In the majority of cancers, TAMs are thought to be activated towards an M2 phenotype due to the various immunosuppressive cytokines that are released by the tumour, polarizing the macrophages towards a trophic, wound healing phenotype (Mantovani et al., 2002). By creating an immunosupressive

environment, the tumour can be seen to 'educate' macrophages to display a supportive 'wound healing', M2 activated phenotype rather than an cytoxic, M1 activated phenotype which may direct the immune system to target the tumour (Mantovani et al., 2002). Presentation of tumour associated antigens is also inhibited by the tumour, mainly though inhibition of mature dendritic cell differentiation, which are the main antigen presenting cell of the immune system (Menetrier-Caux et al., 1998). Release of growth factors such as M-CSF and IL-6 from both the tumour and TAMs are implicated in this aspect of immunosupression and this indirectly also prevents recruitment of cells of the adaptive immune system such as T cells which may have lead to immune recognition of the tumour, this process is shown in figure 1.8.

In support of the views described above, large numbers of infiltrating TAMs correlates with poor prognosis in a variety of cancers including most human breast, prostate, ovarian and cervical cancers and this also often correlated with high local levels of M-CSF (Kacinski, 1997; Lin et al., 2002; Bingle et al., 2002; Smith et al., 1995). Overall, 80 % of malignancies are associated with large numbers of infiltrating TAMs which give a poor patient prognosis (Bingle et al., 2002) and as described previously, mice that are deficient for macrophage production show resistance to metastasis (Pollard 2004).

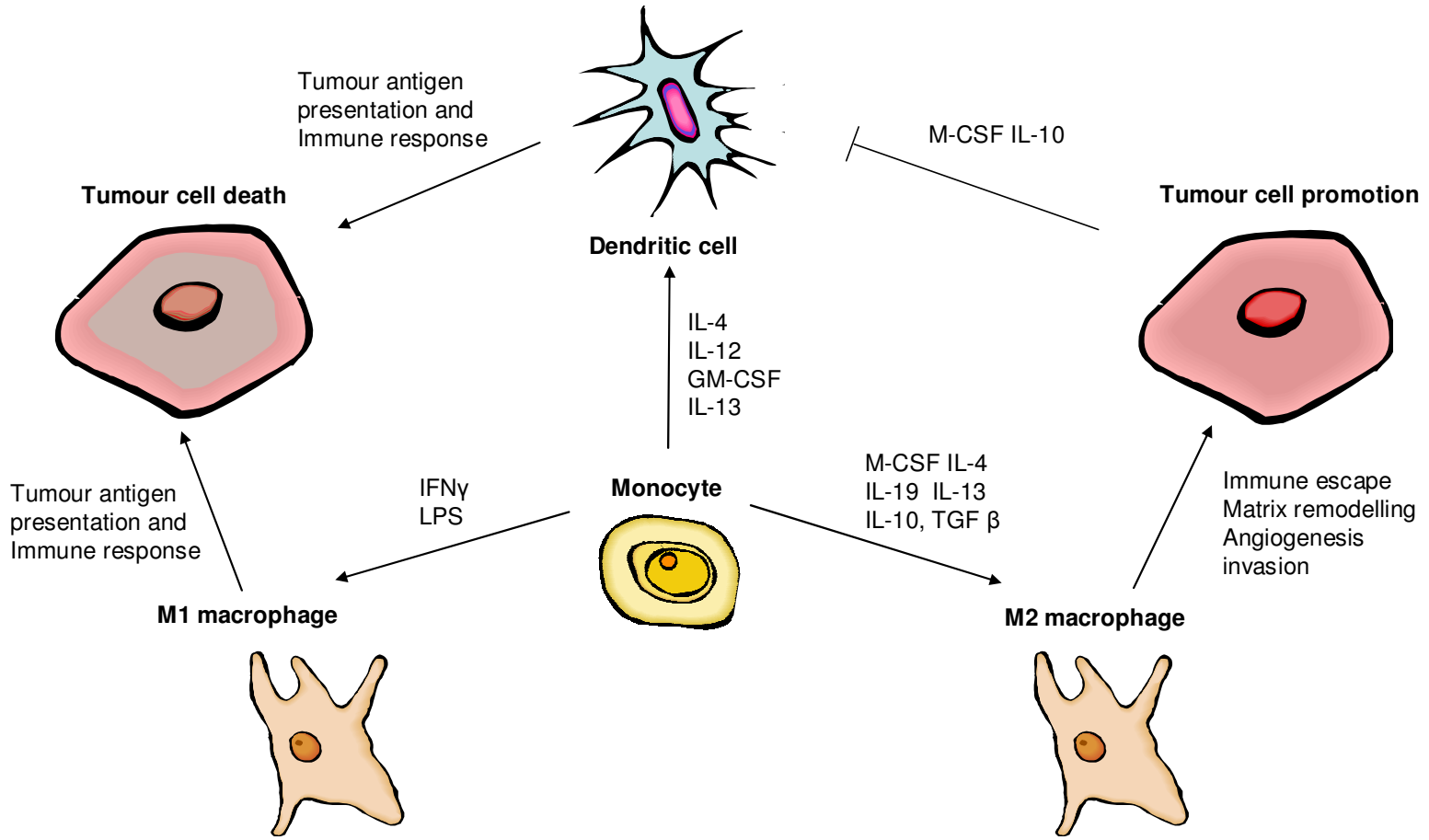


Figure 1.8. Interplay between tumour cells and innate immune cells. Tumours are heavily influenced by their populating immune cells and in return the tumour can 'educate' these cells to create a tumour supporting environment and evade immune detection. Many tumour microenvironments are rich in growth factors such as M-CSF, IL-4 and TGF β which support tumour growth and angiogenesis through appropriately polarised macrophages. In contrast to this, some tumours are incapable of immune evasion and the tumour becomes a target of the immune system mediated through M1 polarised macrophages and mature dendritic cells.

By contrast, if macrophages were to become activated towards a more classical M1 phenotype, it would be expected that they may direct the immune system to attack the tumour. There are a few examples of studies which have shown a correlation between infiltrating macrophages and good patient prognosis (Shimura et al., 2000; Kerr et al., 1998). Because of this various studies have investigated whether *ex vivo* autologous macrophages which had been activated *in vitro* towards an M1 phenotype could be used to target various cancers. These studies yielded disappointing results and the macrophages did little to effect the tumour progression and the authors suggested that the tumour environment may have 're-educated' these macrophages to become immunosupressed (Andreesen et al., 1998).

1.3.1 Tumour associated macrophages in tumour angiogenesis

It is well known that tumours require angiogenesis to grow beyond a critical size, due to limiting oxygen and nutrient supplies. This requires that a number of angiogenic factors including monocyte chemoattractant protein-1, VEGF, bFGF and TGFβ are released and stimulate angiogenesis within the tumour. Macrophages are capable of producing these factors and are in particular known to be key producers of VEGF in the tumour environment (Lewis et al., 2000; Leek et al., 2000). VEGF is the ligand for the VEGF receptors and is a key growth factor involved in angiogenesis and vasculargenesis. Hypoxia is a major regulator of VEGF signaling in tumours and as the tumour grows beyond the capacity of its vascular supply, hypoxia related transcription factors such as HIF cause the production of angiogenic factors such as VEGF from macrophages. This recruits surrounding stromal cells to increase vascularisation and thereby allows tumour growth which is unrestricted by its vascular supply (Hanahan and Folkman, 1996; Shweiki et al., 1992). This is

supported by a mouse model of cervical cancer in which an inhibitor of MMP-9 was used to target macrophages and prevent VEGF production by macrophages. In this model, both tumour volume and vascularisation was significantly reduced (Giraudo et al., 2004). Furthermore, the depth of invasion and angiogenesis of primary cutaneous melanoma is thought to be mediated by TAMs (Torisu et al., 2000) and in a study of primary invasive breast carcinomas, angiogenesis was associated with macrophage 'hotspots' which stained positive for the angiogenic transcription factor HIF-2β (Leek et al., 2002).

1.3.2 Tumour associated macrophages in matrix remodeling and metastasis

Tumour associated macrophages are also important in tumour metastasis as they are implicated in assisting matrix remodeling and invasion of tumour cells. One view is that infiltrating monocytes and macrophages provide pathways for tumour cells to migrate into blood vessels without the need for any matrix remodeling and in effect migrate out of the tumour environment following the path that the macrophages made to migrate into the tumour (Opdenakker and Van Damme, 1992). A more popular view is that the macrophages behave as a 'guide' for tumour cells and that the two cell types migrate together whilst secreting proteases and matrix remodeling chemokines along the way. In a mouse model of breast cancer, two paracrine loops were established between macrophages and tumour cells. Macrophages secreted EGF, which stimulated chemotaxis in tumour cells through the EGF receptor and in parallel to this, tumour cells secreted M-CSF, which stimulated chemotaxis in the macrophages through the FMS receptor (Wyckoff et al., 2004). Therefore, both the tumour cells and the macrophages adopt an exaggerated migratory phenotype and co-

migrate, allowing the tumour cells to benefit from the matrix remodeling properies of the macrophages (Yamaguchi et al., 2006).

1.4 Small Molecule Kinase Inhibitors

The first link between the oncogenic mutation of cell signalling components and human cancer was made in the early 1980s when the sequence similarity between EGF receptor and the v-erbB and the PDGF receptor and v-sis retroviral oncogenes were noted (Downward et al., 1984; Doolittle et al., 1983). Shortly after this, Bishop and Varmus noted that animal retroviral oncogenes such as v-src and v-ras may have oncogenic counterparts in human cancers and this solidified the idea that cancer was in part caused by gain of function mutations in oncogenes (Ullrich et al., 1984; Varmus and Bishop, 1986). Oncogenic gain of function mutations of receptor tyrosine kinases or intracellular kinases have since been discovered in many human cancers and there has been much success in developing inhibitors which inactivate these enzymes.

There are two main types of inhibitor; monoclonal antibodies which target cell surface receptors or their ligands and small molecule inhibitors which usually compete with ATP to bind to the kinase domains of cell surface receptors or of intracellular signalling molecules. Table 1.2 shows that there are many of these inhibitors currently in advanced clinical development against a range of kinases although this list is not exhaustive (Dancey and Sausville, 2003).

Table 1.2. Summary of advanced therapeutic agents which target kinases.

Target	Inhibitor	Company	Type of inhibitor	Clinical trial status	Reference
EGFR	Gefitinib; Iressa	AstraZenica	Small Molecule	FDA Approval	(Ranson and Wardell, 2004)
EGFR	OSI-774; erlotinib	OSI-Pharmaceuticals	Small molecule	FDA Approval	(Cohen et al., 2005)
HER-2	Herceptin	Genentech	Monoclonal antibody	FDA Approval	(Vogel et al., 2002)
KIT	Imatinib	Novartis	Small molecule	FDA Approval	(Joensuu et al., 2002)
BCR-ABL	Imatinib	Novartis	Small molecule	FDA Approval	(Druker et al., 2001b)
BCR-ABL	Nilotinib	Novartis	Small molecule	FDA Approval	(Kantarjian et al., 2006a)
Ras	ISIS 2503	Isis Pharmaceuticals	Antisense oligonucleotide	Phase II	(Alberts et al., 2004)
Raf	ISIS 5132	Isis Pharmaceuticals	Antisense oligonucleotide	Phase II	(Mullen et al., 2004)
PKC	PKC412	Novartis	Small molecule	Phase II	(El Fitori et al., 2007)
PKC-β	LY333531; Ruboxistaurin	Eli Lilly	Small molecule	Phase II	(2007)
PDK1	UCN-01	Kyowa Hakko Kogyo	Small molecule	Phase II	(Welch et al., 2007)
Raf	Sorafenib; BAY43-9006	Onyx/Bayer	Small molecule	Phase III	(Procopio et al., 2008)

Imatinib (Imatinib Mesylate; Gleevec; Glivec; STI571) was amongst the first of the small molecule tyrosine kinase inhibitors to be used successfully and is now the first line treatment for chronic myeloid leukaemia (CML). Imatinib is an ATP analogue which binds to the ABL tyrosine kinase domain of the BCR-ABL fusion protein and figure 1.9 shows the crystal structure of imatinib bound to the ABL kinase domain ((Schindler et al., 2000).

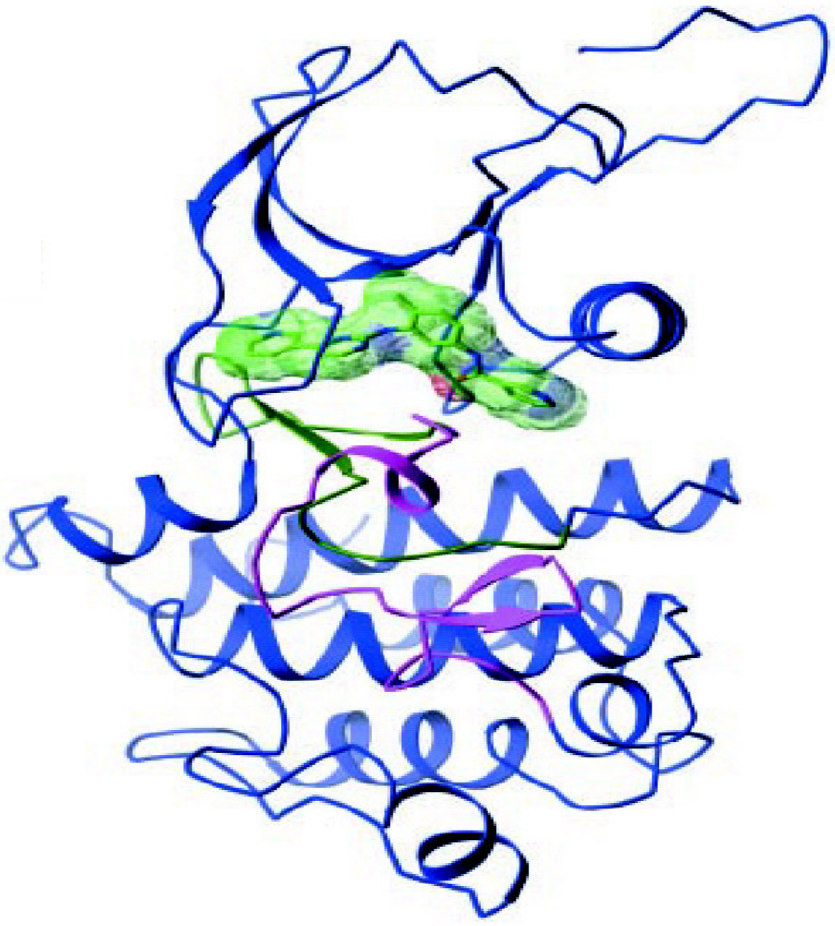


Figure 1.9. Crystal structure of the kinase domain of ABL tyrosine kinase (blue) with imatinib bound to the ATP binding pocket (green) (Schindler et al., 2000).

BCR-ABL is the result of a reciprocal translocation between the gene coding for the breakpoint cluster region (BCR) on chromosome 22 and the gene encoding for the abelson (ABL) tyrosine kinase on chromosome 9. This chromosome fusion is known as the Philadelphia chromosome because it was first discovered in 1960 by Peter Nowell in this State and it encodes the constitutively active BCR-ABL tyrosine kinase. BCR-ABL is present in nearly all cases of CML and 15-30% of acute lymphoblastic leukemia and is sufficient for the initial development of these leukaemias (Daley et al., 1990; Heisterkamp et al., 1990; Kelliher et al., 1990).

Imatinib is also an inhibitor of the PDGF receptor and the KIT receptor (PDGF receptor IC50 = 0.25 μM and the KIT receptor IC50=0.1 μM (Buchdunger et al., 2000). This is probably a reflection of the striking similarity in the structure of the kinase domains of ABL and the PDGF family (Mol et al., 2004b). As a result imatinib has been used to successfully treat gastrointestinal stromal tumours (GISTS) which are a tumour of the gastrointestinal tract and are identified by expression of the KIT or PDGF receptors. These kinases have usually suffered deletions of the JMR which results in mutant receptors that are fortunately much more sensitive to imatinib that the wild type receptor (Chen et al., 2003; Frost et al., 2002; Ma et al., 2002).

FLT-3 and FMS were initially thought to be insensitive to imatinib despite being closely related kinases to the KIT and PDGF receptors (Buchdunger et al., 2000; Dewar et al., 2003). FLT3 is resistant to imatinib due to a phenylalanine residue at position 691 which blocks imatinib binding and when this residue is mutated to a threonine, the mutant FLT-3 receptor becomes sensitive to imatinib (Bohmer et al., 2003). The equivelent mutation of KIT threonine 670 to isoleucine results in an

imatinib resistant KIT receptor and is common in GISTS (Tamborini et al., 2006) and the same mutation in ABL, T351I is a major cause of imatinib resistance in CML (Hofmann et al., 2004). This conserved threonine is located on the edge of the ATP binding pocket and is thought to make critical hydrogen bonds between the kinase and imatinib, nilotinib and dasatinib. When this residue is mutated to an isoleucine, this critical stabilising bond is not made and the inhibitor is also sterically blocked from binding by the larger side chain of isoleucine (O'Hare et al., 2005a; O'Hare et al., 2005b). The corresponding residue in the FMS receptor is a threonine (T663) and this residue is conserved in the imatinib sensitive kinases PDGFR (T674), KIT (T670), ABL (T315) (Daub et al., 2004).

It therefore seemed unlikely that FMS would be resistant to imatinib considering the sequence similarity between the FMS and KIT receptor kinase domain (Dibb et al., 2004). We and another group have more recently shown that FMS is in fact sensitive to imatinib (Taylor et al., 2006; Dewar et al., 2005a) and Taylor et al found that FMS is probably as sensitive to imatinib as the KIT and PDGF receptors, giving an IC50 value for inhibition of FMS of 0.3 μM in comparable assays to ABL and KIT.

CML is normally characterised by three main phases of disease; chronic stage, accelerated stage and blast crisis (Savage et al., 1997). The chronic stage lasts for several years and is associated with a slow increase in the number of myeloid cell precursors in the haematopoetic system. This is followed by an accelerated phase which lasts 4-6 months and during this time, the CML cells become more genetically unstable and this results in the accumulation of immature cells with various genetic and karyotypic abnormalities (Kantarjian et al., 1988). This is followed by a blast

stage where the haematopoetic system becomes over whelmed by immature blast cells, this stage lasts only for one or two months (Calabretta and Perrotti, 2004).

Imatinib is only able to bind to ABL when it is in an inactivated state and it is therefore ineffective against mutations which permanently lock the ABL kinase domain into an activated position (Schindler et al., 2000). Resistance to imatinib is therefore common in patients who are treated in the more advanced and aggressive blast stage of CML where there are excessive numbers of BCR-ABL positive blasts. Some of these blasts inevitably carry imatinib resistance mutations due to their high number and are selected for by imatinib treatment and clonally expanded. Patients treated in the early chronic phase of the disease on average achieve a 96-97% haematological response within 12 months (Hochhaus and La Rosee, 2004) whereas for patients treated in the late accelerated phase of the disease this is reduced to 40% (Talpaz et al., 2002) and less than 10% in the more advanced blast stage (Sawyers et al., 2002).

This problem with imatinib resistance has stimulated the development of a number of other kinase inhibitors which target the common imatinib resistant mutations. Nilotinib (AMN107, Tasigna) and dasatinib (BMS-354825, Sprycel) have been the most successful in treating imatinib resistance and both of these drugs have FDA approval for treatment of imatinib resistant CML (Cortes et al., 2006; Kantarjian et al., 2006a).

1.4.1 FMS Inhibitors

Recently, there has been interest in developing small molecule inhibitors which target the FMS receptor (Irvine et al., 2006; Conway et al., 2005; Murray et al., 2003; Ohno et al., 2006; Brownlow et al., 2008a; Brownlow et al., 2008b; Taylor et al., 2006; Meegalla et al., 2008). The FMS receptor is an important signalling molecule for macrophages, osteoclasts and microglia and as described above these cell types are implicated in a number of diseases, particularly chronic inflammatory diseases and tumour metastasis making FMS an interesting target. One such inhibitor, Ki20227 has been shown to be useful in treating mouse models of collagen induced arthritis, osteolytic bone metastasis and multiple sclerosis progression, mainly by inhibiting macrophage and osteoclast survival (Ohno et al., 2006; Ohno et al., 2008; Uemura et al., 2008). Another FMS inhibitor, SU11248 has been tested in a mouse model of bone metastasis where it inhibited osteolytic bone destruction and also more surprisingly inhibited tumour growth (Murray et al., 2003).

Imatinib has been reported as a FMS inhibitor by us and another group (Taylor et al., 2006; Dewar et al., 2005a) and has been suggested as a treatment for osteoclast related bone diseases due to its potency against osteoclasts (Dewar et al., 2006). Consistently, an altered bone and mineral metabolism has been reported in some patients who were treated with imatinib for extended periods (Berman et al., 2006) and there is also a report of occasional bone thickening in patients who are treated with imatinib, based on bone trephine staining. In this study the authors implicate an inhibition of osteoclastogenesis due to inhibition of the FMS receptor and also promotion of osteoblastogenesis due to inhibition of the PDGF receptor in osteoblast precursor cells (Fitter et al., 2007).

Due to the massive success of imatinib and other such small molecule inhibitors in targeting kinases, many more of these inhibitors currently being trialled and some examples of these are shown in table 1.2. Most of these inhibitors bind to the ATP binding pocket of the kinase domain thereby inhibiting kinase activity. Many of the approximately 500 known kinases have similar structures, particularly around the ATP binding pockets so these inhibitors inevitably have other targets than their intended primary target (Manning et al., 2002). The kinase inhibitors which are used routinely in the clinic are generally well tolerated by patients and this gives an opportunity to treat diseases which are associated with other kinases that are also sensitive to these FDA approved kinase inhibitors. A good example of this in practice is imatinib. Imatinib was originally designed as an inhibitor of the PDGF receptor, inspired by the therapeutic importance of this receptor in treating some inflammatory diseases. It was then shown later that imatinib is also a particularly potent inhibitor of BCR-ABL and it has since become the front line therapy for treatment of CML (as discussed above) (Druker et al., 1996). It was later discovered that imatinib also targets the KIT receptor and it has also since been very useful for treating GISTS where activating KIT mutations are implicated (van Oosterom et al., 2001; Joensuu et al., 2001). As an extension of this, imatinib is currently in clinical trials for treatment of a large number of malignancies, a few examples are shown in table 1.3.

Table 1.3. Current clinical trials involving imatinib.

Disease	**Target**
Head and neck squamous cell cancer	PDGF receptor
Ovarian Cancer	PDGF and KIT receptors
Uterine serous papillary carcinoma	PDGF, KIT, ABL
Rheumatoid arthritis	PDGF receptor
HIV-associated Kaposi's sarcoma	General tyrosine kinase inhibition
Metastatic breast cancer	General tyrosine kinase inhibition

Information taken from www.clinicaltrials.gov.

So imatinib is a good example of a kinase inhibitor which has proven extremely useful in treating several diseases.

1.5 Project Aim

1. To identify FMS inhibitors. At the beginning of this project there were no commercially available inhibitors which targeted the FMS receptor and therefore the main aim of this project was to find some FMS inhibitors and to assess their potential to target diseases in which FMS activity is implicated. Given the ability of imatinib to inhibit both ABL and KIT kinases it seemed likely that FMS, which is closely related to KIT might be also be a target of imatinib, despite reports to the contrary (Buchdunger et al., 2000; Dewar et al., 2003). I therefore assayed various clinically advanced, well tolerated small molecule inhibitors of BCR-ABL and the the FLT-3 receptor, which is also closely related to FMS. I found that imatinib (Taylor et al., 2006), nilotinib (Brownlow et al., 2008a), tandutinib (Brownlow et al., 2008b) and dasatinib (Brownlow et al, submitted) are all reasonable inhibitors of FMS as assessed by various biochemical and cell based assays.

2. To assess the potential of these inhibitors to target macrophages and osteoclasts. To do this I needed to develop assays which could be easily used to test these inhibitors *in vitro* upon primary macrophage and osteclast cell types. I have successfully developed various useful assays of osteoclastogenesis, macrophage colony formation and tumour associated macrophage production from ovarian cancer ascites and used these to assess the therapeutic potential of these inhibitors to target these cell types.

3. To use the kinase inhibitors as research tools to investigate FMS receptor activation and downregulation. I also set out to further establish a previous finding that the degradation of FMS is independent of intrinsic receptor kinase activity. The

FMS inhibitors that I identified here proved to be very useful in addressing this problem and I have used them to provide evidence that the mechanism of FMS degradation is dependent on a structural change in the receptor and not receptor kinase activity. The kinase inhibitors also revealed that at least two tyrosine autophosphorylation sites of FMS can still be phosphorylated in the absence of FMS kinase activity. This implies that other kinases may be involved in FMS activation.

CHAPTER 2. MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 General cell culture

All culture dishes (Invitrogen, Gibco) and flasks (Triple Red) were suitable for tissue culture and all serological plugged pipettes were purchased from Costar. All experiments were carried out in a Class II flow cabinet and cells were grown at 37°C in 5% CO_2 (v/v). All adherent cell lines including Rat-2 fibroblasts and N9 microglia were grown in Dulbeco's Modified Medium (DMEM) supplemented with 10% (v/v) heat inactivated Foetal Calf Serum (FCS), 50 U/ml penicillin/streptomycin (Invitrogen, Gibco) and 200 µM glutamine (Invitrogen, Gibco). Cells were passaged twice weekly by washing in PBS and then incubating with 1 ml of 0.25% trypsin (Invitrogen, Gibco) for 5 minutes. The cells were re-suspended in 9 ml of DMEM supplemented with 10% (v/v) heat inactivated FCS, 50 U/ml penicillin/streptomycin (Invitrogen, Gibco) and 200 µM glutamine (Invitrogen, Gibco) and plated to the required density. Suspension cell lines including all FDC-P1 cell lines were grown in RPMI 1640 supplemented with 10% (v/v) heat inactivated FCS, 50 U/ml penicillin/streptomycin, 200 µM glutamine and 10% (v/v) Wehi 3B conditioned medium (containing IL3). Suspension cells were grown in 6 well trays and passaged weekly by putting 50 µl, 100 µl and 250 µl into three wells containing 4 ml of RPMI 1640 supplemented with 10% (v/v) heat inactivated FCS, 50 U/ml penicillin/streptomycin, 200 µM glutamine and 10% (v/v) Wehi 3B conditioned medium which contains IL-3.

The cell lines FDC-P1 (Wheeler et al., 1987), FD-cfms (Dibb et al., 1990), FD-V Fms (Dibb et al., 1990; Wheeler et al., 1987), FD-D802V (Morley et al., 1999), Rat-2cfms

(Baker et al., 1994), Rat-2V Fms (Baker et al., 1994), Rat-2 D802V (Morley et al., 1999), Rat-2 D802VY708F (Morley et al., 1999), Bac1.2F5 (Morgan et al., 1987), K562 (Klein et al., 1976) have been described previously. Rat-2T633I was made as described in section 2.5.

2.1.2 Freezing cell lines

Cells were grown in T75 flasks until confluent. Adherent cells were incubated with 1 ml of 0.25% trypsin, and then the trypsin was inactivated by adding 9 ml of DMEM with 10% (v/v) heat inactivated Foetal Calf Serum (FCS), 50 U/ml penicillin/streptomycin and 200 µM glutamine. The cells were pelleted by spinning for 5 minutes and re-suspended in 4 ml of DMEM supplemented with 10% (v/v) heat inactivated FCS, 50 U/ml penicillin/streptomycin (Invitrogen, Gibco) and 200 µM glutamine (Invitrogen, Gibco) (adherent cells) or RPMI 1640 supplemented with 10% (v/v) heat inactivated FCS, 50 U/ml penicillin/streptomycin, 200 µM glutamine (suspension cells) with 10% DMSO and aliquoted into freezing vials (0.75 ml per vial). The cells were cooled slowly in freezing apparatus containing propan-2-ol at -80°C overnight then stored in liquid nitrogen.

2.1.3 Cell counting

Cells were counted using disposable haemocytometers (Hycor Biomed) down a light microscope (Olympus CK2). This value was converted into cells per ml based on the volume of the counting chamber. Each experiment was done in triplicate.

2.1.4. Primary cell assays

2.1.4.1 Isolation of non adherent progenitor cells from mouse bone marrow

Hind legs from 8 week old Black 6 mice were kindly donated by A. Chandrashekran. The femur and tibia were removed, excess muscle tissue removed and bone marrow was aspirated with alpha MEM (Invitrogen, Gibco) and a 25 gauge needle (Hycor Biomedicals). The bone marrow was collected, strained using a 70 μM Nylon Cell Strainer (Beckton Dickinson) and spun at 1000 rpm for 5 minutes. The bone marrow cells were then incubated in alpha MEM with 10% (v/v) heat inactivated FCS, 50 U/ml penicillin/streptomycin, 200 μM glutamine and 5000 U/ml M-CSF to deplete fibroblasts or mature macrophage and non-adherent mononuclear cells were re-plated after 24 hours.

2.1.4.2 Normal human and mouse osteoclasts

Normal human osteoclasts were produced from G-CSF mobilised human peripheral blood which was obtained from Hammersmith Hospital Stem Cell facility with informed consent and was a kind donation from S. Marley and M. Gordon. Mononuclear cells were purified from total peripheral blood using lymphoprep tubes (Axis-Shield) as per manufacturer's protocol. 1 X 10^6 mononuclear cells were cultured overnight in alpha-MEM + 10 % FCS and 50 IU/ml penicillin/streptomycin and 200 μM glutamine on tissue culture plastic in 24 well trays and adherent cells were discarded. Primary mouse progenitor cells which contain monocytes were obtained as described above (section 2.1.4). For normal human and mouse osteoclast cultures, 5 x 10^6 mononuclear cells per well in a 24 well tray or 1 x 10^6 cells were cultured in a 96 well tray with or without dentine slices in the bottom of each well. Both human and mouse primary osteoclasts were cultured in alpha Modified Eagles

Medium (Invitrogen, Gibco) with 10% FCS and penicillin/streptomycin, 50 IU/ml and glutamine 200 μM. This media was supplemented with 5000 U/ml rHuM-CSF (First Link) and 40 ng/ml rHuRANKL (First Link) and changed every 3 days for 2 weeks.

2.1.4.3. Macrophage colony formation

Primary mouse and normal human macrophages were produced by culturing mononuclear cells as described above in methylcellulose semi-solid media (Stem Cell Technologies) with 10% FCS, 200 μM L-Glutamine and 5000 U/ml RHuM-CSF for assessment of macrophage colony formation. Macrophage colonies were counted after 2 weeks and identified based on their morphology with advice from S. Marley (Hammersmith Hospital Haematology Dept.).

2.1.4.4 Tumour associated macrophages and osteoclasts

For experiments in which human ovarian cancer ascites was used, 2mls of total ascites was cultured overnight in 24 well trays and adherent cells were used as precursor cells for both macrophages and osteoclasts. Osteoclasts were produced from this material after 3 weeks of culture with alphaMEM with 10% FCS and 50 IU/ml penicillin/streptomycin, and 200 μM glutamine. Osteoclasts were identified based on TRAP staining and each cell containing more than 3 nuclei. TAMs were produced by culturing this material with 5000 U/ml rHuM-CSF for 1 week. Tumour associated macrophages were identified based on immunofluorescence staining with the macrophage marker CD 68 and normally a ~ 99 % pure cell population was achieved in this system.

2.1.5 FDC-P1 and K562 Growth Assay

Various FDC-P1 or K562 cell lines were grown in RPMI media supplemented with 10% FCS and 200 μM L-Glutamine, 50 IU/ml penicillin/streptomycin and FDC-P1 cells were also cultured with 10% (v/v) Wehi 3B conditioned medium as a source of IL-3. FDC-P1 cells that expressed the FMS receptor as a result of retroviral infection (Dibb et al., 1990) could be grown with either IL-3 or M-CSF. These cells were then seeded into 24-well plates at $1x10^4$ cells/ml with increasing concentrations of the appropriate inhibitor with or without M-CSF (5000 U/ml). Cells were grown for 1 week at 37 °C, 5% CO_2 and were counted using a haemocytometer.

2.2 Staining

2.2.1 TRAP assay

Primary osteoclasts were fixed in 4% paraformaldehyde (Fluka) diluted in PBS for 20 minutes and stained for Tartrate Resistant Acid Phosphatase (TRAP) using the TRAP kit from Sigma Aldrich. TRAP solution (made according to manufacturers protocol, see section 2.7.2) was heated to 37 °C and added to fixed cells for 1 hour at 37 °C. After this period the cells were washed in PBS and mounted in water based mount (DAKO faramount) by placing a 13 mm^2 coverslip over the cells. The exposed edges were sealed with clear nail varnish.

2.2.2 Pit assay

Primary osteoclasts were grown on dentine discs (IDS Ltd) in the bottom of 96 well trays. After 3 weeks of culture the discs were removed and placed in an eppendorf tube with 1ml of water and sonicated for 10 seconds. The slices were then stained for 10 minutes with 0.5% (w/v) toluidine blue and washed with water. The pits were

viewed using a Nikon Eclipse E600 microscope with a Nikon Digital camera DXM1200. The pit area was measure using the 'measure area' function on the Lucia G V.4.61 software.

2.2.3 Bone trephine staining

Bone trephines were stained by the histopathology department, Hammersmith Hospital using a standard Masson trichrome stain. Trephine width was measured blindly at 10 random places and the distance between two points was measured using the 'measure width' function on the Lucia G V.4.61 software.

2.2.4 Phalloidin and Giemsa staining

For phalloidin and giemsa staining of transformation assays, Rat-2 fibroblasts were grown on glass coverslips to 70% confluence, treated with the appropriate inhibitor for 24 hours and fixed with 4% w/v paraformaldyhyde (Fluka) for 20 minutes, washed in PBS and incubated at room temperature in either TRITC labelled phalloidin (1:1000 dilution in PBS) (Sigma-Aldrich) or Giemsa stain (1:10 dilution in PBS) (BDH biosciences). The coverslips were washed in PBS and mounted in aqueous mounting media (DAKO faramount,).

2.2.5 TUNNEL apoptosis assay

For TUNEL apoptosis assays, mature osteoclasts were cultured on 13 mm diameter glass coverslips and were fixed in 4% (w/v) paraformaldehyde (Fluka) for 20 minutes and DNA strand breaks were labelled using an *in situ* cell death detection kit (Roche) according to the manufacturer's protocol. The assay is based on the preferential binding of the FITC-dUTP to terminal deoxynucleotidyl transferase to 3' OH ends of

the DNA. The coverslips were mounted in Vectashield mount with antifade and DAPI (Vector laboratories). The number of apoptotic nuclei (within an osteoclast if applicable) and total nuclei were counted in 3 fields of view at x 200 magnification using a Nikon Eclipse E600 microscope. The number of apoptotic nuclei was calculated as a percentage of total nuclei.

2.2.6 Flow cytometry

Flow cytometry was carried out by C. Hayford. Ascites samples were centrifuged at 1000 g for 10 minutes and the supernatant was discarded. The cell pellet was diluted in culture media (RPMI 1640 + 10% FCS + Pen/ Strep and Glutamine) and the mononuclear cells were isolated using Histopaque-1077 (Sigma Aldrich). FACS analysis was performed by using direct FITC-labelled antibody against CD14 (AbD Serotec; 1:50 dilution). Cells were analysed by flow cytometry (FACS Canto, BD Biosciences) using FlowJo software (Ashland, United States). Large granular cells were gated and analysed for expression of the monocyte/macrophage marker CD14.

2.2.7 Fluorescence Microscopy

Cells were grown on 13 mm diameter glass coverslips (VWR) in the bottom of a 24 well tray overnight. After the appropriate drug treatment, the cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilzed with 5% Triton X-100/PBS for 5 minutes, washed in PBS and blocked for 20 minutes in the appropriate blocker (see section 2.7.1). The primary antibody (see section 2.7.1) was diluted in the same blocker and incubated with the cells for 1 hour at room temperature. This was washed and incubated with the appropriate Alexa-fluor conjugated secondary antibody in the

dark diluted in blocker for 1 hour. The coverslips were then washed in PBS for 5 minutes, removed from the 24 well tray, dipped once in deionised water and mounted onto a glass slide using vecta shield mountant with DAPI and antifade (Vector Laboratories). These slides were then visualised using a Zeiss fluorescent microscope.

2.3 Conditions used for inhibitors

For both western blotting and immunofluorescence studies, kinase inhibitors were incubated with live cells at 37 °C for 3 hours. Imatinib was stored in deionised water at 10 mM. Nilotinib (Novartis), tandutinib (Millennium pharmaceuticals), Dasatinib (Bristol Myers Squib), SU6656 (Calbiochem) and Staurosporine (Calbiochem) were diluted in DMSO to a stock concentration of 10 mM. Methylamine was diluted in deionised water to a stock concentration of 1M. All inhibitors except for methylamine were stored at -20 °C. Methylamine was stored at 4 °C.

2.4 Protein Assays

2.4.1 Sample preparation

For both immunoprecipitation and western blotting assays, Rat-2 fibroblasts, N9 microglia or Bac1.2F5 macrophages were plated into 10 cm diameter plates and grown for 48 hours or until confluent. Cells were washed in 1 x PBS then lysed in 1ml of RIPA (see section 2.7.2) directly on the plate. Using a scraper, the lysates were transferred into eppendorfs and pelleted at 13 000 x g in a microfuge for 5 minutes at 4 °C and pellets were discarded. 50 µl Laemlli lysis buffer (see section 2.7.2) was added to each western blotting lysate. Samples were stored at -20 °C.

2.4.2 Immunoprecipitation of the FMS receptor

Samples lysed in RIPA were thawed and spun for 5 minutes at 4°C and 13000 x g in a microfuge, and the supernatant was added to 10 µl of FMS (prep792) rabbit polyclonal antibody, that was raised against a C-terminal peptide. The lysates were transferred into new eppendorfs which were rolled at 4 °C for 1.5 hours. The samples were again spun for 5 minutes at 4°C and 13000 x g in a desktop microfuge and the supernatant was added to 20 µl of protein-A-beads (Autogen bioclear) and rolled for 2 hours at 4 °C. The beads were washed three times in 1 ml of RIPA (see section 2.7.2). After the third wash the beads were re-suspended in 10 µl of laemlli lysis buffer and stored at -20 °C.

2.4.3 SDS-PAGE

Cell lysates which were to be probed for FMS or phospho-FMS were run on a 8% (w/v) resolving and 4% (w/v) stacking acrylamide gel (see section 2.7.2) and lysates to be analysed for MAPK or AKT were run on a 12% (w/v) resolving and 4% (w/v) stacking acrylamide gel (see section 2.7.2). Glass plates (18.5cm x 20cm) were assembled using 1 mm spacers at the sides and the bottom. These were held together using bulldog clips and sealed using 1% molten agarose. The gel was placed into a vertical gel slab apparatus. The bottom and top atrium of the tank were filled with 1 x running buffer (see section 2.7.2). The gel was run at 100 volts for 1 hour then 150 volts for 5 hours.

2.4.4 Western blotting transfer system

Gels were transferred using a wet-blotting system (Biorad). The gels were taken out of the apparatus and the glass plates were carefully removed. The gel was layered in to the apparatus with a sponge layer, then 2 sheets of Whatman chromatography paper, then the nitrocellulose sheet (0.2 μm pore size) (Amersham), then the gel, then a further 2 sheets of whatman blotting paper (Whatman) and finally another sponge layer. The whole sandwich of layers was soaked in a dish filled with transfer buffer (see section 2.7.2) and a stripette was rolled across the sandwich to remove air bubbles. The apparatus was closed and slotted into the transfer tank, which was filled with transfer buffer. The proteins were then transferred for 5 hours at 200 mA.

2.4.5 Immunoblotting

Nitrocellulose membrane was stained with Ponseau S (see section 2.7.2) to see the total protein loading. This membrane was scanned and saved for record. The membrane was then washed in 1XTBS / 0.15 %TWEEN-20 (TBS-T) for 10 minutes and blocked in the appropriate blocker (see section 2.7.1) for 30 minutes and incubated in the appropriate primary antibody (see section 2.7.1) diluted in blocker for 12 hours at 4 °C. The membrane was then washed in TBS-T for 15 minutes with 1 change and then the appropriate secondary antibody was added (see section 2.7.1) for 2 hours at room temperature. After this incubation the membrane was again washed in TBS-T for 15 minutes with 1 change.

All membranes were developed using an ECL plus system (Amersham). The ECL plus was left on the membrane for 5 minutes before wrapping the membrane to cling

film and placing in a film case. The membrane was developed to GRI biomax film using a film developing machine (Kodak).

2.4.6 *In vitro* kinase Assay

FMS was immunoprecipitated from Rat-2 cfms cells using the FMS792 antibody as described above. The precipitated FMS receptor was washed 3 times in RIPA (see section 2.7.2) and, following this washed 3 times in 50 mM Tris pH7. The 50mM Tris pH7 was then aspirated using a 20 gauge needle and resuspended in 17 µl of kinase buffer (see section 2.7.2) per tube. This mixture was then incubated with 0.5 µl (0.185 MBq per reaction) of gamma ATP^{32} and incubated at 30°C for 20 minutes. The reaction was stopped by adding 20 µl of Laemmli buffer (see section 2.7.2) and the tubes were stored at -80 ºC in a Perspex box.

2.5 Molecular Biology Techniques

2.5.1 Site-directed mutagenesis

The FMS T633I mutant was made by oligo-directed mutagenesis following the scheme shown in figure 2.1. Vsn-2 c-fms is a retroviral construct containing the entire cDNA of 3.1 Kb human c-fms cloned into the retroviral vector vsn-2 (Dibb et al.,1990; fig 2.1).

Initially, two short fragments were amplified by PCR by using the following primer pairs; forward primer and mutant primer 2 and reverse primer and mutant primer 1 (see section 2.5.3 for primer sequences). Product 1 contained a Xho1 restriction site and the T633I mutation and product 2 contained a BsrG1 restriction site and the

T633I mutation. These 2 products were E gel purified and ethanol precipitated and resuspended in 30 µl of TE buffer (see section 2.7.2). 1 µl of each of these initial products was used in a second PCR reaction using the forward and reverse primers (see section 2.5.3 for sequences) which generated a larger product which contained both of the restriction sites, Xho1 and BsrG1 and the required mutation. This product was again E gel purified and ethanol precipitated and resuspended in 30 µl of TE buffer. 2 µl of this product and 1 µg of the original vsn-2 c-fms plasmid were restriction digested with Xho1 and BsrG1. These products were again E gel purified and ethanol precipitated and resuspended in 10 µl of TE buffer and then ligated to reform a circular plasmid which contained the required mutation. The plasmid was transformed into DH5 alpha E. coli and 12 plasmid mini preps were made and sequenced to confirm the presence of only the required mutation.

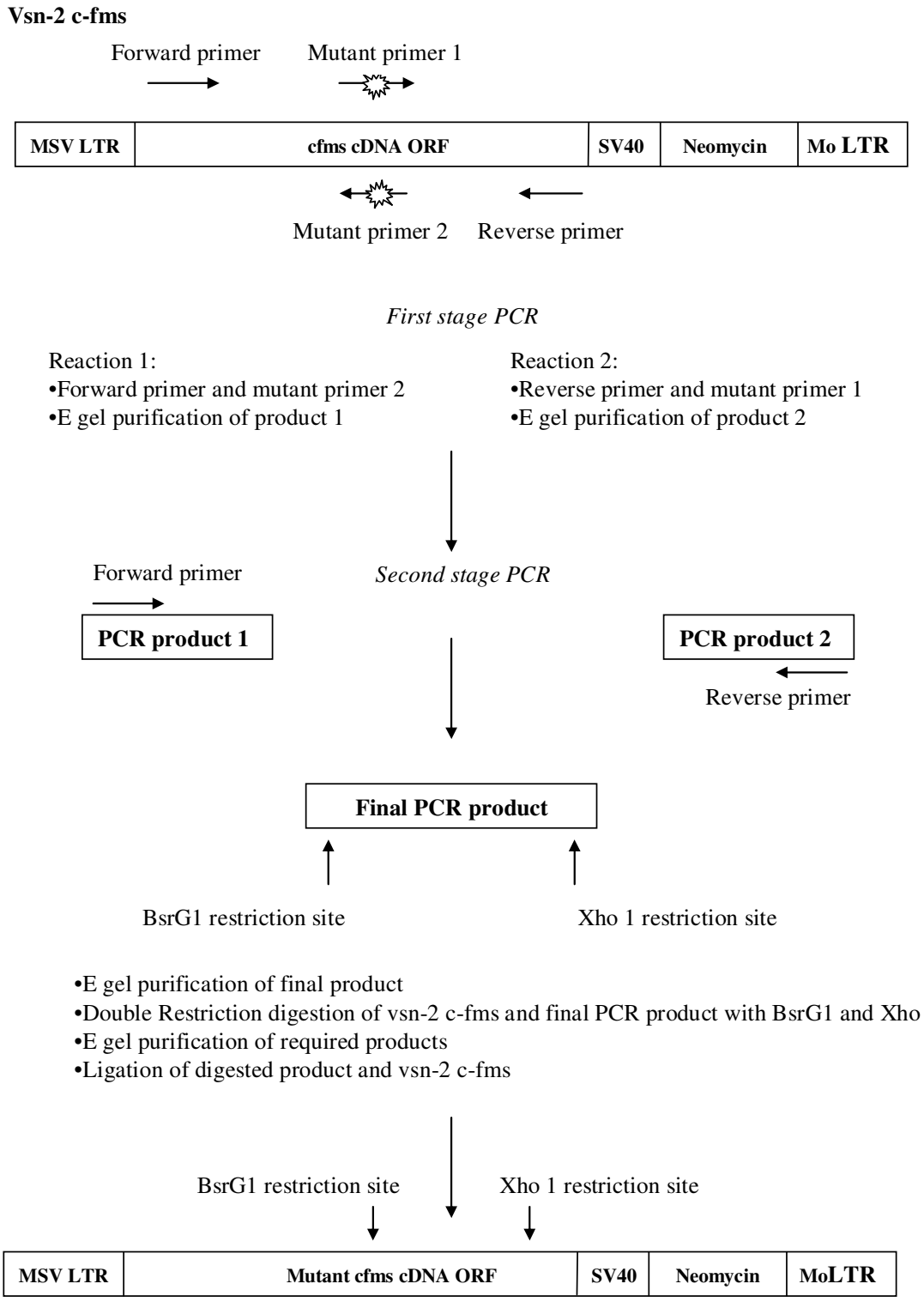


Figure 2.1. Schematic diagram of cloning strategy used to make FMS T663I mutation.

2.5.2 Designing oligonucleotides for mutagenesis

Forward and reverse primers were complementary to the vsn-2 c-fms sequence except for the required base mutations. Primers were also designed upstream and downstream of the mutation encompassing enzyme sites that were then used to clone the fragment back into the vsn-2 cfms construct (Figure 2.1). The primers were designed between 18-40 bases in length.

2.5.3 PCR primer sequences

Mutant Primer 1 (forward): GTACTGGTCATCATCGAGTACTGT

Mutant Primer 2 (reverse): ACAGTACTCGATGATGACCAGTAC

Forward Primer (ND218):ATCGGAATTCCGGGCCGTCCATCCTCCTT

Reverse Primer (ND206): AGGTGACGGTCCATAGCCTGCTG

Sequencing primer 1(ND97): GAGCCCTTCTACAAGGTGACG

Sequencing primer 2(ND220): TGCGAATTCCGGAGCAGATCTGCTGGAAGGT

2.5.4 Polymerase Chain reaction (PCR)

All PCR cycling was carried out on a Hybaid PCR express machine. The following conditions were used for all PCR reactions (primers had similar annealing temperatures):

94 °C – 3 mins (denaturing)

60 °C – 30 secs (annealing)
72 °C – 1 min (extension) } 30 cycles
94 °C – 30 secs (denaturing)

Hold – 4 °C

Each PCR reaction was carried out in 20µl of water and contained 5 µM forward and reverse primers (stock solutions at 100 µM); 1µg of vsn-2 cfms (or 1µl of each of the first round DNA fragments); 0.2 mM dNTP's (Abgene); 2µl of 10x PCR buffer with $MgCl_2$ (Roche) and 0.8 µl of Taq polymerase (Expand TM High Fidelity PCR system, Roche). With each reaction a positive and negative (primers alone) control, no taq polymerase, and one control with no template DNA was used.

2.5.5 TBE agarose electrophoresis

TBE agarose gels were routinely used for checking PCR product sizes as well as restriction enzyme digestions of maxi and mini preps (see below). Agarose was dissolved in 1x TBE (see section 2.7.2) by heating the solution in the microwave and left to cool slightly before adding 0.1µg/ml ethidium bromide (Sigma-Aldrich). The concentration of the agarose gels varied from 0.7 to - 2% (w/v) depending on the size of the DNA fragments. The cooled agarose was poured into a small horizontal gel tank (Flowgen). Once the gel had set the metal plates were removed and the tank was filled with 1x TBE. The samples were prepared by adding $1/10^{th}$ the volume of orange loading dye (5 x orange G, see section 2.7.2) and then loaded into the wells of the gel with a 1 Kb ladder (Gibco-BRL) to assess the size of the DNA bands. Gels were run at 50 volts for 1-3 hours. The bands were visualised using a UV light box.

2.5.6 Preparation of inserts and vectors for cloning by restriction enzyme digestion

Restriction enzyme digests were performed using NEB restriction enzymes (Biolabs) in the recommended buffers and at the recommended reaction temperatures. The reactions were carried out in a final volume of 20µl and contained 1µl of plasmid prep DNA (2µg) or 5µl of PCR product, 2µl enzyme buffer and 1µl enzyme and were incubated for a minimum of 1.5 hours at the suggested temperature. The reactions were stopped by adding orange loading dye (5 x orange G) and the DNA fragments isolated on an E-gel (see section 2.5.7).

2.5.7 E-Gel extraction and ethanol precipitation

Digested DNA samples intended for isolation were run onto thin (3 mm x 75 mm x 75 mm) 1% (w/v) agarose TAE gels (containing 0.1µg/ml ethidium bromide). Sample loading buffer (5 x orange G) was added to each of the digested DNA samples then loaded into the E-gel leaving a lane between each sample. Gels were run at 30 volts for two to three hours in 1x E-buffer. To isolate the required fragment, a cut was made in the gel under the DNA band using the UV light box to visualise the DNA. The gel was the placed back in the tank on a raised block. All the empty wells were filled with E-buffer. The hole was filled with about 40µl of 1x E buffer and the gel was run for 15 seconds (small DNA fragment) or 20 seconds (larger DNA fragment) at 150volts. During this step the DNA enters the cut well and can be removed by using a pipetteman. This step was repeated four times until all the required DNA was removed as seen under UV light. The tube was spun for 2 minutes and the supernatant removed to a fresh tube. The DNA was ethanol precipitated by adding $1/10^{th}$ of the

volume of NaAc pH 5.2 and 2.5 times the volume of ethanol (-20°C) and the samples were left on ice for 30 minutes (or overnight at -20°C) and spun for 10 minutes at 13000g in a microfuge. The supernatant was removed and tubes washed with 70% ethanol (room temperature). Pellets were air dried before being re-suspended in 30µl of TE buffer (see section 2.7.2).

2.5.8 Ligation of inserts into vectors

Ligation reactions were set up in 1.5ml eppendorf tubes as follows;

	1	2	3	4	5
Digested vector DNA	1µl	1µl	1µl	1µl	-
Digested insert DNA	-	-	1µl	3µl	1µl
T4 DNA ligase	-	0.5µl	0.5µl	0.5µl	0.5µl
10 x 1igase buffer	1µl	1µl	1µl	1µl	1µl
Water	8µl	7.5µl	6.5µl	4.5µl	7.5µl

The tubes were left at room temperature for 5 hours before transforming into competent DH5α cells (see section 2.5.11).

2.5.9 Small scale/ large scale plasmid purification

Plasmids were purified after transformation of the appropriate plasmid into DH5 alpha E.coli (see section 2.5.11). For small scale plasmid purification, colonies of transformed E. coli were inoculated into 1 ml of LB media containing 100 µg/ml ampicillin and placed in a heated shaker overnight. E coli were spun at 13 000 x g for

5 minutes. Plasmids were purified using the QIAprep miniprep plasmid Purification Kit following manufacturers guidelines (Qiagen).

For large scale plasmid purification, DH5 alpha cells which had been transformed with the appropriate plasmid and grown on an agar plate containing 100 µg/ml ampicillin overnight were scraped into 250 mls of TY media containing 100 µg/ml ampicillin. This was placed in a 37 °C shaker overnight and the plasmid was harvested by spinning down the E. coli at 13 000 x g for 10 mins and using the QIAGEN Maxi plasmid purification kit following manufacturers guidelines (Qiagen). The OD_{260} was measured and the DNA concentration was adjusted to 2 mg/ml.

2.5.10 Sequencing double stranded DNA to screen for mutations

Plasmids were checked for the appropriate mutation by sequencing with a suitable primer. Sequencing reactions were prepared in microtubes as follows: 1µl (maxi prep) or 2µl (mini prep) DNA, 1µl primer (100 µM), 8µl Big Dye terminator (Applied Biosystems) and made up to 20 µl with water. The samples were run on a PCR machine with a program of 95°C for 1min then 95°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes (35 cycles). These reactions were cleaned by an isopropanol precipitation method. 80µl of 75% isopropanol was added to each reaction, mixed and left at room temperature for 15 minutes. These tubes were then spun at 13000g in a mircocentrifuge for 15 minutes after which the supernatant was removed. Next, 100µl of 70% isopropanol was added, mixed and incubated at room temperature for 10 minutes. The tubes were spun at 13000g for 10 minutes, the supernatant removed and the precipitates left to air dry for 30 minutes. These were

then stored at –20°C until ready to be sequenced by the in house service at the IRDB. Samples were reconstituted back into 30 µl of Hi-dye then denatured for 5 minutes at 96°C and left on ice. Sequences were analyzed using the 3100 Genetic analyser, 16 capillary model (Applied Biosystems) using standard programmes for DNA sequencing. Data was collected automatically using the DNA sequencing analysis software version 3.7 (Applied Biosystems). This system can successfully read sequences of up to 560-600 bases.

2.5.11 Transformation of DH5α cells with plasmid DNA

Competent DH5 alpha cells were transformed with 1-10 ng of plasmid DNA. DNA was added to 100µl of cells and the tubes were placed on ice for 45 minutes before being heat shocked for 90 seconds at 37°C. Cells were transferred to tubes containing 1 ml of 2 x TY and shaken for 30 minutes at 37°C. Following this 100 µl of each culture was plated onto TY agar plates containing 100 µg/ml ampicillin and incubated at 37°C overnight in an inverted position.

2.5.12 Stable retroviral transfection

Rat-2 fibroblasts were grown to approximately 40% confluence in T25 flasks. Polybrene (Sigma) was added to the flask to a final concentration of 4ng/µl and incubated for 1 hour at 37°C, 5% CO_2. During this incubation supernatant containing the virus was removed from the previously transiently transfected (see section 2.5.13) PA317 cells and filtered through a 0.45 micron filter into a 50ml tube and stored in the fridge. After the hour the media was removed from the cells and 1.5ml of viral supernatant was added along with polybrene (final concentration of 4ng/µl). After 24

hours, fresh DMEM supplemented with 10% (v/v) heat inactivated Foetal Calf Serum (FCS), 50 U/ml penicillin/streptomycin (Gibco) and 200 µM glutamine (Gibco) was added to the cells containing G418 to a final concentration of 1mg/ml.

2.5.13 Transient transfection

The cell line to be transfected was grown to 80 % confluence and the growth media was changed to DMEM with penicillin/streptomycin (Gibco) 50 IU/ml and glutamine (Gibco) 200 µM. Cells were transfected with ESCORT V lipid based transfection reagent as per manufacturer's protocol. Briefly, for transfection of plasmid DNA into a 6 well plate, 300 µl of Escort V buffer was mixed with 3.5 µg of plasmid and in a separate tube, 300 µl of Escort V buffer was mixed with 10 µl of Escort V transfection reagent. These two mixtures were combined, inverted 3 times and incubated at room temperature for 25 minutes. The mixture was added to cells drop wise and incubated for 48 hours before assaying.

2.6 Molecular modelling

Molecular modelling of nilotinib was carried out by J. Murray and the modelling for dasatinib was carried out by C. Mol. The molecular model for the kinase domain of FMS was constructed from the homologous (72% amino acid identity) kinase domain of activated c-Kit (PDB code: 1PKG (Mol et al., 2003) by manual replacement of non-homologous residues, and visual inspection of the model using the XtalView molecular graphics program (McRee, 1999). This model structure was energy-minimized using REFMAC (Murshudov et al., 1997) within the CCP4 suite of programs. A model for the binding of dasatinib or Nilotinib to active FMS was

derived by superposition and comparison with the crystal structure of dasatinib bound to active Abl kinase (Tokarski et al., 2006).

2.7 Reagents

2.7. 1. Antibodies

Antibody	Source	Application	Dilution	Blocker
Rabbit anti-FMS C-Terminus polyclonal (Ab792)	N. Dibb	WB, IP	1:1000	5% non fat milk/TBS 0.15% TWEEN 20
Rabbit anti-phospho-Tyrosine723	Cell Signalling	WB	1:1000	5% BSA fraction V /1mMNaVO_3/TBS 0.15% TWEEN 20
Rabbit anti-phospho-Tyrosine708 polyclonal	Santa Cruz Biotech.	WB	1:1000	55% non fat milk/1mMNaVO_3/TBS 0.15% TWEEN 20
Mouse anti-phospho-AKT monoclonal	Cell Signalling	IF, WB	1:100 (IF) 1: 1000 (WB)	10% FCS/ PBS 5 % milk/TBS-T
Rabbit anti-phospho-MAPK polyclonal	Cell Signalling	IF, WB	1:100 (IF) 1: 1000 (WB)	10% FCS/ PBS 5 % milk/TBS-T
Rabbit anti-FMS N-Terminus (3-4A4) Mouse/rat specific	Santa Cruz Biotech.	IF	1:100	10% FCS/ PBS

Mouse anti-FMS N terminus clone AFS98 Human specific	Ebioscience	IF	1:100	10% FCS/ PBS
Alexa 488 conjugated Goat anti- Rabbit IgG	Invitrogen Molecular Probes	IF	1:500	10% FCS/ PBS
Alexa 568 conjugated Goat anti- Mouse IgG	Invitrogen Molecular Probes	IF	1:500	10% FCS/ PBS
Peroxidase conjugated Goat anti-mouse IgG	Sigma Aldrich	WB	1:10 000	5% non fat milk/TBS 0.15% TWEEN 20
Peroxidase conjugated Goat anti-rabbit IgG	Sigma Aldrich	WB	1:10 000	5% non fat milk/TBS 0.15% TWEEN 20
Mouse anti-CBL (A-9)	Santa Cruz	WB	1:1000	5% non fat milk/TBS 0.15% TWEEN 20
Mouse anti CD68 (KP1)	Santa Cruz	IF	1:1000	10% FCS/ PBS

Western blotting(WB), Immunoprecipitation (IP), Immunofluorescence (IF)

2.7.2. Table of commonly used solutions.

Laemmli lysis buffer 20% Glycerol 2% SDS 0.1M Tris pH 6.8 10% ß-Mercaptoethanol 7M Urea	**10x Running Buffer** 121g Tris base 578g Glycine 40g SDS Water to 4 litres
Coomassie Stain 0.25% Coomassie Brilliant Blue 45% Methanol 10% Acetic Acid Made in water	**Kinase Buffer** 10mM $MnCl_2$ made in 20mM HEPES/20mM PIPES buffer
Fixer for radioactive gels 10% MeOH 5% Glycerol Made in water.	**10x PBS** (1) 0.5M NaH_2P0_4 (30g/500ml) (2) 0.5M Na_2HP0_4 (35.5g/500ml) Add (2) to (1) until pH 7.2 this gives stock solution. Take 200ml of this stock and add 8.76g NaCl and make up to 1 litre with distilled water.

RIPA/SDS		**Protease inhibitor mix**	
1%	Nonidet P-40	20mg/ml	phenyl methyl sulfonyl fluoride (PMSF)
1%	Triton X-100	20mg/ml	1-10 phenanthroline
1%	Sodium deoxycholate	20mg/ml	Benxamine
0.1%	SDS	Dissolved in ethanol and stored at -20°C	
150mM	NaCl		
10mM	Tris pH 8.0		
2nM	NaF		
RIPA/SDS was stored at 4°C and 10µl per ml of protease inhibitor mix and Aprotinin was added before use			
Transfer Buffer		**TRAP Solution**	
25mM	Tris base	44ml Water	
0.2M	Glycine	2 ml Acetate solution (Sigma cat 386-3)	
20%	Methanol	2ml Naphthol AC-BI Phosphoric acid (Sigma cat no. 386-4	
0.05%	SDS	2ml Tartrate solution (Sigma Cat No. 386-2)	
		1 capsule Fast Garnet GBC Salt (Sigma Cat 386-15)	
		Filter through Whatman No.54 before use.	

Polyacrylamide 8% gel	**Polyacrylamide stacking gel**
16.95 ml Deionised water	6.8 ml Deionised water
13.4 ml 30% Acrylamide / Bis solution (Biorad)	1.7 m 30% Acrylamide / Bis solution (Biorad)
18.75 ml 1 M Tris (pH 8.8)	1.25 ml 1 M Tris (pH 6.5)
0.5 ml 10 % SDS	0.1 ml 10 % SDS
0.5 ml 10 % APS	0.1 ml 10 % APS
30 µl TEMED	10 µl TEMED
Polyacrylamide 12% gel	**TBE**
10.15 ml Deionised water	432 g Tris
20 ml 30% Acrylamide / Bis solution (Biorad)	220 g Boric acid
18.75 ml 1 M Tris (pH 8.8)	37.2 g EDTA
0.5 ml 10 % SDS	Water to 3 litres
0.5 ml 10 % APS	
30 µl TEMED	
2xTY	**2xTY + AGAR**
16g Tryptone	Same as 2xTY but with 15g of Bacto-agar add per litre.
10g Bacto-yeast extract	
5g NaCl	
Water to 1 litre	

10x TBE 432g Tris 220g Boric acid 37.2g EDTA Water to 4 litres	**20x TAE (E-Buffer)** 0.8M Tris 0.4M Sodium acetate 20mM EDTA pH 8.0 with glacial acetic acid
TE Buffer 10 mM Tris-HCL pH 7..4 1 mM EDTA	**Ponceau S** 0.2 % Ponceau Red 5 % Acetic acid
5 X Orange G loading buffer 0.1 % Orange G powder (SigmaAldrich) Dissolved in TAE buffer.	

CHAPTER 3. Imatinib and Nilotinib inhibition of the FMS receptor and human osteoclastogenesis.

3.1 Introduction

Many diseases are linked to the over activation or aberrant signalling from kinases, ranging from cancer to inflammatory diseases. Over the past 15 years there has been much success in treating these diseases with various kinase inhibitors which compete with ATP for binding to the kinase domain. One of the first of these inhibitors was imatinib mesylate (Gleevec, Novartis). Imatinib has been very successful in treating Philadelphia chromosome-positive chronic myelogenous leukemia (CML) and is currently the first line treatment after having a significant impact on outcome in patients newly diagnosed with CML (Branford et al., 2003; Kantarjian et al., 2003; O'Brien and Deininger, 2003). Patients treated in an advanced stage of the disease do however tend to develop resistance to imatinib (see introduction, page 48).

This problem has led to the development of a second generation of kinase inhibitors against BCR-ABL with the view to overcome imatinib resistance. Nilotinib (AMN107, Tasigna, Novartis) is a new derivative of imatinib that has greatly enhanced affinity for the ATP binding site of the Bcr-Abl kinase (Manley et al., 2005). It is more potent against CML cells *in vitro* than imatinib and it is also active against 32/33 mutations of ABL that confer resistance to imatinib in cell lines (Weisberg et al., 2005). In a phase 1 trial of patients who had previously been treated with imatinib and become resistant, 75 percent of patients with the accelerated phase and 39% in the blast crisis achieved complete haematological response (Kantarjian et al., 2006a). Despite this success, the problem of drug resistance remains as treatment with

nilotinib simply further selects for mutations which are resistant to both imatinib and nilotinib. A particularly common mutation of this class is a mutation of a threonine residue at the 'gatekeeper position' (see chapter 1, Introduction) in the ATP binding site which is particularly necessary for both imatinib and nilotinib to bind to ABL (Gorre et al., 2001).

Imatinib was developed from an inhibitor of the PDGF receptor (Buchdunger et al., 1996; Buchdunger et al., 1995) and both imatinib and nilotinib are also active against other members of this receptor family including KIT but not FLT-3 (Buchdunger et al., 2000; Bohmer et al., 2003; Dewar et al., 2005a; Taylor et al., 2006; Lacey et al., 1998; Manley et al., 2005) although the increased sensitivity of ABL to nilotinib compared to imatinib does not correspond to an increased sensitivity to the PDGF receptor family (Weisberg et al., 2005). Imatinib is particularly active against KIT receptors that have oncogenic juxtamembrane mutations (Longley et al., 2001; Frost et al., 2002) and this provides a novel and effective treatment for patients who have gastrointenstinal stromal tumours (GISTs) where this oncogenic mutation is common (Joensuu et al., 2002). However, imatinib is ineffective against another common oncogenic mutation of the PDGFR family that changes an Asp residue to a hydrophobic amino acid (usually Val) within the kinase domain of this receptor family (Frost et al., 2002; Ma et al., 2002). This oncogenic mutation is found at high frequencies in mast cell leukaemia, acute myeloid leukaemia, testicular cancer and a subset of GISTs (Dibb et al., 2004). There are conflicting reports regarding the sensitivity of nilotinib to this mutation (Verstovsek et al., 2006; von Bubnoff et al., 2005).

Imatinib is well-tolerated by most patients with CML although it has been observed that some patients who were treated with higher doses of imatinib showed an association with altered bone and mineral metabolism (Berman et al., 2006; Joensuu et al., 2002; Joensuu and Reichardt, 2006). These findings indicate a reduction in osteoclast number or function due to imatinib treatment.

Here we use a number of assays to show that both imatinib and nilotinib are effective inhibitors of FMS signalling at concentrations above 600 nM and 250 nM respectively. In confirmation of this we found that mutation of Asp 802 to Val described above, conferred resistance to both imatinib and nilotinib. In the majority of FMS-based assays, nilotinib was only a slightly more effective inhibitor of FMS signalling compared to imatinib, however we observed that nilotinib (IC50 50 nM) was a notably more effective inhibitor than imatinib (IC50 > 500 nM) of human osteoclast production in primary cultures. The therapeutic implications of these results are discussed at the end of this chapter.

3.2. Results

The proliferation of the haemopoietic cell line FDC-P1 is normally dependent upon IL-3 (Dexter et al., 1980; Hapel et al., 1984), however, M-CSF can also be used to stimulate the growth of cells that express recombinant FMS (Rohrschneider and Metcalf, 1989; Dibb et al., 1990) . FDC-P1 cells which express the oncogenic FMS receptors V-Fms (Dibb et al., 1990) and FMS D802V (Glover et al., 1995) proliferate independently of IL-3 and M-CSF due to the constitutively active FMS receptor.

Figure 3.1 shows that both imatinib and nilotinib inhibited FMS receptor stimulated cell proliferation and that a mutation of Asp 802 to Val (D802V) conferred resistance. This is in accordance with previous reports of imatinib and nilotinib inhibition of the equivalent mutation of the KIT receptor (Frost et al., 2002; Verstovsek et al., 2006) although contrary to another report which shows that KIT D816V is sensitive to nilotinib (von Bubnoff et al., 2005). Figure 3.1A shows that the growth of FD cfms cells was fully inhibited at 2 μM by imatinib (figure 3.1A; stripes) and accordingly the growth of FD V Fms was also fully inhibited at this concentration (figure 3.1A; Black). The IC50 for imatinib inhibition of the FMS receptor was calculated to be 0.6 μM from this figure. In contrast, the proliferation of FD D802V was resistant to imatinib (figure 3.1A; no fill). The growth of FD-CP1 cells with IL-3 was unaffected by imatinib as expected (data not shown). In accordance with these results, the murine macrophage cell line Bac1.2F5, which expresses endogenous FMS receptor and is dependent on M-CSF for survival and proliferation (Boocock et al., 1989) was sensitive to imatinib (figure 3.1B; circles). Infection of these cells with a retrovirus expressing FMS D802V conferred resistance to imatinib in this cell line (figure 3.1B; squares).

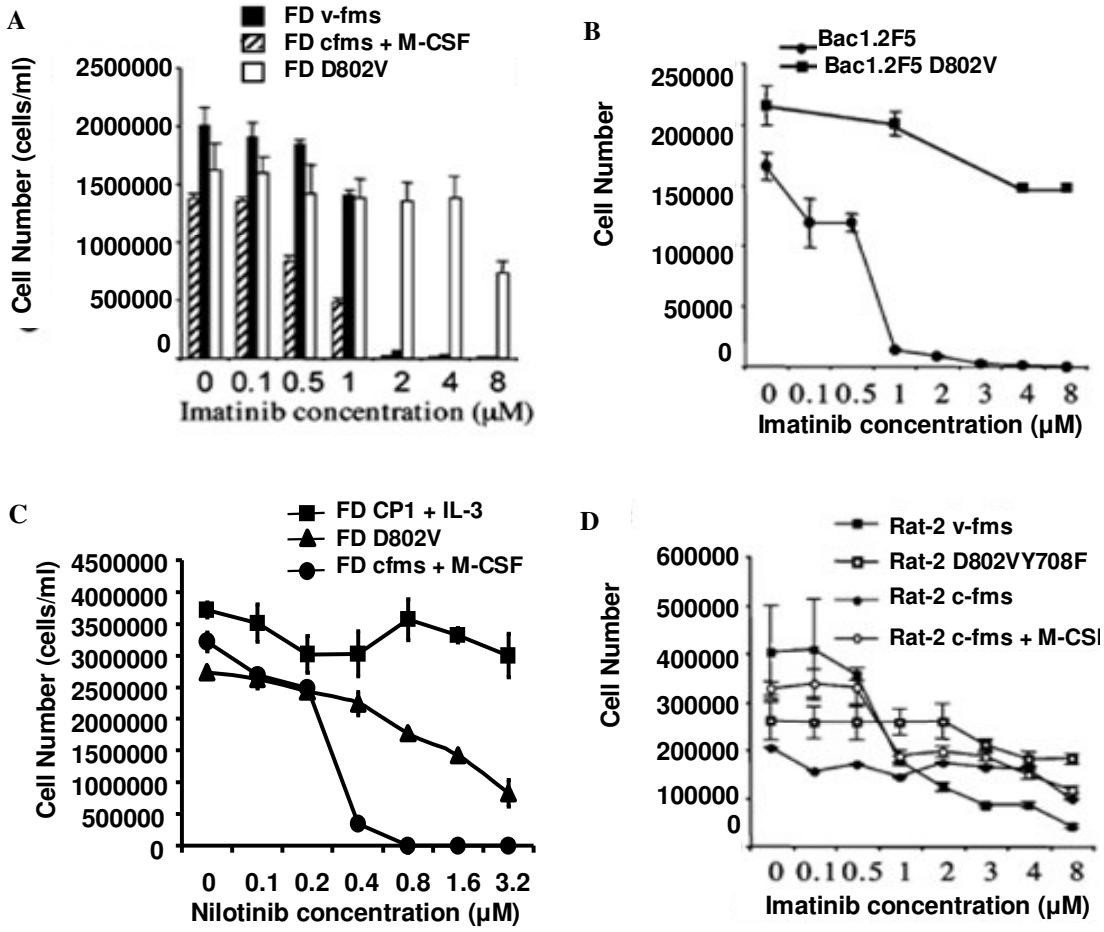


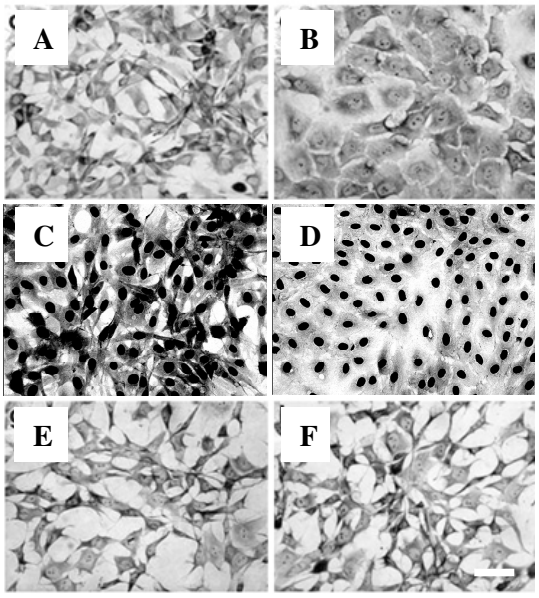
Figure 3.1. Imatinib and nilotinib inhibit FMS dependent cell proliferation and a mutation of asp 802 to val confers resistance. (A) FDC-P1 cells that express FMS (FD c-fms) V-fms (FD v-fms) or D802V (FD D802V) were plated at 1x104 cells/ml in RPMI + 10% FCS in 24-well plates and grown for 7 days in the indicated concentrations of imatinib. FD c-fms cells were grown with 5000 U/ml M-CSF. (B) Bac1.2F5 macrophage cell line grown with M-CSF (diamonds) or expressing oncogenic FMS receptor FMS D802V (squares) grown with increasing concentrations of imatinib. 1 x 10^4 Bacl.2F5 cells were plated in DMEM+10% FCS+5000 U/ml M-CSF in 24-well trays and grown for 4 days in the indicated concentrations of imatinib. (C) FD c-fms cells were grown in either 10 ng/ml IL-3 (squares) or 5000 U/ml M-CSF (circles), along with the indicated concentrations of nilotinib. FD D802V were grown in the absence of both M-CSF and IL-3 (triangles), and cell numbers were counted after 5 days. (D) Rat-2 fibroblasts expressing V-fms, (Rat-2 V-Fms) FMS (Rat-2 cfms) and FMS D802VY708F (Rat-2 D802VY708F) were plated in DMEM 10% FCS and grown for 5 days in the indicated concentrations of imatinib. All experiments were repeated at least 3 times and done in triplicate each time. Viable cell numbers were counted using a haemocytometer and triplicate assays were averaged. Error bars show standard error of the mean (SEM). (A) Courtesy of J. Taylor.

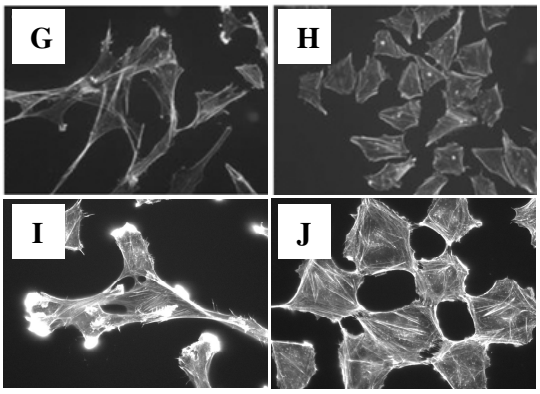
A similar result was also shown with nilotinib inhibition of FMS. Figure 3.1C shows that M-CSF dependent proliferation of FD cfms cells was totally inhibited by nilotinib at 0.8 μM (figure 3.1C; circles) and the IC50 value was calculated from figure 3.1C as 0.3 μM. The D802V mutation confers marked but not complete resistance to nilotinib, shown in figure 3.1C (triangles). The growth of FDC-P1 cells with IL-3 was unaffected by nilotinib as expected showing that the inhibition of proliferation seen in this assay is specific to the FMS receptor pathway (figure 3.1C; circles).

I next assessed the ability of imatinib and nilotinib to inhibit Rat-2 fibroblast transformation. Normally, these cells proliferate when grown in media with FCS but show contact inhibition when they become confluent. When they ectopically express the FMS receptor and are stimulated with M-CSF they become transformed, lose contact inhibition and grow to a higher cell number (Baker et al., 1994; Roussel et al., 1987). Figure 3.1D shows that when Rat-2 c-fms cells were stimulated with M-CSF , the final cell number increased by about 75% (figure 3.1D; open versus closed circles). This effect was inhibited by 1 μM imatinib treatment, which resulted in similar cell numbers to Rat-2 cfms grown without M-CSF or imatinib (figure 3.1D). Similarly, proliferation stimulated by the oncogenic receptor V-Fms was also inhibited by imatinib (figure 3.1D; closed squares). Expression of FMS D802V only partially transforms Rat-2 fibroblasts and only when combined with a second mutation of Tyr 708 to Phe. This is due to an enhancement of receptor stability (Morley et al., 1999). In accordance with this; proliferation of Rat-2 D802VY708F (figure 3.1D; open squares) is enhanced compared to Rat-2 cfms alone but not to the extent of Rat-2 V-

Fms proliferation (figure 3.1D; closed squares). The transforming effect of FMS D802VY708F, was less inhibited by imatinib, in accordance with Figures 3.1A-C.

The data in figure 3.1D is confirmed in figure 3.2 which shows that both imatinib and nilotinib inhibited the morphological transformation of Rat-2 fibroblasts that is caused by FMS signalling. Figure 3.2A and 3.2C, show the transformed morphology of Rat-2 V-Fms fibroblasts and figure 3.2B, D show that this was suppressed by both 1 μM imatinib (figure 3.2B) and 1 μM nilotinib (figure 3.2D) and that contact inhibition is restored. Figure 3.2E, F shows that the transformed morphology of Rat-2 FMS D802VY708F was resistant to imatinib. Figure 3.2G and 3.2I show F-Actin staining of Rat-2 -cfms cells, which illustrates the presence of extended actin cables. This feature was suppressed by both 1 μM imatinib (figure 3.2H) and 1 μM nilotinib (figure 3.2J) as expected. The specificity of imatinib to the FMS signalling pathway in these cells is demonstrated by Figure 3.2K-N. Figure 3.2K shows Rat-2 V-Src cells which have a similar transformed morphology to Rat-2 V Fms (figure 3.2A, C). However, 1 μM imatinib was unable to reverse the v-Src induced transformation (figure 3.2L) of Rat-2 fibroblasts but transformation was reversed by the Src kinase inhibitor SU6656 at 1 μM (figure 3.2N).





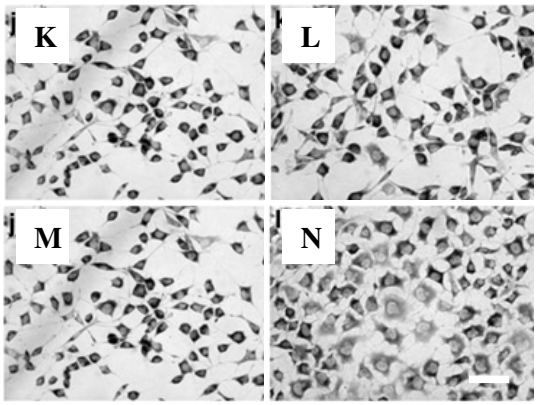


Figure 3.2. Imatinib and nilotinib both inhibit Rat-2 fibroblast transformation by FMS. (A-F) Giemsa staining of Rat-2 V Fms or Rat-2 FMS D802VY708F treated overnight with or without imatinib or nilotinib. (A) Rat-2 V Fms (B) Rat-2 V Fms + 1 μM imatinib (C) Rat-2 V Fms (D) Rat-2 V Fms + 1 μM nilotinib(E) Rat-2 FMS D802VY708F (F) Rat-2 FMS D802VY708F+ 1 μM imatinib. (G-J) Phalloidin staining of F-Actin of Rat-2 V Fms treated with or without imatinib or nilotinib overnight. (G) Rat-2 V Fms (H) Rat-2 V Fms + 1μM imatinib (I) Rat-2 V Fms (J) Rat-2 V Fms + 1 μM nilotinib. (K-N). Giemsa staining of Rat-2 V-Src treated with or without imatinib or SU6656 overnight. (K) Rat-2 V-Src (L) Rat-2 V-Src + 1 μM imatinib (M) Rat-2 V-Src (N) Rat-2 V-Src + 1 μM SU6656. Ruler = 100 μm**.**

Figure 3.3A shows that M-CSF addition caused the phosphorylation of the mature form of human FMS expressed by Rat-2 fibroblasts (compare figure 3.3A; lanes 1 and 2) and that pre-treatment with 0.25 or 0.5 μM nilotinib largely inhibited this process. The major phosphorylated band of figure 3.2A is the mature FMS receptor and the higher molecular weight smearing is due to receptor ubiquitination (Taylor et al., 2006). The antibody used for the immunoprecipitation is shown as a loading control (3A; lower panel). Unlike normal FMS, both the immature and mature forms of the oncogenic receptor FMS D802VY708F are phosphorylated in Rat-2 fibroblasts and this occurs constitutively in the absence of M-CSF (Morley et al., 1999). Figure 3.3B shows that the phosphorylation of FMS D802VY708F was not inhibited by nilotinib. Ponceau staining of part of the nitrocellulose membrane is shown as a loading control (figure 3.3B; lower panel). Similarly, FMS D802VY708F also showed greater resistance to nilotinib than wild type FMS in an *in vitro* kinase assay (figure 3.2C,D). Densitometry readings of kinase assays are shown in figure 3.3D. Similar results for imatinib inhibition of FMS receptor phosphorylation and kinase activity were found (data not shown) (Taylor et al., 2006).

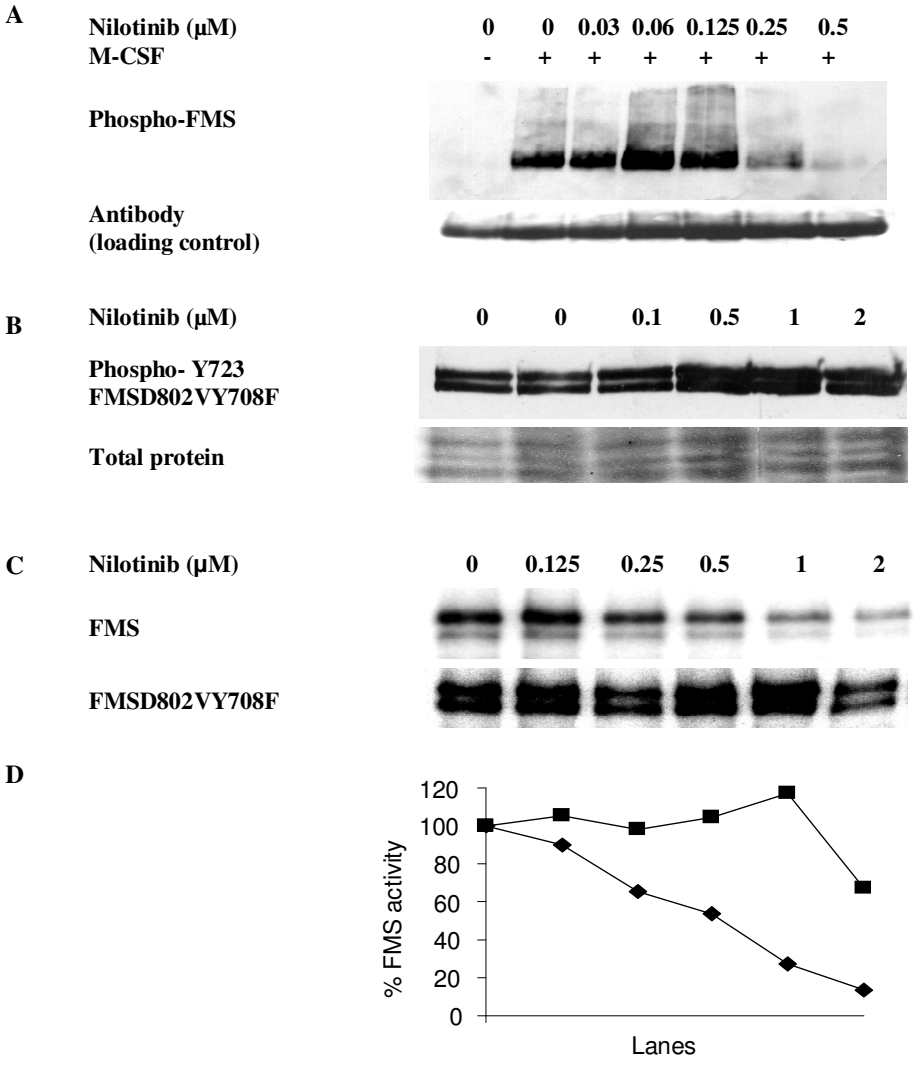


Figure 3.3 Nilotinib inhibits M-CSF induced FMS phosphorylation and the FMS D802VY708F mutation confers resistance. (A) Phosphotyrosine blot of FMS expressed by Rat-2 fibroblasts that were pretreated with the indicated concentrations of nilotinib for 1 h before the addition of 5000 U/ml M-CSF for 5 mins. Total lysates were immunoprecipitated with an FMS-specific rabbit polyclonal (FMS Ab 792) and blotted with the anti-phosphotyrosine antibody PY99. (B) Phosphotyrosine blot of Rat-2 FMS D802VY708F treated similarly to (A), except that total lysates were western blotted with an antibody specific to FMS phosphotyrosine 723. (C) *In vitro* kinase assays of immunoprecipitated FMS or FMSD802VY708F receptors subsequently treated with the indicated concentrations of nilotinib. Densitometry readings of the *in vitro* kinase assays are shown for FMS (diamonds) and FMSD802VY708F (squares).

I also assessed the ability of imatinib and nilotinib to inhibit two downstream signalling molecules of the FMS receptor; p42/p44 MAPK (ERK) and AKT (figure 3.4). Figure 3.4A shows that imatinib inhibited M-CSF induced p42/p44 MAPK phosphorylation in the FD cfms cell line. When these cells are starved of Foetal Calf Serum (FCS), background p42/p44 MAPK phosphorylation is reduced (figure 3.4A; upper panel, lane 1). Phosphorylation of MAPK can be stimulated through the FMS receptor by adding M-CSF (figure 3.4A; lane 2). MAPK phosphorylation was completely inhibited at 2.5 μM imatinib (figure 3.4A; upper panel). Figure 3.4A, lower panel shows total p42/p44 MAPK protein loading. Similarly, figure 3.4B, upper panel shows that starving Rat-2cfms fibroblasts of FCS for 5 hours reduces AKT phosphorylation considerably and that this can be re-stimulated by adding M-CSF (figure 3.4B; upper panel, lane 1). Phospho-AKT was completely inhibited at 2.5 μM imatinib and figure 3.4B, lower panel shows total AKT as a control for even loading. Comparable results were obtained for nilotinib inhibition of p42/p44 MAPK and AKT phosphorylation in Rat-2 cfms fibroblasts (figure 3.4 C-J). Figure 4C and 4G show an absence of p42/p44 MAPK phosphorylation and Akt phosphorylation respectively when Rat-2c-fms fibroblasts were starved of FCS for 24 hours. Phosphorylation of both p42/p44 MAPK (figure 3.4D) and Akt (figure 3.4H) was stimulated by M-CSF and partially inhibited at 0.5 μM nilotinib (figure 3.4E, 3.4I) and totally inhibited at 1 μM nilotinib (figures3.4F, 3.4J).

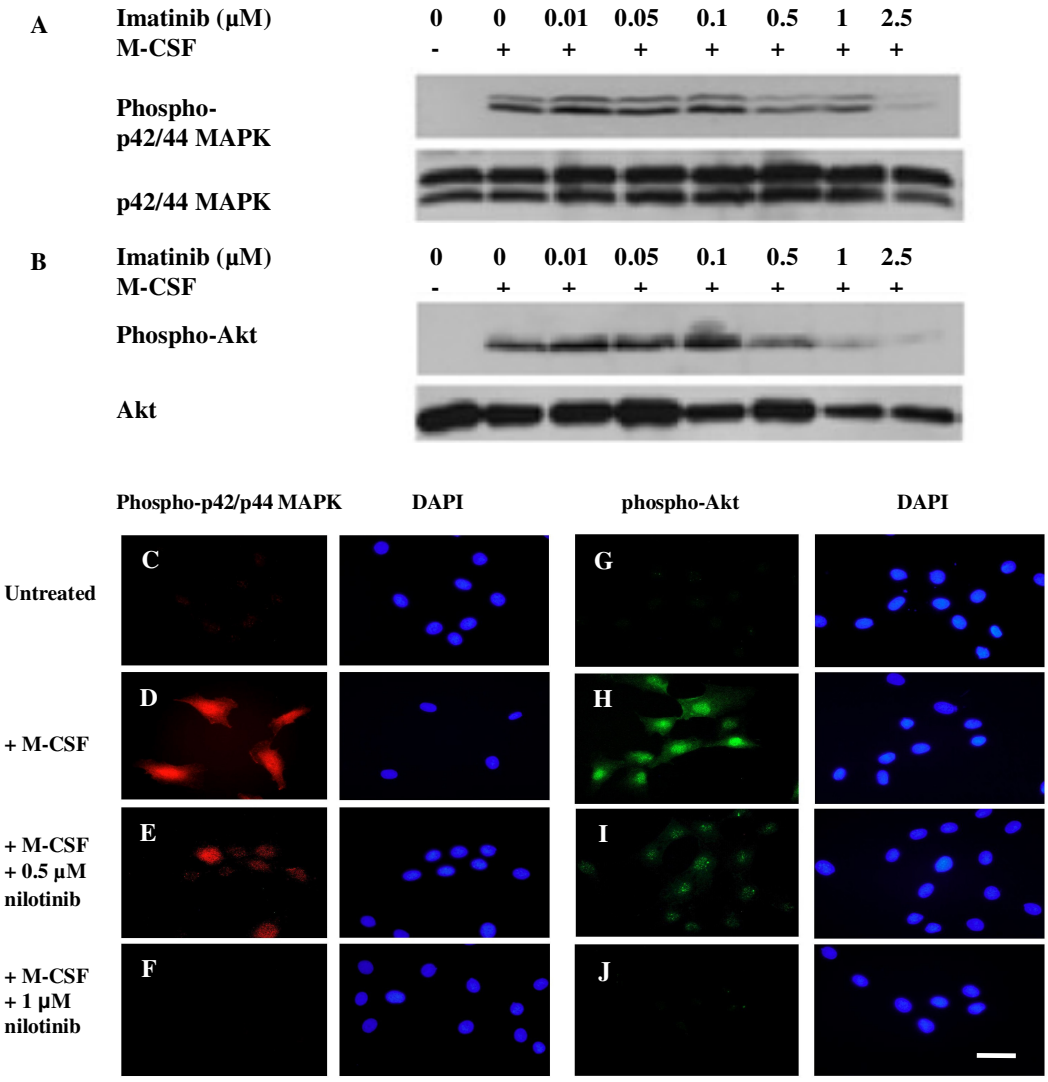


Figure 3.4. Imatinib and nilotinib inhibit M-CSF induced p42/44 MAPK and AKT phosphorylation. (A,B) 5 x 10^6 FDc-fms cells were starved of FCS for 5 h and then incubated with the indicated concentrations of imatinib for 3 h and then stimulated with 5000 U/ml M-CSF for 2 mins. Cell lysates were analysed by Western blotting and probed with antibodies to p42/44 MAPK, Phospho-p42/44 MAPK, Akt or Phospho-Akt as indicated. (C-J) Rat-2 cfms cells were grown to semi-confluence on 1 cm diameter glass coverslips in DMEM and then starved of FCS for 24 hours and pre-treated with nilotinib for 1 hour prior to stimulation with M-CSF (5000 U/ml) for 2 minutes. Coverslips were probed with antibodies for either phospho-p42/44 MAPK (C-F) or phospho-AKT (G-J) and visualised with a fluorescent secondary antibody. Panels to the right show DAPI staining of the nucleus. No treatment (C, G); M-CSF stimulation only (D, H); M-CSF + 0.5 μM nilotinib pre-treatment (E, I); M-CSF + 1 μM nilotinib pre-treatment (F, J) Ruler = 100 μm. (A,B) Courtesy of J. Taylor.

I also investigated the inhibitory effect of imatinib and nilotinib upon osteoclastogenesis from human mononuclear progenitor cells mobilised from bone marrow. Osteoclasts can be readily generated from peripheral blood samples of patients who have had their bone marrow mobilised by G-CSF for the purpose of bone marrow transplantation. These samples were collected with the appropriate informed consent. Osteoclasts generated in this system are dependent on stimulation by both recombinant M-CSF and RANKL which is replenished in the media every 3 days. No osteoclasts were formed in media lacking M-CSF and RANKL in agreement with previous findings (Susa et al., 2004; Matsuzaki et al., 1998). Table 3.1A shows that nilotinib inhibited osteoclastogenesis completely at 500 nM with an IC50 value of 50 nM, similar IC50 values were obtained in two further experiments. By contrast, in the same experiments imatinib had an IC50 value of 600 nM for the inhibition of osteoclastogenesis (Table 3.1), which is in accord with a previous report (Ando et al., 2006).

Table 3.1. Osteoclastogenesis from human mobilised peripheral blood.

Nilotinib (nM)	**Mean osteoclast number ± SEM**
0	125.3 ± 14.2
31	79.7 ± 3.7
62	52.3 ± 8.4
125	16.0 ± 2.1
250	14.0 ± 3.5
500	0
1000	0
Imatinib (nM)	
0	295.6667 ± 48.0
100	272 ± 23.4
500	264 ± 20.8
1000	9.0 ± 2.0

Figure 3.5A shows a representative photograph of human osteoclasts which can be seen to have characteristic multiple nuclei and a distinctive actin ring. Figure 3.5B shows tartrate resistant acid phosphatase (TRAP) staining of osteoclasts which is a marker of mature osteoclasts (Minkin, 1982) and figure 3.5C illustrates the inhibitory effect of 1 μM nilotinib upon osteoclast formation. Figure 3.5D shows that human osteoclasts cultured upon dentine slices cause pit formation as shown by toluidine blue staining and figures 3.5E, F show that pit formation was completely inhibited by 1 μM nilotinib or imatinib, as expected. The inhibitory potential of both imatinib and nilotinib on macrophage colony formation was also investigated using murine bone marrow. Macrophage colonies formed in methylcellulose semisolid media when stimulated with M-CSF. Figure 3.5G shows that imatinib and nilotinib had similar profiles of inhibition of macrophage colony formation in response to M-CSF with IC50 values of approximately 0.5 μM This is in agreement with a previous report (Dewar et al., 2003).

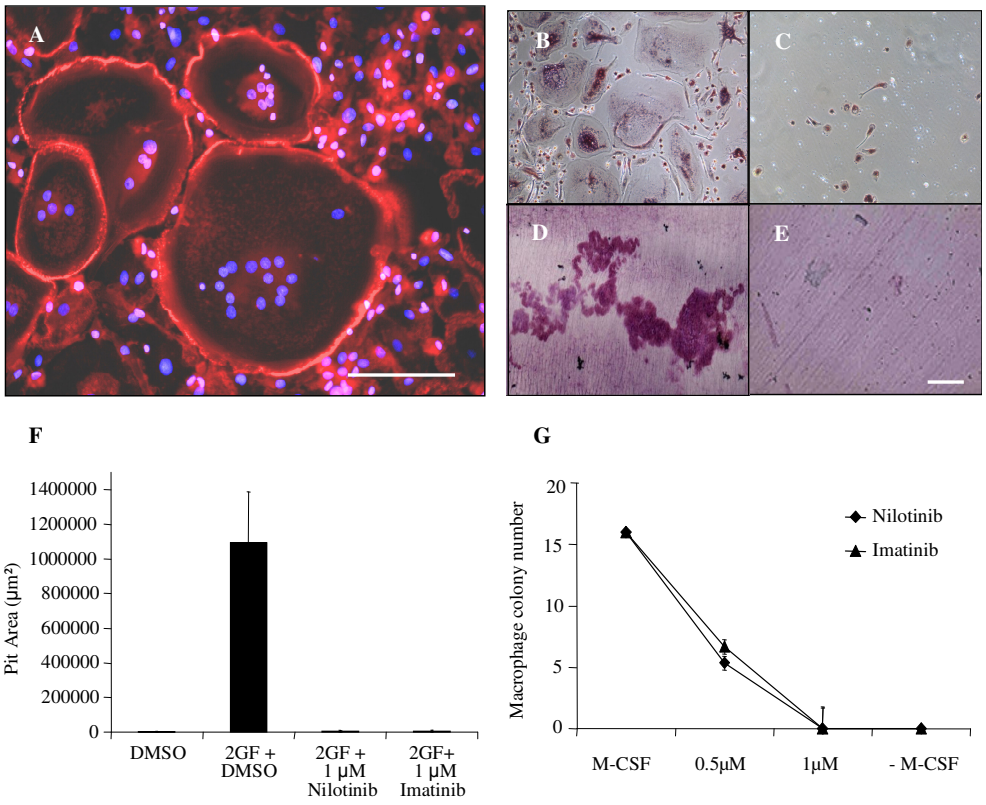


Figure 3.5. Imatinib and nilotinib inhibit osteoclastogenesis from human mononuclear cells and also inhibit murine macrophage colony formation (A-F) Human mononuclear cells were cultured with 5000 U/ml rHuM-CSF and 40 ng/ml rHuRANKL (2GF) for 3 weeks in alpha MEM with 10% FCS. (A) Representative photograph of the osteoclasts with F-Actin staining shown in red and DAPI stained nuclei shown in blue. (B-E) BMM were cultured as above with (C,E) or without (B,D) 1 µM nilotinib on either tissue culture plastic (B,C) or dentine slices (D,E). After the culture period the cells were stained for TRAP to show TRAP positive osteoclasts (B,C) or stained with Toluidine blue to show pit formation (D,E). Pit area was measured using the Lucia G V.4.61 software (F). (G) Murine bone marrow was cultured in methylcellulose with 10 % FCS and 5000 U/ml rHuM-CSF for 2 weeks with 0.5 or 1 µM nilotinib or imatinib. Macrophage colonies were counted after 2 weeks. Scale bars = 50 µM

Nilotinib was able to induce mature osteoclast death by apoptosis, this is shown in figure 3.6 by tunnel staining of DNA fragmentation. Figure 3.6A, 1st panel shows that before nilotinib was added, there are no apoptotic nuclei and figure 3.6B, 1st panel shows many apoptotic nuclei after a 24 hour incubation with 1 μM nilotinib. Figure 3.6C shows the number of apoptotic nuclei compared to non apoptotic nuclei at various concentrations of nilotinib and at 1 μM nilotinib 35% of the nuclei within osteoclasts was apoptotic.

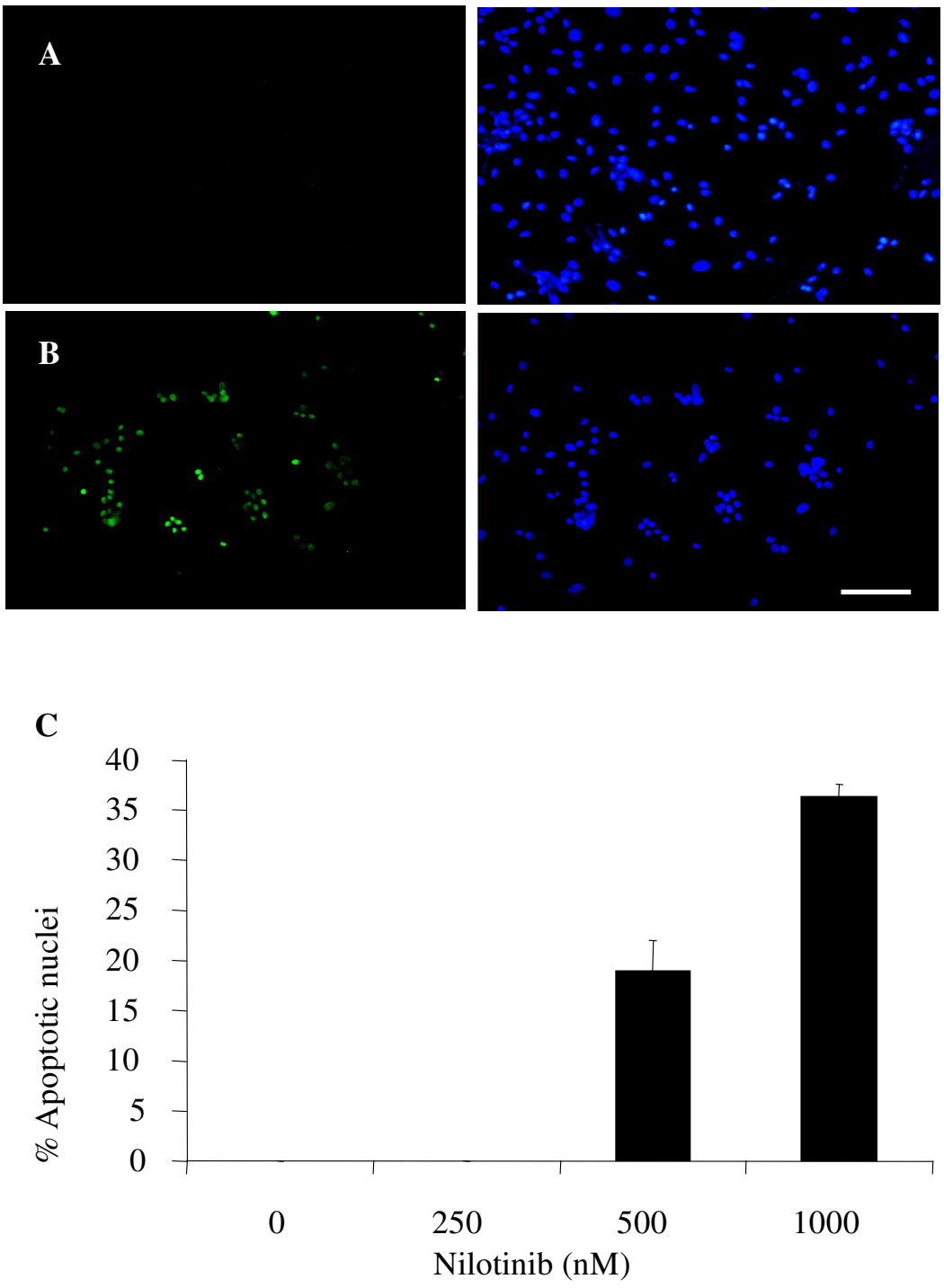


Figure 3.6. Nilotinib induces apoptosis of mature osteoclasts. Mature osteoclasts were incubated overnight with the indicated concentrations of nilotinib and the number of apoptotic nuclei contained within an osteoclast was counted based on TUNNEL staining and a percentage of apoptotic compared to non-apoptotic nuclei was calculated. (A,B) Representative photographs showing TUNNEL staining in green and DAPI stained nuclei in blue. (A) Untreated, (B) 1 µM nilotinib (C) Quantization of apoptotic nuclei. Ruler = 100 µm

Figure 3.7 models the docking of nilotinib into the recently determined structure of the inactive kinase domain of FMS (Schubert et al., 2006). This model is supported by the fact that it predicts hydrogen bond interactions between nilotinib and the kinase domain of FMS that are similar to those seen between imatinib and KIT (Mol et al., 2004a), which is to be expected as there is extensive overlap between the structures of nilotinib and imatinib and between the structures of KIT and FMS (Mol et al., 2004a; Schubert et al., 2006; Cowan-Jacob et al., 2007). Molecular modelling was done by J. Murray.

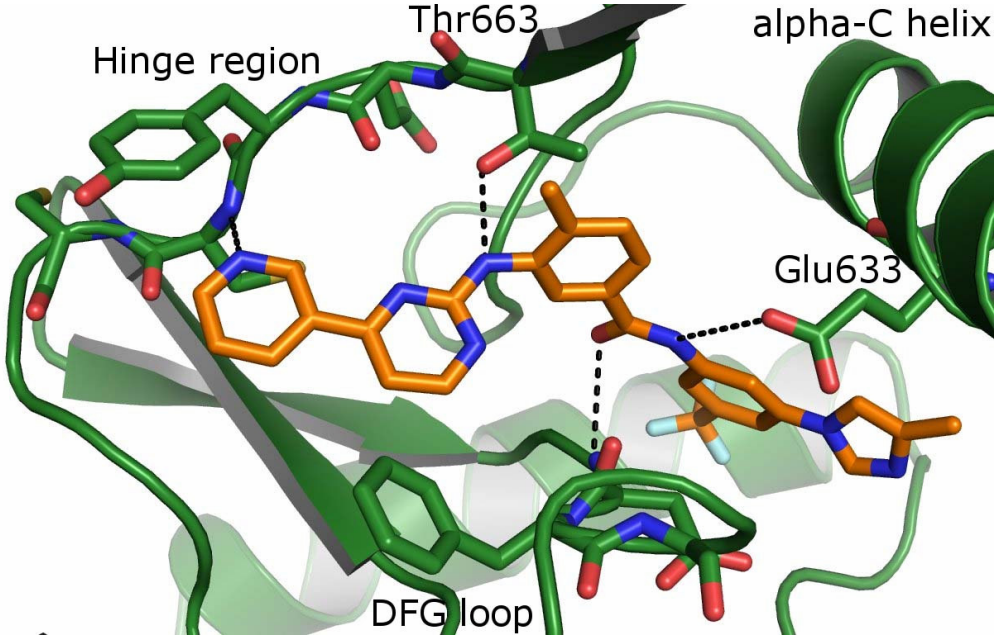


Figure 3.7. Docking model of nilotinib in FMS kinase domain. The protein backbone trace is shown in the region of the active site with residues Glu633, Thr663-Cys666 (Hinge region), and Asp796-Gly798 (DFG loop) depicted in stick representation. The co-ordinates from the crystal structure of FMS (RSCB code 2i1m) were used (25). Nilotinib is shown in stick representation with carbon atoms coloured orange. Dashed lines indicate hydrogen bond interactions. Numbering is based on Genebank NM_005211. **Courtesy of J. Murray.**

3.3. **Discussion**

Both imatinib and nilotinib inhibited the phosphorylation and kinase activity of the normal FMS receptor and its activation of the downstream signalling molecules Akt and p42/44 MAPK. In confirmation that imatinib and nilotinib are specific inhibitors of FMS activity, I found that neither inhibit IL-3 dependent growth of the FDC-P1 cell line (figure 3.1, data not shown) and that a mutation of Asp 802 to Val of FMS confers resistance (figure 3.1A). This is in accord with reports that the equivalent mutation of the KIT receptor, KITD816V is not sensitive to either imatinib or nilotinib (Frost et al., 2002; Verstovsek et al., 2006). The IC50 values for the inhibition of M-CSF dependent growth of FDc-fms by nilotinib and imatinib are 0.3 and 0.6 μM respectively (figure 3.1;(Taylor et al., 2006)). This agrees with previous reports that the >20 fold increase in inhibition of Bcr-Abl by nilotinib compared to imatinib is not accompanied by a similar increase in the inhibition of the PDGF or KIT receptors (Weisberg et al., 2005).

Imatinib and nilotinib inhibited Rat-2 fibroblast transformation induced by V-Fms (figure 3.2A-D) but not FMS D802VY708F (figure 3.2E-F) or by V-Src induced transformation (figure 3.2K-L), showing specific inhibition of the FMS signalling pathway in fibroblasts. Nilotinib inhibited FMS phosphorylation with an IC50 value of 0.2 μM (figure 3.2C) and inhibited the kinase activity of the wild type receptor with an IC50 value of around 0.5 μM (figure 3.3C) which is similar to the value of 0.3 μM for imatinib (data not shown) (Taylor et al., 2006; Dewar et al., 2005a).

Osteoclastogenesis of primary human progenitor cells was more sensitive to nilotinib (IC50 approx 50 nM) than to imatinib (IC50 greater than 500 nM) (Table

3.1), which is in contrast to the similar effects of imatinib and nilotinib upon the other assays reported here. The relatively low IC50 value for the inhibition of osteoclastogenesis by nilotinib suggests a target other than FMS. The Ephrin receptor is reported to be particularly sensitive to nilotinib whereas this receptor is resistant to imatinib (Melnick et al., 2006). This could provide a possible explanation for this data as the Ephrin receptor is an important receptor for osteoclast formation and function (Zhao et al., 2006). Nilotinib and imatinib showed a similar profile for the inhibition of the growth of macrophage colonies from murine bone marrow (figure 3.5G). This is in accord with previous reports of the inhibitory effects of imatinib and other FMS inhibitors upon macrophage production and function (Dewar et al., 2005b; Dewar et al., 2003; Conway et al., 2005; Irvine et al., 2006).

Recently there has been much interest in inhibitors of the FMS receptor which can inhibit osteoclastogenesis *in vivo* (Murray et al., 2003; Ando et al., 2006; Dewar et al., 2006; El Hajj Dib et al., 2006; Ohno et al., 2006). I find that nilotinib is more effective at inhibiting osteoclastogenesis than imatinib. There are reports of altered bone and mineral metabolism in patients who have been treated with imatinib for extended periods at high doses (Berman et al., 2006; Joensuu and Reichardt, 2006; Fitter et al., 2007), which implies that this may be a more serious issue if patients were to be treated with nilotinib for extended periods. It is necessary to consider that nilotinib is a much more effective inhibitor of BCR-ABL than imatinib, and that the concentration of nilotinib necessary to inhibit BCR-ABL *in vivo* may be much lower than that necessary to inhibit osteoclastogenesis *in vivo*. The plasma levels achieved in patients treated for CML with imatinib and nilotinib are 1.5 - 4.5 μM (Kantarjian et al., 2006a) and 1.7 - 3.6 μM (Peng et al., 2004a; Peng et al., 2004b) respectively.

These concentrations would be expected to inhibit the FMS receptor based on our findings, although there is likely to be much more signalling redundancy *in vivo* so this may not necessarily be significant.

There is interest in the literature in finding FMS inhibitors to treat diseases where macrophages, osteoclasts and microglia are implicated. These cell types are involved in a variety of diseases including macrophage mediated diseases such as metastasis (Pollard, 2004; Irvine et al., 2006) and renal disease (Zoja et al., 2006; Le Meur et al., 2002), neurological diseases where microglia are implicated (Aldskogius, 2001; Perry, 2004) and both metastatic and inflammatory bone diseases where osteoclasts are involved (El Hajj Dib et al., 2006; Mundy, 2002; Murray et al., 2003). The extensive range of diseases in which the FMS receptor is implicated support the requirement for many new FMS inhibitors. Imatinib and nilotinib therefore form a strong platform for development of further FMS inhibitors, and their optimisation for specific therapeutic applications.

CHAPTER 4. Dasatinib inhibition of the FMS receptor and tumour associated macrophages and osteoclasts.

4.1. Introduction

4.1.1. Dasatinib

Since the success of targeting ABL in BCR-ABL positive leukaemias, there has been much interest in developing new inhibitors which target a broader range of imatinib resistant mutations of BCR-ABL (see section 1.4 for more detail). Dasatinib (BMS-354825, Sprycel) is a second generation BCR-ABL kinase inhibitor which also has activity against various other kinases, including most of the SRC family kinases, KIT, Ephrin receptor (EPH) and PDGFR (Lombardo et al., 2004; Shah et al., 2004; Fabian et al., 2005; Schittenhelm et al., 2006). Dasatinib has a broader spectrum of activity against ABL mutations compared to imatinib and is able to inhibit to all of the common imatinib resistant Abl mutants with the exception of the T315I mutation, which also confers resistance to imatinib and nilotinib (Shah et al., 2004).

Dasatinib inhibits the KIT D816V receptor, which is resistant to both imatinib and nilotinib and so gives exciting treatment possibilities for systemic mastocytosis where KIT D816V is an important oncogenic mutation (Shah et al., 2006; Pardanani et al., 2003). Dasatinib is active against a larger range of kinase mutants partly due to its ability to bind to both the active and inactive forms of the kinase domain, so unlike imatinib, dasatinib binding is unaffected by mutations which force the kinase into its active position (Mol et al., 2004b; Tokarski et al., 2006). As a result dasatinib has less molecular restrictions for binding and so targets many more kinases than imatinib (Karaman et al., 2008). Regardless of dasatinib's apparent promiscuity, clinical trials have shown that it is tolerated reasonably well by patients and dasatinib has now been

approved for use in patients with CML or acute lymphoblastic leukaemia (ALL) who have developed imatinib resistance or intolerance (Kantarjian et al., 2006b). Dasatinib is therefore an interesting drug with other potential clinical applications.

4.1.2. The tumour microenvironment

It has recently become clear that tumours are not independent of their surroundings and that their immediate microenvironment has wide ranging effects on both tumour phenotype and disease prognosis (Albini and Sporn, 2007; Kuppers, 2004). A major contributor to the tumour micro-environment is the tumour associated macrophage (TAM) (Condeelis and Pollard, 2006; Allavena et al., 2007). Macrophage infiltration of solid tumours and tumour microenvironment often correlates with poor prognosis and an aggressive disease (Takanami et al., 1999; Condeelis and Pollard, 2006; Yao et al., 2001; Tsutsui et al., 2005). As a result, TAMs are a valid therapeutic target intended to complement traditional regimes in treating solid tumours (Joyce, 2005).

A number of different approaches have already been investigated to specifically target TAMs. These include a natural inhibitor, Yondelis (Trabectidine) which is derived from the marine tunicate *E. turbinate* and interferes with transcription and inhibits macrophage differentiation *in vitro* (Allavena et al., 2005), a DNA-based vaccine against leguimain, which is over-expressed on the surface of macrophages (Luo et al., 2006) and zoledronic acid, which inhibits MMP-9 production by macrophages (Giraudo et al., 2004).

Tumours are also known in turn to influence their microenvironment. Osteolytic bone lesions are often associated with tumour metastasis to the bone marrow, causing

excessive osteoclastogenesis and bone absorption (Mancino et al., 2001; Roato et al., 2005; Johnson et al., 1989). Notably, in multiple myeloma, where bone metastasis is particularly common, osteolytic lesions only occur in areas directly associated with myeloma cells (Roodman, 2001) and in metastatic breast cancer, the main mediator of bone metastasis is thought to be a parathyroid hormone-related protein which is secreted from tumour cells and stimulates osteoblasts to release increased levels of RANKL, thereby stimulating local osteoclastogenesis (Mundy, 2002; Powell et al., 1991). Currently, the main therapy for bone metastasis is the use of bisphosphonates which reduce osteoclast activity by causing apoptosis and in some cases cause dangerous side effects such as renal toxicity (Diel et al., 2007). There are also various new compounds progressing through clinical trials for osteolytic bone metastasis, mainly targeting the RANK pathway (Mundy, 2002).

Since the FMS receptor activity is essential for macrophage and osteoclast differentiation and survival (Wiktor-Jedrzejczak et al., 1990), I hypothesized that FMS may be a valuable therapeutic target for diseases where these cell types are involved. Here I show that dasatinib targets the FMS receptor at low nanomolar concentrations (IC50 = 0.5 nM) in various cell based assays. I have also investigated the potential of dasatinib to inhibit the differentiation and survival of tumour associated macrophages and osteoclasts using ovarian cancer ascites as a source of haematopoetic cells from the tumour microenvironment and have shown dasatinib to be a potent inhibitor of both of these cell types *in vitro*.

4.2. Results

4.2.1. Dasatinib inhibits the FMS receptor in Rat-2 fibroblasts and FDC-P1 haematopoetic cell lines.

The murine haematopoetic cell lines FDC-P1, FD c-fms and FD FMSD802V were used initially to establish that FMS is sensitive to dasatinib (assays also described in chapter 3). Fig 4.1A shows that dasatinib inhibited M-CSF stimulated growth of FD c-fms cells with an IC50 value of 0.5 nM, as calculated from figure 4.1A, circles. The D802V mutation of FMS confers resistance to imatinib (see figure 3.1) (Taylor et al., 2006) but not to dasatinib (Fig 4.1A; triangles) and has a similar IC50 to the wild type receptor. This is in accordance with previous reports that KITD816V is sensitive to dasatinib (Schittenhelm et al., 2006; Shah et al., 2006). As expected, IL-3 stimulated growth of FD c-fms cells was unaffected by dasatinib (Fig 4.1A; diamonds).

Figure 4.1B shows that Rat-2 fibroblasts that express the oncogene V-Fms have a transformed morphology (Rettenmier et al., 1987a), and this was suppressed by 1 nM dasatinib (Fig 4.1C). Consequently, dasatinib has a potent inhibitory effect upon FMS signalling in fibroblasts as well as haematopoietic cell lines.

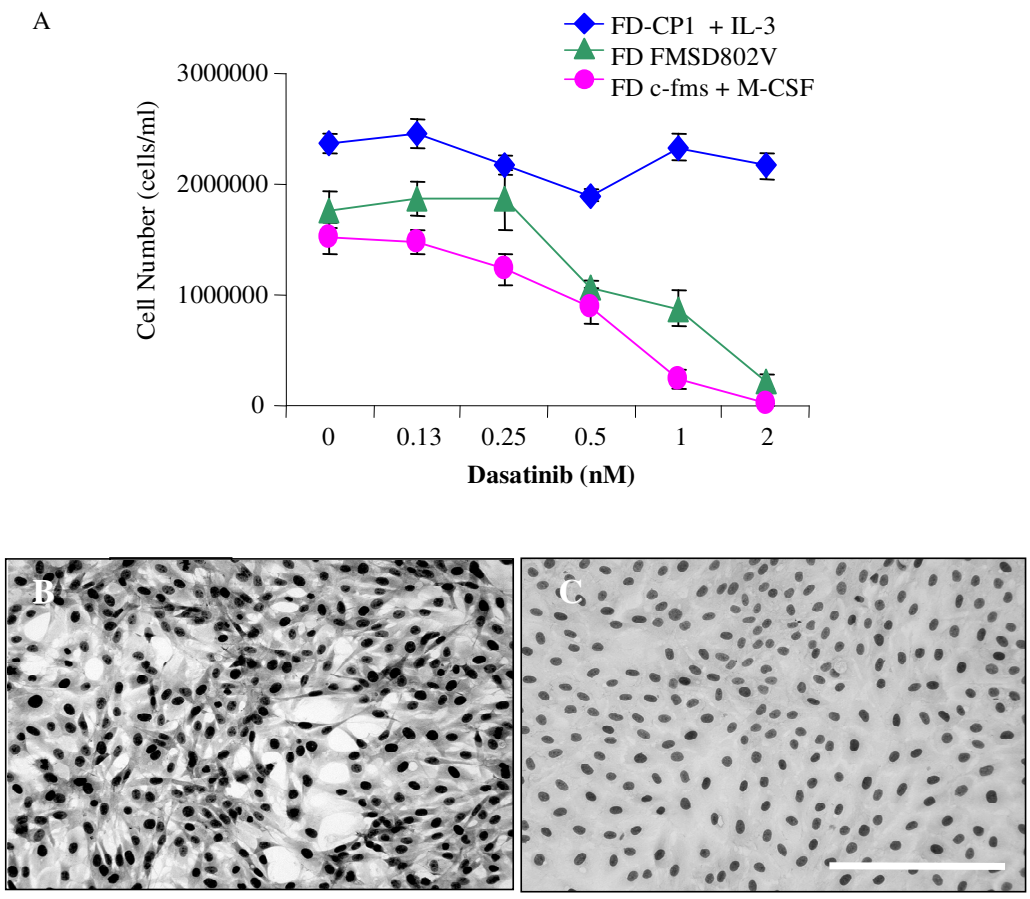


Figure 4.1. Dasatinib inhibits growth of the FDcfms haematopoetic cell line by M-CSF and inhibits v-fms induced fibroblast transformation. (A) All cells were seeded at 5 x 10^4 cells/ml in RPMI and 10% FCS and cells expressing c-fms were grown with 10 ng/ml IL-3 (diamonds) or 5000 U/ml M-CSF (circles) and cells expressing oncogenic FMSD802V were grown without IL-3 or M-CSF (triangles) with the indicated concentrations of Dasatinib. Viable cell numbers were counted after 5 days. (B, C) Giemsa staining of Rat-2 V Fms fibroblasts (B) and Rat-2 V Fms treatment for 24 hours with 1 nM Dasatinib (C). Scale bar = 200 µm.

Figure 4.2 A, B show that 1 nM dasatinib inhibited the *in vitro* kinase activity of FMS and FMS D802VY708F receptors, but was ineffective against a T663I FMS receptor so establishing that dasatinib is a potent inhibitor of wild type and oncogenic FMS but not of FMS with a mutation in the Thr 663 'gatekeeper residue' . This is in accordance with reports that the equivalent mutations of ABL (T315I) and KIT (T670I) also confer resistance to dasatinib (Guo et al., 2007; Bradeen et al., 2006). Figure 4.2B shows densitometry readings of the *in vitro* kinase assays.

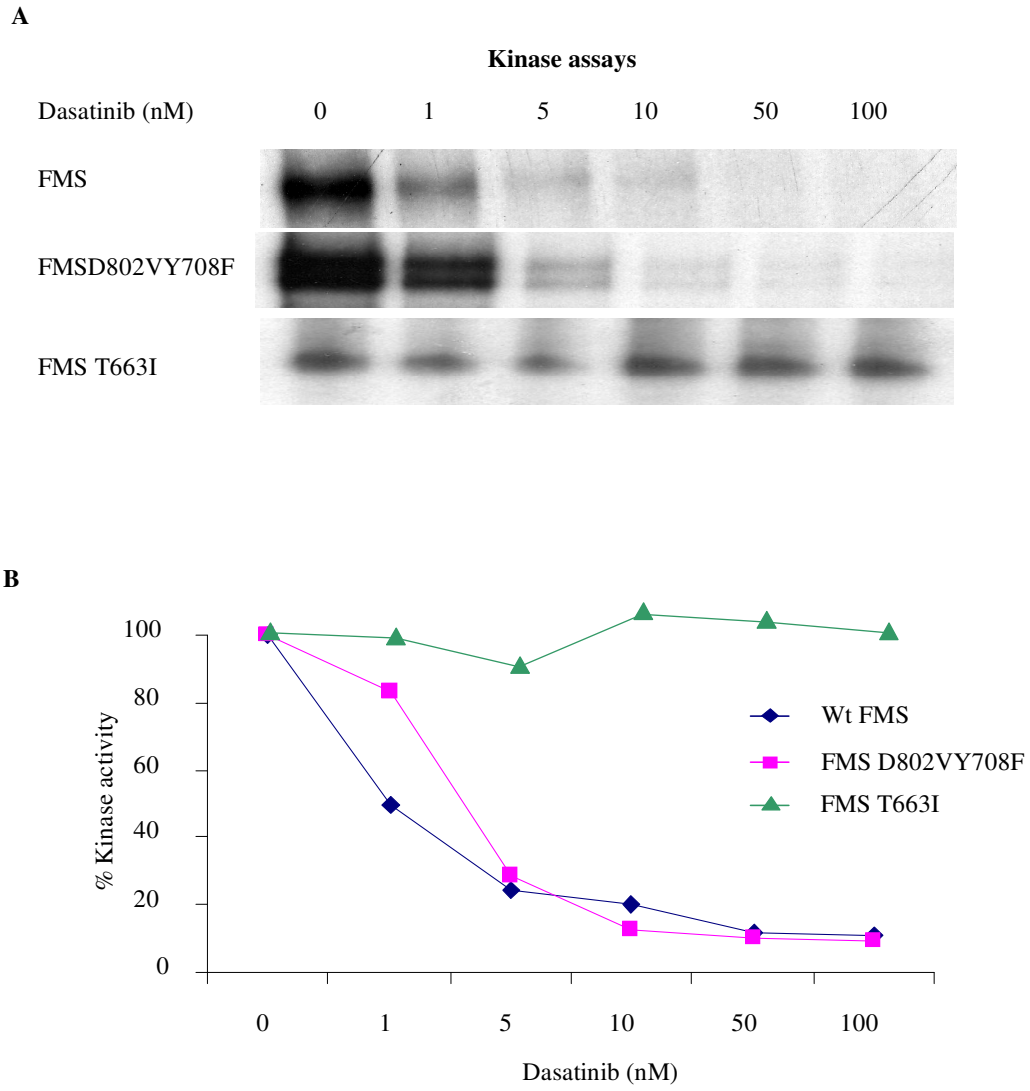


Figure 4.2. Dasatinib inhibits FMS kinase activity *in vitro*. (A) *In vitro* kinase assays of immunoprecipitated FMS (A, upper panel), FMSD802VY708F (A, middle pannel) or FMS T663I (A, lower panel). FMS was immunoprecipitated from Rat-2 fibroblasts expressing wild type FMS, FMS D802VY708F or FMS T633I using a rabbit polyclonal antibody against the C-terminal tail (Ab792) and the purified receptor was pretreated with the indicated concentrations of dasatinib for 30 minutes. *In vitro* kinase activity was assessed by incubating samples with gamma P^{32} labelled ATP. (B) Densitometry readings of the *in vitro* kinase assays (see A).

I have also confirmed these findings by analysing whether dasatinib can inhibit the phosphorylation of the FMS receptor and the phosphorylation of downstream signalling molecules p42/p44 MAPK and Akt in response to M-CSF stimulation (Fig 4.3A-E). Figure 4.3A shows that stimulation of Rat-2 cfms fibroblasts with M-CSF caused phosphorylation of the mature form of the receptor. High molecular weight smearing is due to ubiquitination (Taylor et al., 2006). Phosphorylation of FMS was inhibited entirely at 0.5 nM dasatinib, whereas the mutant receptor FMS T663I was resistant (Fig 4.3B). Dasatinib inhibited both M-CSF induced p42/44 MAPK (Fig 4.3C) and Akt (Fig 4.3D) phosphorylation with a large effect at concentrations as low as 0.1 nM. Figure 4.3E confirms that FMST633I is resistant to dasatinib, which does not inhibit M-CSF induced Akt phosphorylation by this receptor. The appropriate non-phosphorylated protein loading controls are shown in the lower panel of figures 4.3A-E.

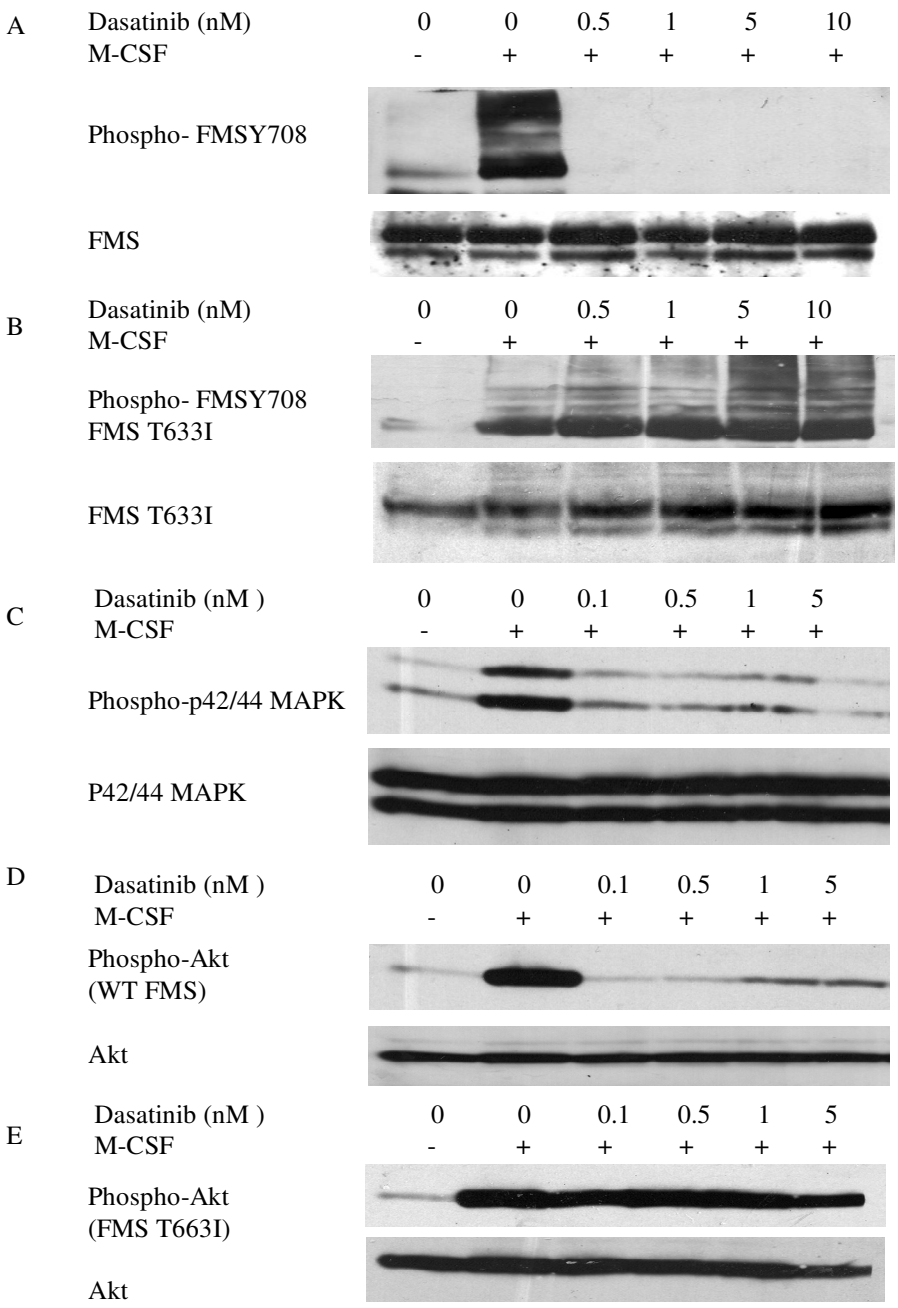
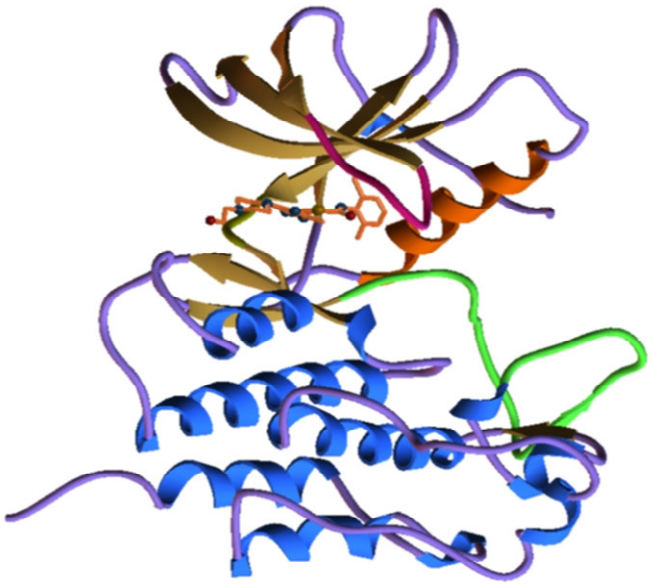


Figure 4.3. Dasatinib inhibits FMS signalling. Rat-2 fibroblasts that express FMS (A, C, D) or FMS T663I (B, E) were pre-treated with the indicated concentrations of dasatinib for 3 hours before 5000 U/ml M-CSF was added for 5 mins. Total lysates were analysed by western blotting with an antibody specific for either FMS phosphotyrosine 708 (A, B, upper panel), Phospho-p42/44 MAPK (C, upper panel), Phospho-Akt (D, E, upper panel). The same western blot samples were also blotted with an antibody specific to the FMS receptor (A. B, lower panel), total p42/44 MAPK (C, Lower panel) or total Akt (D,E lower panel) to show even loading.

4.2.2. Model of dasatinib binding to the FMS kinase domain.

Figure 4.4 models the binding of dasatinib to the ATP-binding pocket of the FMS kinase domain (Walter et al., 2007). In this model the hydrogen-bonding between dasatinib and the hinge region of FMS is similar to the known hydrogen bonding between dasatinib and the kinase domain of Abl and includes a hydrogen bond with the key gatekeeper residue, Thr 663 (Fig 4.4B). The activation loop of FMS is modelled in its active position in order to illustrate that dasatinib would be expected to bind to the active as well as the inactive FMS kinase structures. Modelling courtesy of C. Mol.

A



B

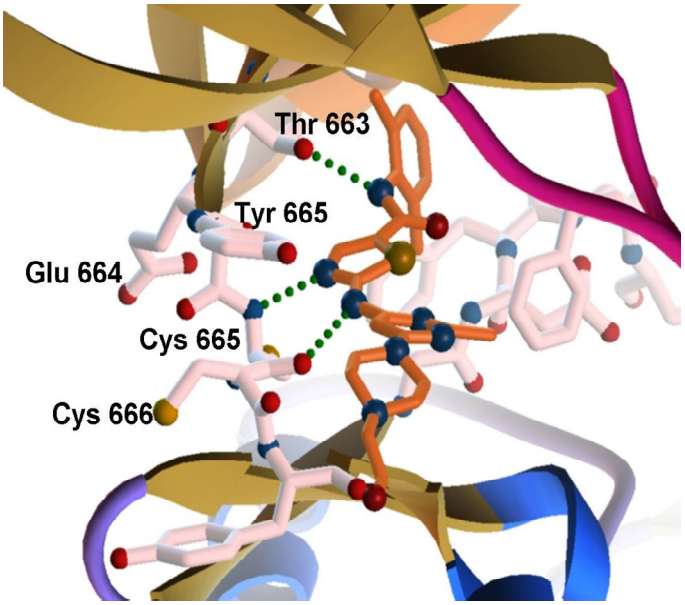


Figure 4.4 Model of dasatinib binding in the ATP pocket of FMS (A). Kinase domain of FMS with the C-helix (orange), activation loop (green) and dasatinib as a stick model (B) Detail of the hydrogen-bonding between dasatinib and the hinge region of the kinase domain of FMS indicated by the dotted lines. **Modelling courtesy of C. Mol.**

4.2.3. Dasatinib inhibits the production of normal human macrophages and ovarian tumour associated macrophages.

I next assessed the effect of dasatinib upon the production of primary macrophages from ovarian tumour ascites samples. Figure 4.5A and B are courtesy of C.Hayford and S. Ghaem-Maghami and shows a representative FACS scatter of an ascites sample, which typically contains a population of lymphocytes and a larger population of large granular cells (circled). This population was gated to include only the granular population (4.5A; circle). On average, about 80% of these granular cells were CD14 positive (Fig 4-5B) (Milliken et al., 2002) and therefore represent a population of cells of the monocytic lineage (n=10) (Ziegler-Heitbrock and Ulevitch, 1993).

Mature TAMs could be produced from this material, shown in figure 4.5C by CD68 staining (red) and as described previously (Hagemann et al., 2006; Allavena et al., 2005). This process was almost completely inhibited by 1 nM dasatinib (4.5D). A comparison of the three panels of Fig 4C (CD68, DAPI and merged) shows that all of the cells present in this assay are macrophages and so a pure population was successfully obtained (4.5C,D; middle and right panel). As expected, the secondary antibody only control failed to stain any cells (data not shown).

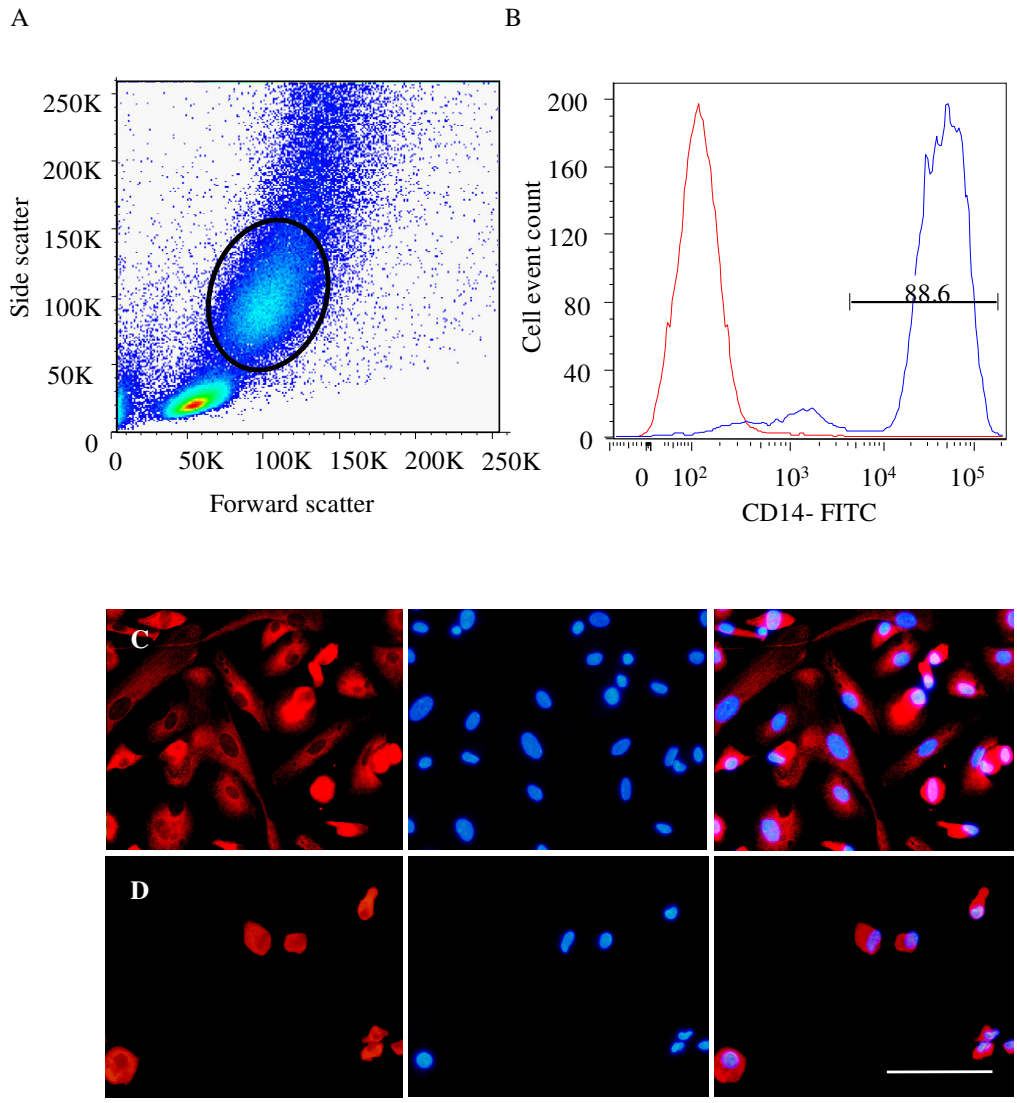


Figure 4.5. Dasatinib inhibits the production and survival of tumour-associated macrophages from ovarian cancer ascites. (A, B) An ascites sample from an ovarian cancer patient was stained for extracellular CD14 and analysed by FACS. (A) FACS scatter plots of ovarian cancer ascites samples showing large granular cell population (black circle). (B) Gated percentage of CD14+ monocyte cells from this population of large granular cells. Red = unstained cells, blue = CD14 stained cells. Similar results were obtained for all samples (n = 10). (C) Macrophages produced in response to M-CSF stimulation of an ovarian tumour ascites samples. Cells were cultured in alpha MEM with 10% FCS and 5000 U/ml M-CSF with the indicated concentrations of Dasatinib for 1 week. Macrophage counts were based on CD68 staining. Similar results were obtained for all tested samples (n=5). (C,D) Representative photographs of TAMs derived from ovarian cancer ascites. TAMs untreated (C) or treated with 5 nM dasatinib for 24 hours (D) CD68 staining (red) DAPI (blue), scale bar = 100 µm. **(A,B) Courtesy of C. Hayford and S. Ghaem-Maghami.**

TAM production was highly dependent on M-CSF addition to the growth media and very few macrophages were produced in its absence (4.6A, B). Figure 4.6A shows that dasatinib potently inhibited the production of tumour-associated macrophages from ovarian cancer ascites samples (n = 7) with an IC50 value of less than 1 nM (figure 4.6A). As expected, TAM production was also sensitive to imatinib (figure 4.6B) with an IC50 value of less than 1 μM (figure 4.6B) which is similar to the concentrations required for imatinib inhibition of FMS (see figure 3.1).

I also assayed the ability of dasatinib to induce TAM death and found that treatment with 5 nM dasatinib killed the vast majority of mature TAMs within 24 hours with an IC50 value of around 1 nM (figure 4.6C). As expected, dasatinib also inhibited the production of colonies of macrophages from normal peripheral blood with complete inhibition at less than 10 nM (data not shown).

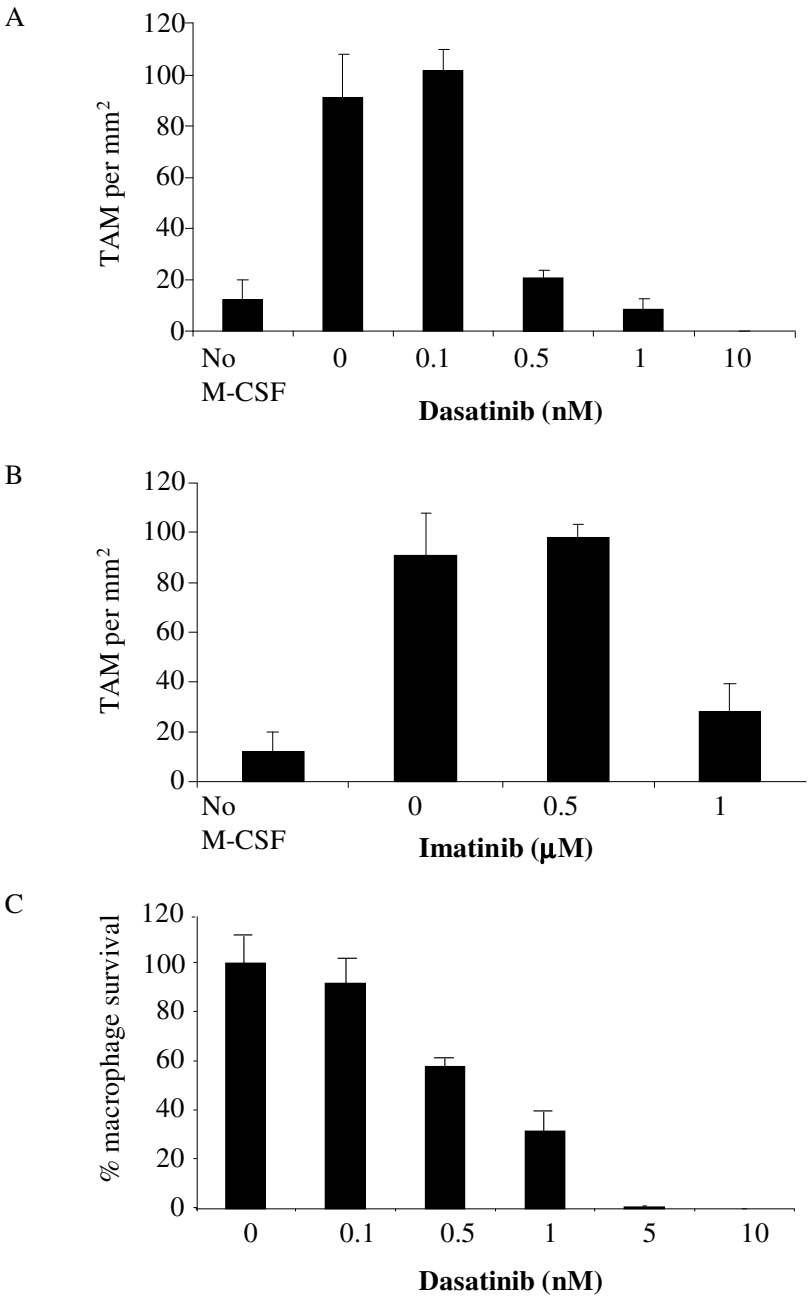


Figure 4.6. Dasatinib and imatinib inhibit the production and survival of tumour-associated macrophages from ovarian cancer ascites. (A,B) Macrophages produced in response to M-CSF stimulation of ovarian tumour ascites samples. Cells were cultured in alpha MEM with 10% FCS and 5000 U/ml M-CSF with the indicated concentrations of Dasatinib (A) or imatinib (B) for 1 week. Macrophage counts were based on CD68 staining. Similar results were obtained for all tested samples (n=5). (C) TAM death assay. Mature macrophages derived from an ovarian cancer ascites sample were cultured with dasatinib for 24 hours in the presence of M-CSF and live cells were counted based on CD68 staining. Similar results were obtained for all tested samples (n=3).

I next investigated the effect of dasatinib on both normal osteoclastogenesis and osteoclastogenesis from ovarian cancer ascites. Normal osteoclastogenesis was modelled using peripheral blood from patients who have given blood for the purpose of bone marrow transplantation. These patients have been given G-CSF and therefore their peripheral blood is enriched with haematopoietic cells from the bone marrow (Gordon et al., 2006).

Table 4.1 and figures 4.7 D, E show that 1 nM dasatinib treatment largely inhibited the production of osteoclasts from both normal peripheral blood and from ovarian tumour ascites samples and that it was completely inhibited at 10 nM. Normal osteoclastogenesis was assayed in the presence of M-CSF and RANKL and was inhibited by dasatinib with an IC50 value of around 0.5 nM. No osteoclasts were formed in the absence of these growth factors as they are both essential for osteoclastogenesis (data not shown) (Kodama et al., 1991a; Kodama et al., 1991b; Kong et al., 1999; Lacey et al., 1998).

Table 4.1. Osteoclastogenesis from normal mobilised peripheral blood and from ovarian cancer ascites samples treated with various concentrations of dasatinib.

Dasatinib (nM)	Osteoclasts from normal peripheral blood (per mm^2) **+M-CSF + RANKL**	Tumour-associated osteoclasts (per mm^2) **- M-CSF - RANKL**
0	28.5 ± 3.0	24.0 ± 1.3
0.1	13.9 ± 0.2	50.9 ± 7.5
0.5	10.2 ± 1.7	41.6 ± 3.9
1.0	2.6 ± 1.6	7.9 ± 2.2
10	0	0

Tumour associated osteoclastogenesis showed a similar sensitivity to dasatinib (Table 4.1) but more surprisingly appeared in the in the absence of both recombinant M-CSF and RANKL. This is in accordance with a previous report of 'spontaneous' osteoclastogenesis from peripheral blood of patients with various solid tumours. (Roato et al., 2005). Figure 4.7A shows a typical photograph of ovarian tumour associated osteoclasts with a distinctive actin ring (4.7A; red), large vacuoles shown by tubulin staining (4.7A; green) and DAPI stained multiple nuclei (4.7A; blue) (Yoshida et al., 1989). Figure 4.7B shows a typical photograph of normal osteoclasts which have been stained for tartrate-resistant acid phosphatase (TRAP). TRAP is used as a marker enzyme for osteoclast differentiation and has a function in cartilage/bone resorption (Minkin, 1982). Figure 4.7C shows that production of these osteoclasts was mostly inhibited at 1 nM dasatinib.

For reasons that are not yet clear, I found that very low concentrations of dasatinib (0.1 and 0.5 nM) enhanced the production of tumour-associated osteoclasts. This is illustrated in both figure 4.7E and table 4.1. Table 4.2 shows that osteoclast production from ovarian cancer ascites was much less sensitive to imatinib than normal osteoclastogenesis. This indicates that tumour associated osteoclasts are physiologically different to normal osteoclasts and that they are not dependent on FMS activity.

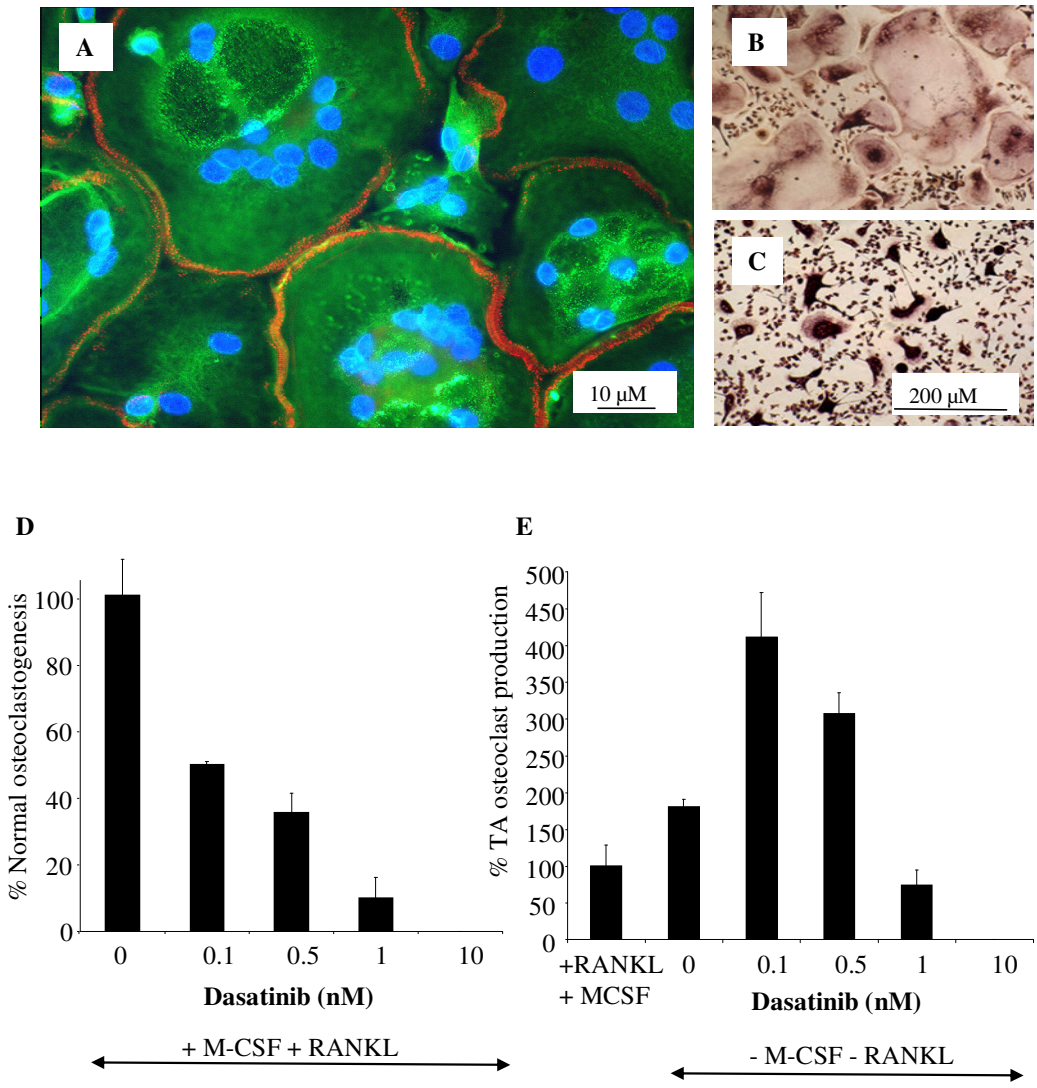


Figure 4.7. Osteoclast production from both normal mobilised peripheral blood and ovarian cancer ascites is inhibited by dasatinib. (A) Representative photograph of ovarian cancer derived ascites osteoclasts which have been grown in alpha 10 for 3 weeks. (B,C) Representative photographs of normal mobilised peripheral blood derived osteoclasts which were grown in alpha 10 + 5000 U/ml M-CSF + 60ng/ml RANKL for 3 weeks with (B) or without (A) 1 µM dasatinib. (D, E) Quantification of osteoclastogenesis assays expressed as percentage for either normal mobilised peripheral blood (D) or ovarian ascites (lE) As indicated cultures were grown in the presence or absence of M-CSF (5000 U/ml) or RANKL (60 ng/ml). Cell numbers are based on osteoclasts being TRAP positive and having 3 or more nuclei. Similar results were achieved for each sample (n=5).

Imatinib (μ M)	% Normal osteoclastogenesis **+ M-CSF + RANKL**	% tumour associated osteoclast production **-M-CSF -RANKL**
0	100 ± 16.0	100 ± 11.5
0.5	89.3 ± 7.0	101.3 ± 16.7
1	3.0 ± 1.5	118.1 ± 5.1
2	0	51.4 ± 14.5

Table 4.1. Osteoclastogenesis from normal mobilised peripheral blood and from ovarian cancer ascites samples treated with various concentrations of imatinib.

Spontaneous osteoclastogenesis was also observed in a sample of pleural effusion fluid from a breast cancer patient (figure 4.8). This could also be inhibited by dasatinib (figure 4.8A) at similar concentrations to ovarian tumour samples. This sample was also sensitive to imatinib (figure 4.8B), unlike osteoclasts derived from ovarian tumour ascites (table 4.2). This observation could not be repeated as we have not yet received further pleural effusion samples.

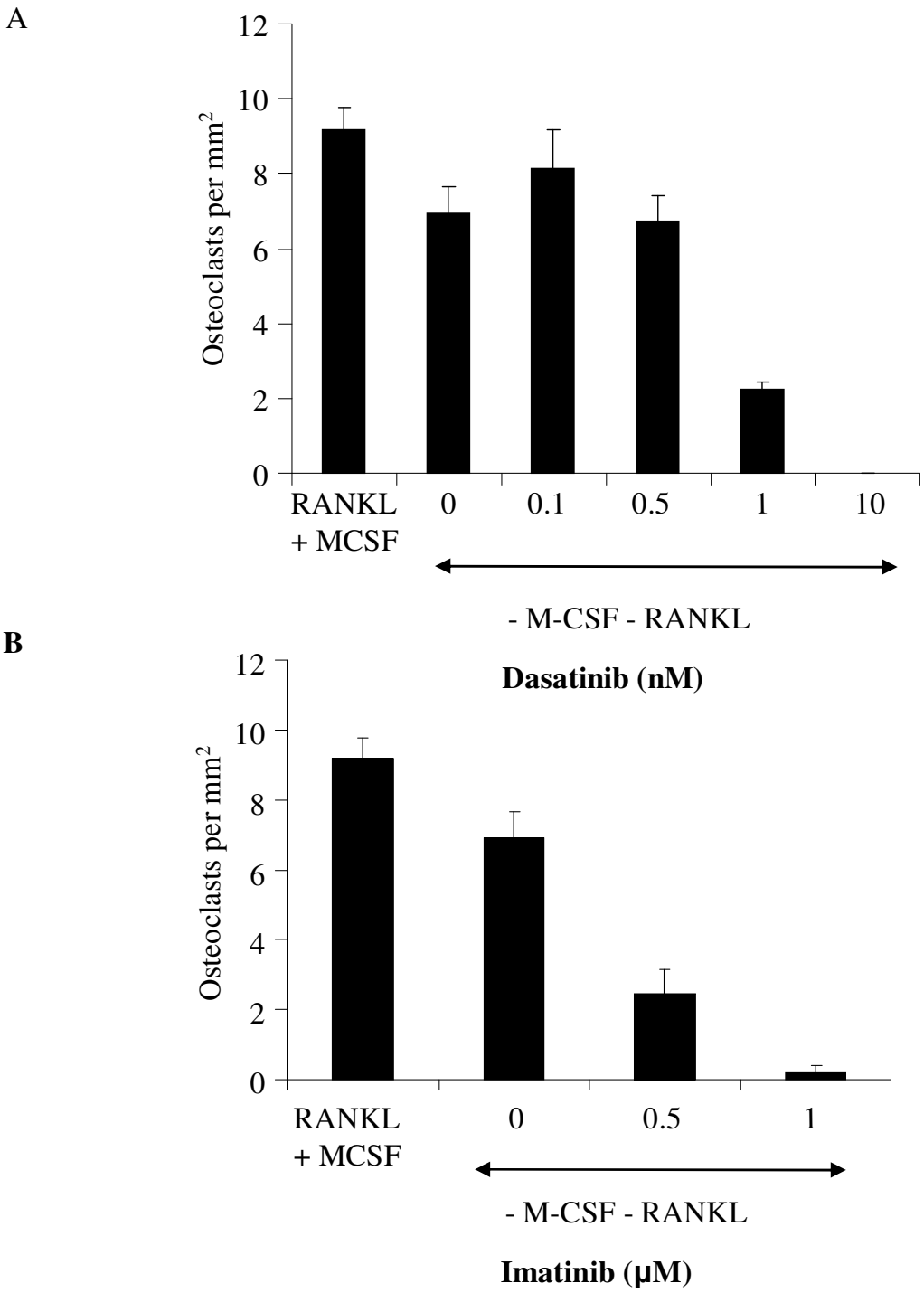


Figure 4.8. Imatinib and dasatinib inhibit osteoclast production from a breast cancer pleural effusion. Pleural effusion fluid was allowed to adhere to tissue culture plastic overnight then cultured with or without 60 ng/ml RANKL + 5000 U/ml M-CSF with various concentrations of dasatinib (A) or imatinib (B) for 2 weeks. Cells were then fixed and stained for TRAP and TRAP positive cells with 3 or more nuclei were counted. Each experiment was done in triplicate (n=1).

I also started a preliminary investigation into whether dasatinib inhibits osteoclastogenesis *in vivo* in patients who are being treated with dasatinib for CML. The patients in this study were being treated with dasatinib after developing resistance to imatinib treatment previously. We were provided with 2 bone trephines taken before dasatinib treatment and 2 taken after dasatinib treatment for each of 6 patients. Figures 4.9A, B show typical trephine sections from before (4.9A) and after (4.9B) dasatinib treatment. Ten random bone measurements from each section were taken and figure 4.9C shows average bone thickness before (4.9C; black) and after (4.9C; stripy) dasatinib treatment. We found no significant effect of dasatinib on bone thickness in this study although a more extensive study would be useful as we did not have access to any patient samples which were either normal and untreated or untreated CML patients.

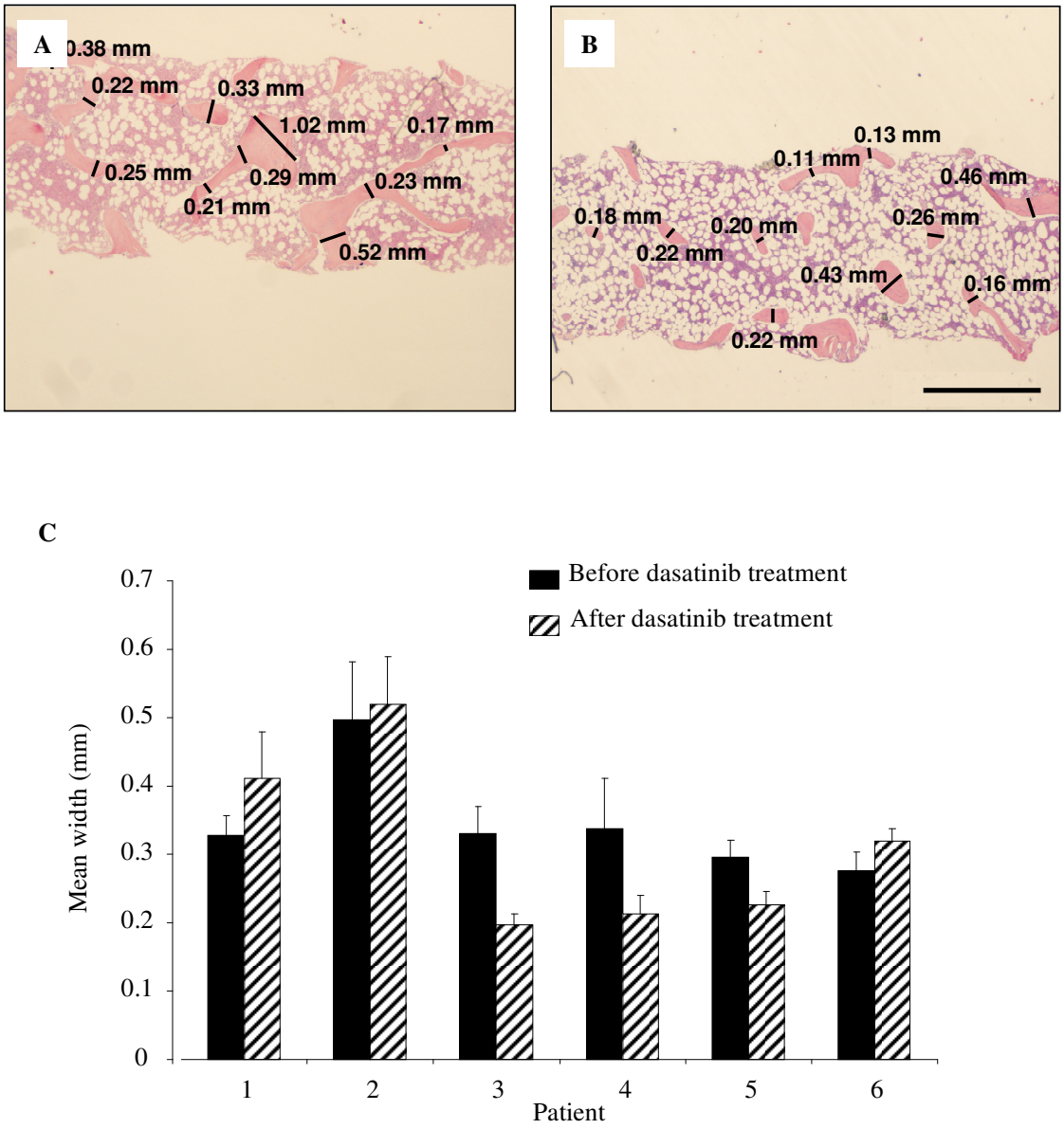


Figure 4.9. Dasatinib has no effect on bone trephine thickness of CML patients treated with dasatinib for at least 1 year in patients who were previously treated with imatinib. (A,B) Representative photographs of bone trephines from a patient before (A) or after (B) dasatinib treatment with 10 random measurements shown. (C) Mean trephine thickness was calculated by blindly measuring bone sections at 10 random points on each trephine and the mean width is shown either before or after dasatinib treatment for at least 1 year. Two trephines were measured both before and after dasatinib treatment. Scale bar = 2mm

There is no current literature stating patient serum levels of dasatinib which are achieved *in vivo*. I therefore designed a system which allows these levels to be estimated. I assayed this by adding serum from patients who are currently being treated with either dasatinib or imatinib to the K562 cell line which is dependent on BCR/ABL activity for survival. I first standardised this experiment by adding dasatinib and imatinib at known concentrations to test the sensitivity of K562 cells to these drugs. Figure 4.10 shows that imatinib (figure 4.10A) and dasatinib (figure 4.10B) inhibited the K562 cell line with IC50 values of 100nM and 0.04 nM respectively. Figure 4.11 shows K562 cells were slightly more sensitive to dasatinib serum than to imatinib serum. The average IC50 values for K562 cell growth inhibition by patient serum calculated from figure 4.11 was $0.54 \pm 0.17\%$ serum for dasatinib and 1.62 ± 0.13 % serum for imatinib treated patients. A total of 6 patients who are being treated with dasatinib were assayed with doses ranging from 50 mg – 70mg twice daily and 7 patients who were being treated with imatinib were assayed with doses ranging from 400 mg to 600 mg once daily.

These results can be used to infer the patient serum levels of these drugs by comparing the IC50 values obtained from figure 4.10 for both dasatinib and imatinib inhibition of K562 cells (0.04 nM and 100 nM respectively) to the IC50 value obtained in figure 4.11 for the amount of patient serum which is required to inhibit the same cell line (0.54 % and 1.62 % respectively). From these figures the patient serum levels can be estimated to be around 6.2 μM for imatinib and 7.4 nM for dasatinib. The serum level estimated here for imatinib is compatible with those found previously in patients treated with imatinib which was recorded as 1.5 - 4.5 μM (Druker et al., 2001).

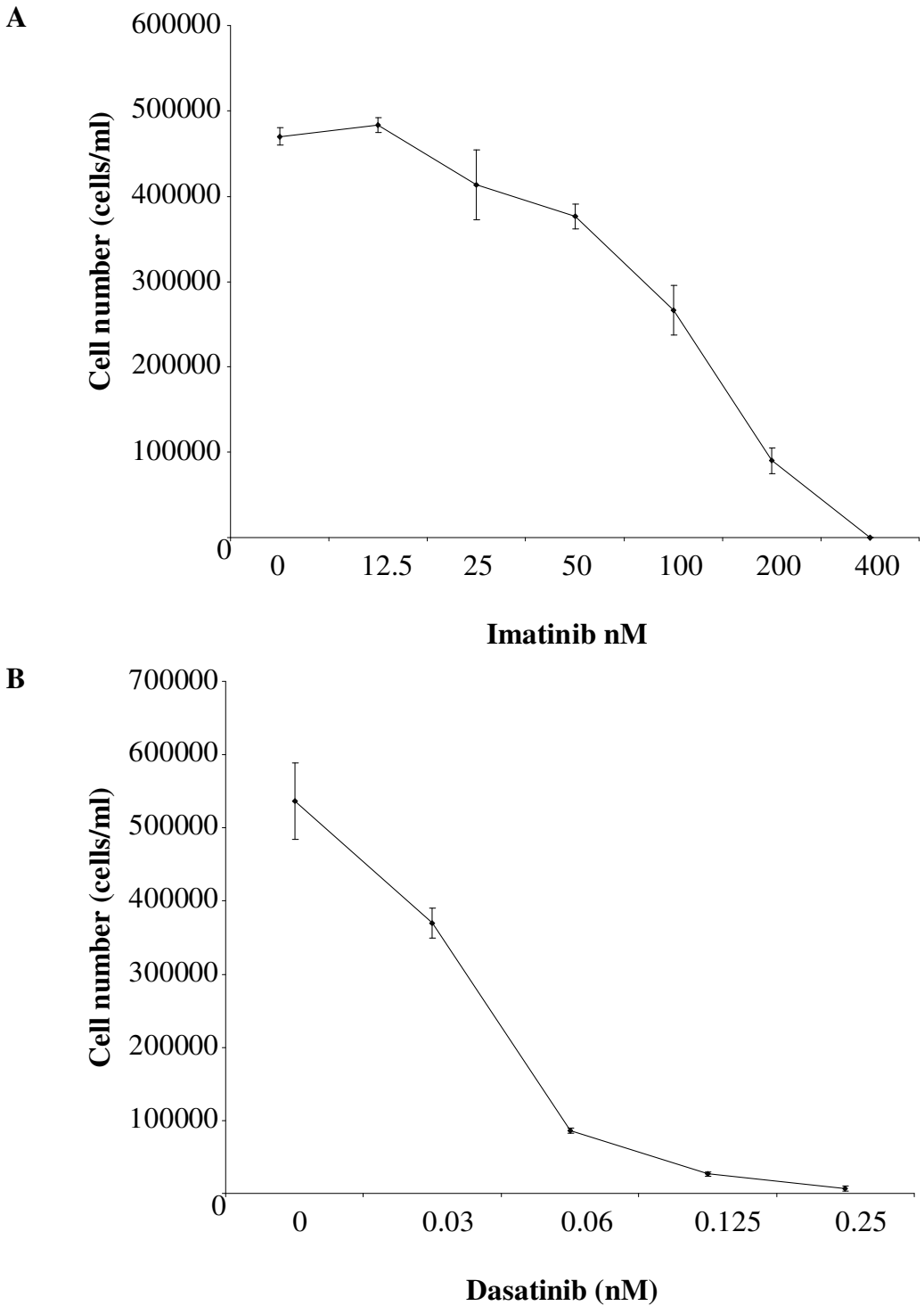


Figure 4.10. Imatinib and dasatinib inhibit BCR-ABL stimulated growth in the K562 cell line. K562 cells were seeded at 5 x 104 cells/ml in RPMI and FCS with the indicated concentrations of either imatinib (A) or dasatinib (B). Viable cell numbers were counted after 5 days of culture and mean cell number per ml is shown. Each experiment was done in triplicate and the error bars represent the SEM.

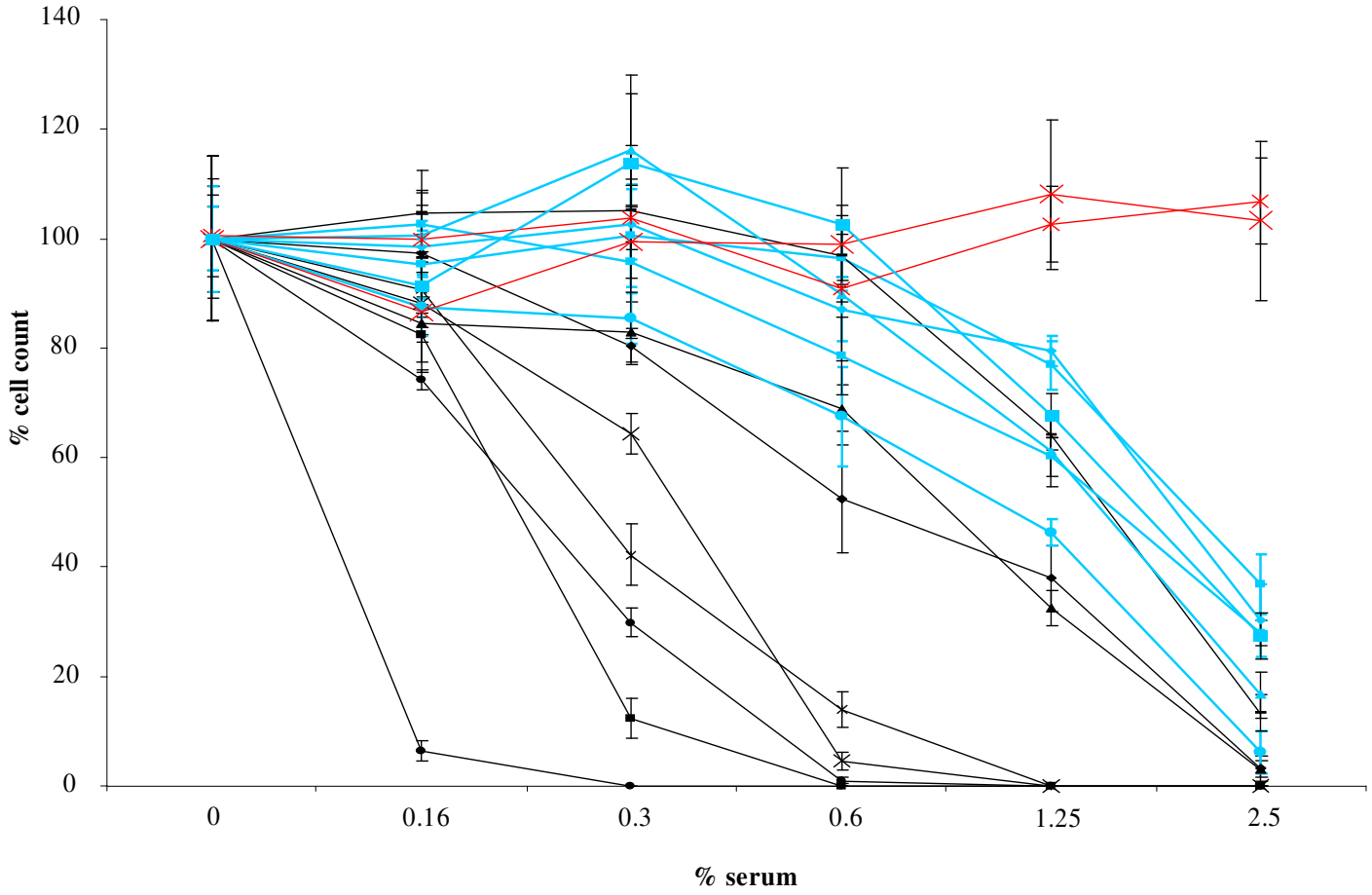


Figure 4.11. Comparison of inhibition of K562 cells by CML patient serum after standard doses of either imatinib or dasatinib. Peripheral blood serum was obtained with informed consent from patients who were being treated for CML with either imatinib (blue lines) or dasatinib (black lines). Untreated controls are shown in red. K562 cells were seeded at 5 x 10^4 cells/ml in RPMI and FCS with the indicated concentrations of patient serum and counted after 5 days of culture (n=14). Each experiment was done in triplicate and SEM is shown for each sample (error bars).

4.3. Discussion

We found dasatinib to be a potent inhibitor of FMS receptor phosphorylation, signalling and FMS induced fibroblast transformation (figures 4.1-4.3). In support of this, the FMS mutation T663I confers resistance to dasatinib (figure 4.2 and 4.3 B,E). The equivalent mutation to this of ABL, T315I, is commonly found in CML patients who have developed resistance to dasatinib (Bradeen et al., 2006). We have also modelled dasatinib binding to the ATP pocket of FMS and this model is consistent with the requirement of Thr 663 to stabilise dasatinib binding through hydrogen bonding (figure 4.4, courtesy of C. Mol.).

Tumour associated macrophages were generated readily from ovarian cancer ascites samples, making them an ideal source of TAMs for this study. Ovarian cancer ascites is in intimate contact with the tumour and contains various immune cells as well as tumour cells. Tumour associated macrophages isolated from this material have previously been shown to display characteristics of M2 activated macrophages and are therefore an ideal model of TAMs from solid tumours (Milliken et al., 2002; Sica et al., 2000; Hagemann et al., 2006). Both dasatinib and imatinib inhibited the production of TAMs in our assays at concentrations consistent with inhibition of FMS (IC50 < 0.5 nM for dasatinib and < 0.5 μM for imatinib) (figure 4.6). Dasatinib treatment also inducted death of mature macrophages, confirming the potency of dasatinib against macrophages *in vitro*.

Osteoclasts could also be easily generated from this material, and in fact arose 'spontaneously' when no growth factors were added to the media (figure 4.7 and table 4.1). This is in agreement with a another report of spontaneous osteoclastogenesis

from peripheral blood of patients with various solid tumours (Roato et al., 2005). In this study, the author suggests that the spontaneous osteoclastogenesis observed may be due to TNF-alpha stimulation and in accordance with this TNF-alpha and RANKL are known to synergise strongly to stimulate osteoclastogenesis (Fuller et al., 2002; Lam et al., 2000).

M-CSF stimulation was an important factor for normal osteoclastogenesis, and accordingly, no osteoclasts formed in its absence or in the presence of FMS inhibitors such as imatinib and nilotinib (see table 3.1) as expected (Hodge et al., 2007; Amano et al., 1998; Fuller et al., 1993). Furthermore, both imatinib and nilotinib stimulate apoptosis of normal mature osteoclasts (see figure 3.6). In contrast to this, FMS signalling was an apparently unimportant factor for ovarian tumour associated osteoclasts as they were resistant to imatinib (Table 4.2). This indicates that the osteoclasts formed from ovarian cancer ascites and those formed from peripheral blood mononuclear cells are phenotypically distinct. The tumour associated osteoclasts were however sensitive to dasatinib and this inhibitory affect was therefore likely to be though inhibition of an unknown kinase (figure 4.7E). Src and ephrin receptors are possible candidates as both of these kinases are sensitive to dasatinib and are important in osteoclastogenesis (Zhao et al., 2006; Soriano et al., 1991; Karaman et al., 2008). Spontaneous osteoclastogenesis seen in the breast cancer samples was inhibited by both imatinib and dasatinib at concentrations consistent with FMS inhibition (figure 4.8). This study is unfortunately incomplete due to the rarity of these samples.

Osteoclasts have well established roles in bone metastasis and therefore the finding that dasatinib inhibits osteoclast production *in vivo* may have great therapeutic value in the complementary treatment of cancers that often metastasise to the bone such as breast cancer and multiple myeloma (Roato et al., 2005; Mundy, 2002; Akhtari et al., 2007). There is already some evidence that FMS inhibitors may target osteoclasts *in vivo*. One report shows altered bone and mineral metabolism in patients treated with high doses of imatinib (Berman et al., 2006) and more recently, bone thickening was also shown in patient bone trephine samples (Fitter et al., 2007).

Macrophage inhibitors could have a significant impact on solid tumour treatment due to the strong association of tumour associated macrophages and tumour metastasis, angiogenesis and aggression (Hagemann et al., 2006; Pollard, 2004; Condeelis and Pollard, 2006). Dasatinib may also be useful in treating many inflammatory diseases where macrophages and osteoclasts are inappropriately activated such as renal inflammatory disease and osteoarthritis (Le Meur et al., 2002; Teitelbaum, 2006; Kitaura et al., 2005; Gravallese, 2002; Hirayama et al., 2002). The potential therapeutic application of macrophage and osteoclast inhibitors is therefore wide ranging and dasatinib forms a good model for development of this type of inhibitor.

CHAPTER 5. Kinase inhibitors as research tools.

5.1 Introduction

In this chapter I establish that the Flt-3 inhibitor, Tandutinib (MLN518; CT53518) also targets the FMS receptor and discuss the therapeutic applications of this finding. I also explore the use of kinase inhibitors of FMS, such as tandutinib, as research tools to study the mechanisms of FMS receptor phosphorylation and downregulation and give evidence that two tyrosine residues which were previously identified as autophosphorylation sites can be phosphorylated by another kinase. I confirm that receptor kinase activity is not required for downregulation of the FMSE633H receptor and that it is most probably a change in structure that promotes accelerated degradation of the FMS receptor (Uden et al., 1999).

5.2 Results

5.2.1 Tandutinib is an effective inhibitor of FMS signalling, osteoclastogenesis and macrophage formation

Tandutinib is a promising quinazoline-based tyrosine kinase inhibitor that is currently in phase II clinical trails for treatment of acute myeloid leukaemia where it targets the Flt-3 receptor (DeAngelo et al., 2006). Flt-3 is constitutively activated by internal tandem duplications in up to 30% of AML patients and a further 5-10% of patients have oncogenic mutations of Asp835 of the kinase domain (Reilly, 2002). Flt-3 is closely related to the KIT, PDGF and FMS receptors and both KIT and PDGF receptors are targets of tandutinib with IC50 values of 170 and 200 nM respectively for *in vitro* kinase assays (Kelly et al., 2002).

In this section I report that tandutinib is an effective inhibitor of the FMS receptor and of both macrophages and osteoclasts *in vitro*. The inhibitory effect of tandutinib was assessed in the FD-cfms or FD FMSD802V cell lines which are dependent on signalling from either FMS, FMS D802V or IL-3 for proliferation and survival (similar assays were used in chapters 3 and 4). Figure 5.1 shows that tandutinib is an effective inhibitor of the FMS receptor (5.1A; circles) but not of FMSD802V (5.1A; triangles). This is in accordance with a previous report that the equivalent mutation of D835 of Flt-3 confers resistance to tandutinib (Clark et al., 2004). The IC50 for FMS inhibition of tandutinib was calculated as 0.4 μM from figure 5.1A. Growth of this cell line in response to IL-3 stimulation was unaffected by tandutinib, confirming specificity of tandutinib to the FMS signalling pathway (5.1A; diamonds). The sensitivity of FMS to tandutinib was also confirmed in a fibroblast transformation assay with Rat-2 fibroblasts which ectopically express v-fms (Baker et al., 1994) (5.1B,C) and the transformed morphology of these cells was inhibited at 1 μM tandutinib (5.1C) (assay described previously in Chapters 3 and 4).

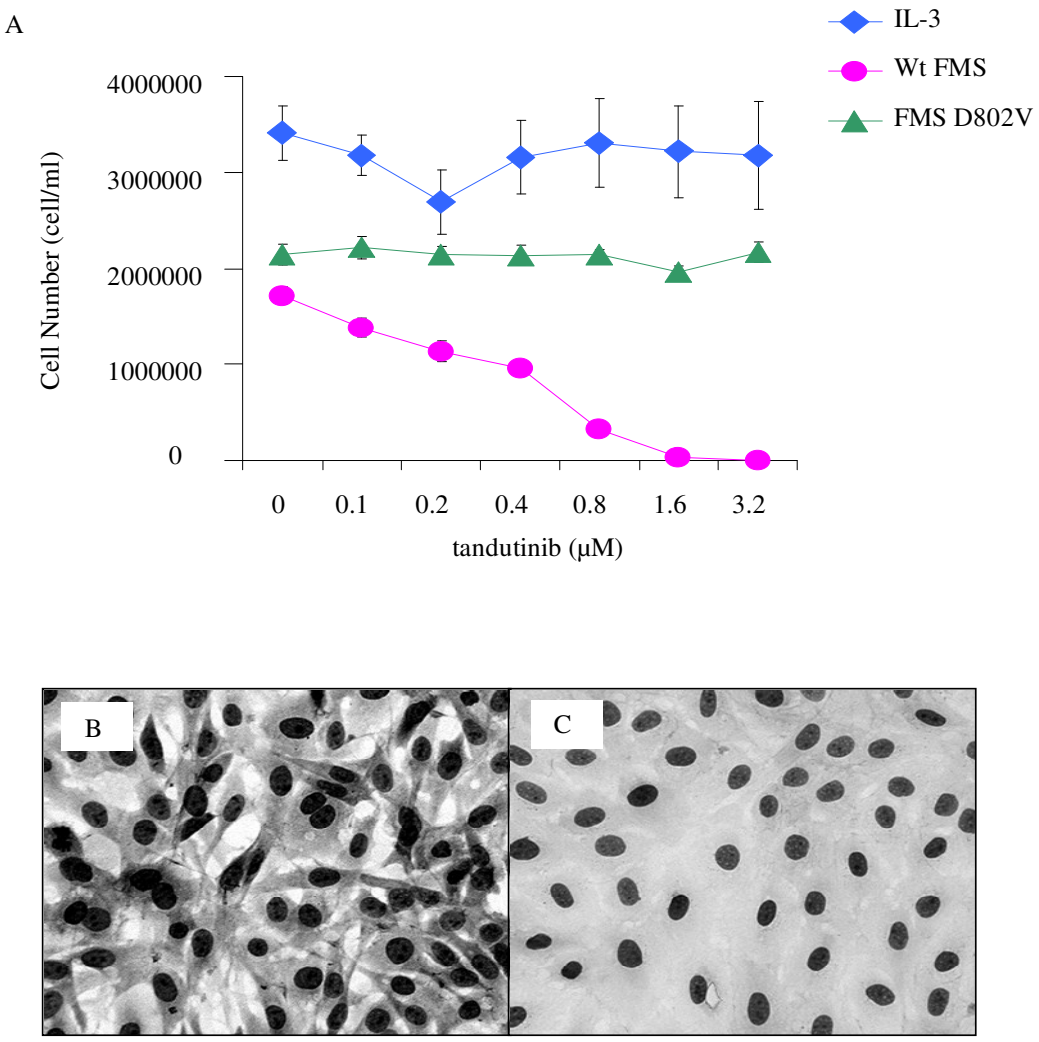


Figure 5.1. Tandutinib inhibits M-CSF induced proliferation and survival of FD-cfms cells and v-fms induced fibroblast transformation (A) FDC-P1 haematopoietic cells that express FMS were seeded at 5 x 104 cells in either 10 ng/ml IL-3 (diamonds) or 5000 U/ml M-CSF (circles) with the indicated concentrations of tandutinib. Similarly, FDC-P1 cells that express the oncogenic receptor FMS D802V were cultured with tandutinib in the absence of both M-CSF and IL-3 (triangles). Viable cell numbers were counted 5 days later. (B, C) Rat-2 V Fms fibroblasts stained with Giemsa following growth in the absence (1B) or presence of 1 μM tandutinib (1C).

Figures 5.2A and B confirm that tandutinib is an inhibitor of FMS phosphorylation (5.2A) and that phosphorylation of FMS D802VY708F was resistant to tandutinib (5.2B). The phosphorylation of the FMS receptor was fully inhibited at 0.25 μM tandutinib which is consistent with the IC50 value observed in figure 5.1A for inhibition of cell growth. Figure 5.2C shows that tandutinib also inhibited the *in vitro* kinase activity of the FMS receptor and that the oncogenic FMS D802V mutation conferred resistance. Densitometry readings of these assays are shown in figure 5.2D.

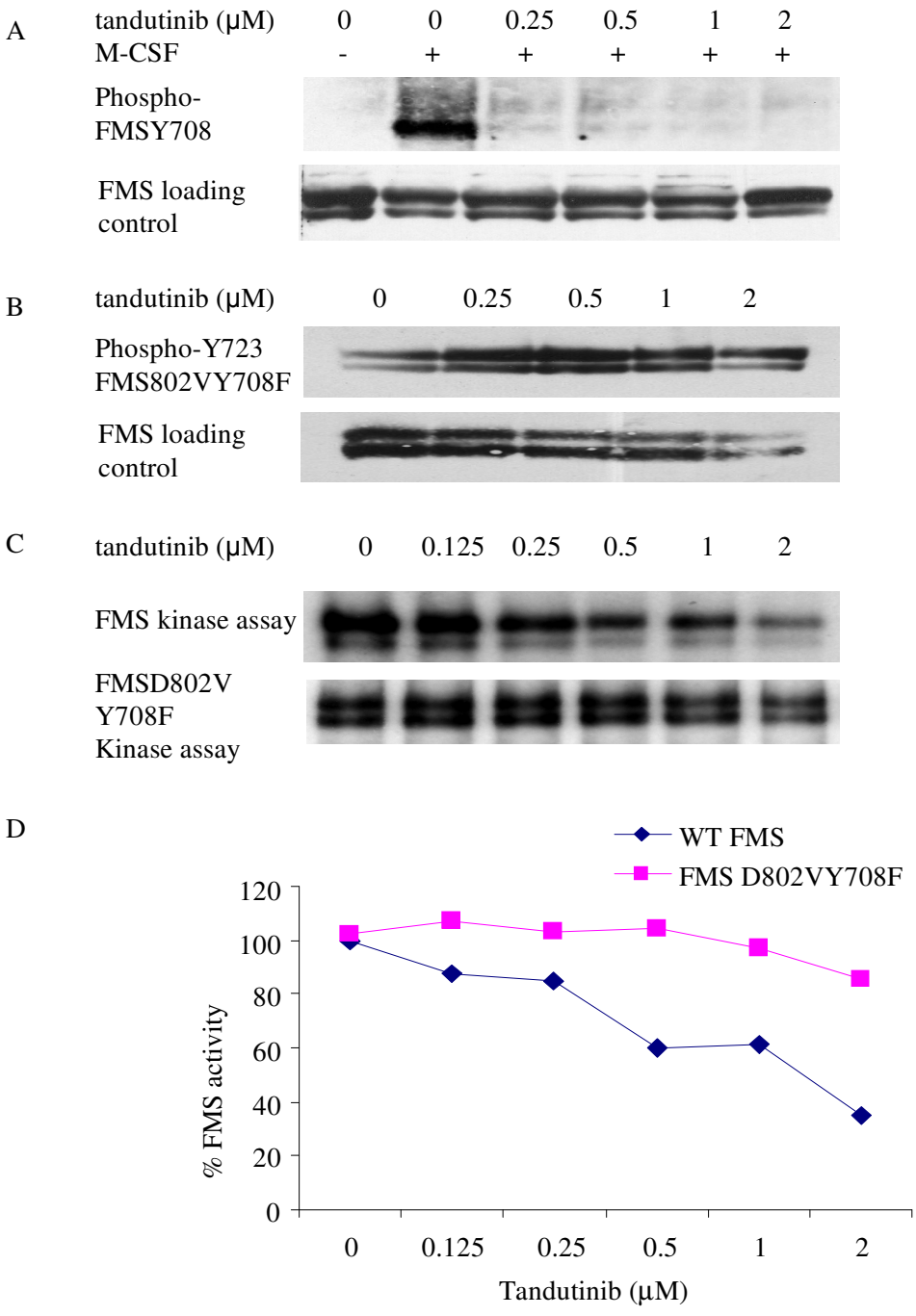
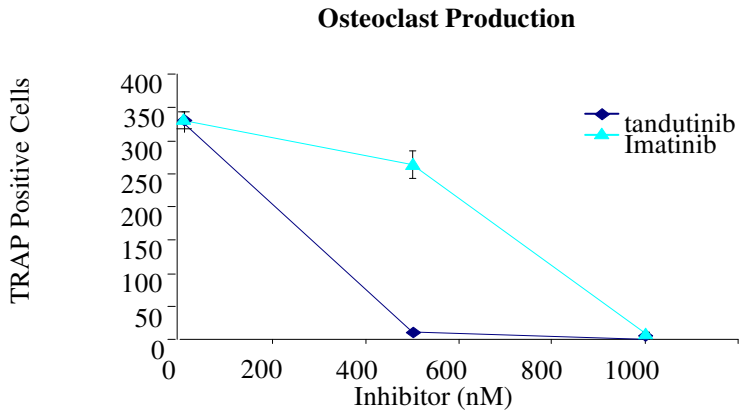


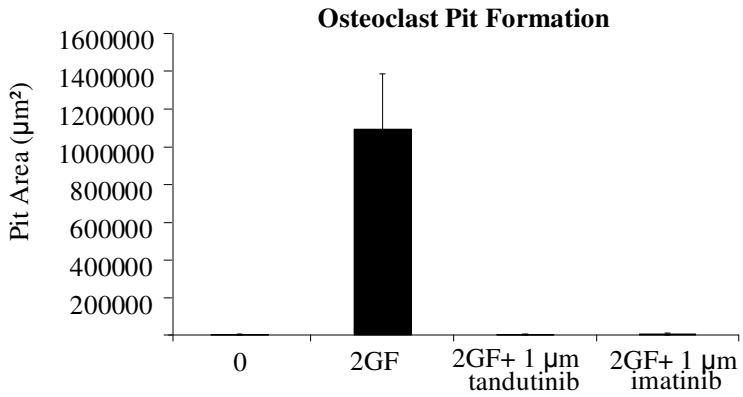
Figure 5.2. Tandutinib inhibits FMS receptor phosphorylation and kinase activity and FMS D802VY708F confers resistance. (A) Tandutinib inhibits M-CSF induced phosphorylation of tyrosine 708 (upper panel), Total FMS receptor is shown as a loading control (FMS Ab 792)(lower panel). (B) The oncogenic receptor FMS D802VY708F confers resistance to tandutinib shown by phosphorylation at tyrosine 723 (upper panel). Total loading of this receptor is shown (lower panel). (C) FMS (upper panel) and FMS D802VY708F (lower panel) *in vitro* kinase assay showing the effect of tandutinib (D) Graph of (C).

Tandutinib inhibited human osteoclastogenesis from mononuclear cells (as described in chapter 3). Figure 5.3A shows a comparison of imatinib and tandutinib inhibition of osteoclast production *in vitro* and shows that tandutinib inhibited osteoclast production slightly more potently than imatinib with an IC50 value of approximately 400 nM compared to an IC50 of approximately 800 nM for imatinib. Figure 5.3B confirms the inhibition of osteoclasts by tandutinib and show that the resorption of dentine slices was completely inhibited by both imatinib and tandutinib at 1 μM. Figure 5.3C shows that tandutinib also inhibits macrophage colony formation from mouse bone marrow cells with an IC50 value of 0.5 μM which is similar to IC50 values shown figures 5.1 and 5.2 for FMS receptor inhibition. No macrophage colonies formed in the absence of M-CSF from this material, as expected.

A



B



C

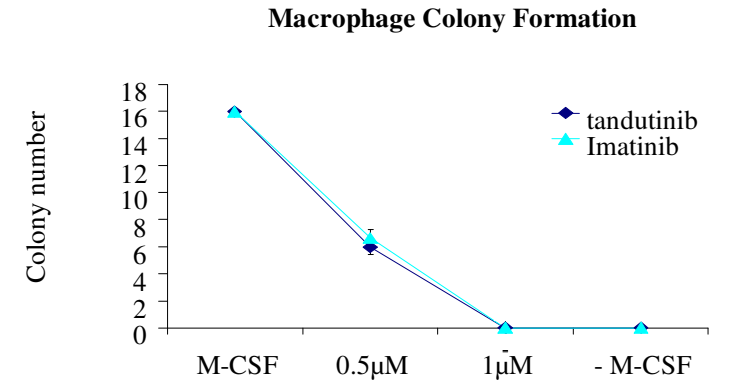


Figure 5.3. Tandutinib and imatinib inhibit osteoclastogensis, osteoclast pit formation and macrophage colony formation. (A) Normal mobilised peripheral blood was cultured for 3 weeks with 5000 U/ml M-CSF and 40 ng/ml RANKL with the indicated concentrations of imatinib or tandutinib. (B) Normal mobilised peripheral blood was cultured as described above on dentine slices and pit formation was measured after 3 weeks. (C) Murine bone marrow cultured in methylecellulose with 5000 U/ml M-CSF and imatinib or tandutinib for 2 weeks and macrophage colonies were counted.

5.2.2 Kinase inhibitors as research tools

5.2.2.1 Anomalous effects of kinase inhibitors upon receptor phosphorylation

I have also assayed the phosphorylation status of FMS tyrosines 723 and 708 when the receptor is exposed to various kinase inhibitors. Figure 5.4A (upper panel) shows that nilotinib was a highly effective inhibitor of FMS phosphorylation at tyrosine 723 with total inhibition at concentrations higher than 0.25 μM. Contrary to this, phosphorylation of the FMS receptor at tyrosine 708 was highly resistant to nilotinib with an IC50 value of greater than 4 μM (figure 5.4A, lower panel). Tandutinib had an opposite effect on FMS phosphorylation to nilotinib. Phosphorylation of FMS tyrosine 723 is resistant to tandutinib up to at least 5 μM (figure 5.4B; upper panel), whereas phosphorylation of tyrosine 708 was inhibited at 0.5 μM tandutinib (figure 5.4B; lower panel). Receptor ubiquitination was inhibited at 0.5 μM tandutinib in both assays, shown by high molecular receptor smearing (figure 5.4A,B, upper panels).

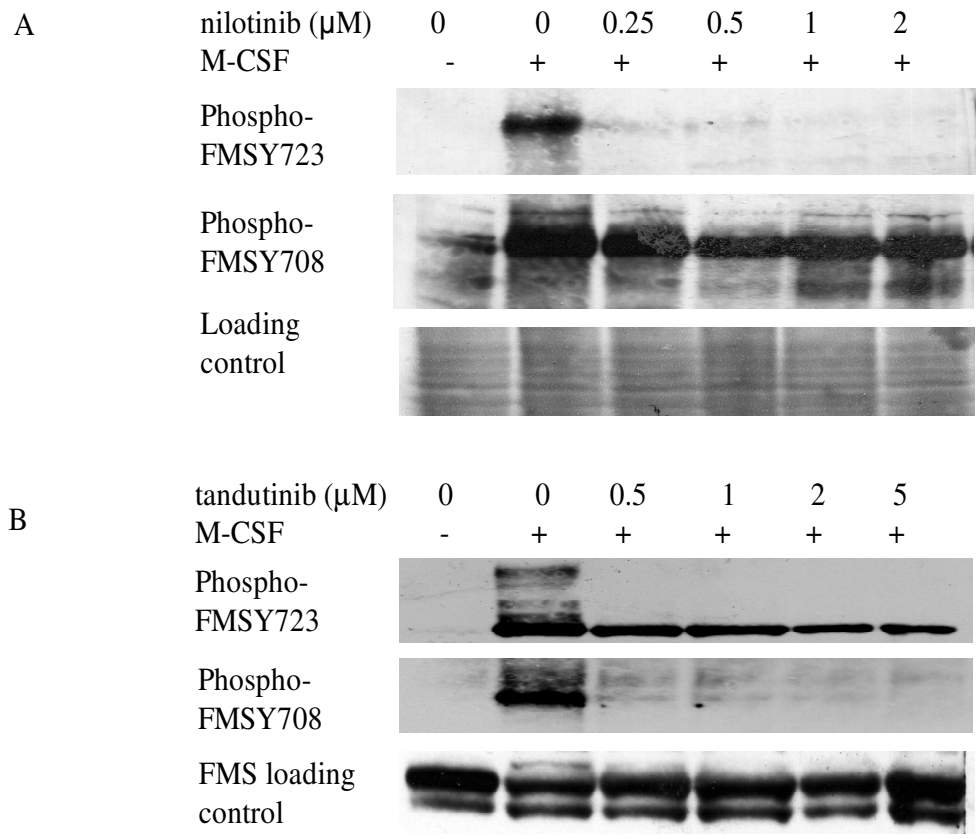


Figure 5.4. Nilotinib and tandutinib have unexpected effects on FMS receptor phosphorylation sites. Western blots of total lysate from Rat-2 cfms cells treated with either nilotinib (A) or tandutinib (B). Rat-2 cfms fibroblasts were pre-treated with the appropriate inhibitor and stimulated with 5000 U/ml M-CSF for 5 mins before being analysed with an antibody to either phospho-FMS tyrosine 723 (A,B; upper panel) or phospho-FMS tyrosine 708 (A, B; middle panel). Either ponseau stained membrane showing total protein loading (A; lower panel) or total FMS receptor (Ab792) (B-E) is shown as a loading control.

Dasatinib had a similar effect on receptor phosphorylation to tandutinib. Phosphorylation of FMS tyrosine 723 was more resistant to dasatinib than phosphorylation of tyrosine 708 and was not completely inhibited at concentrations as high as 10 nM (figure 5.5A; upper panel). Conversely, phosphorylation of tyrosine 708 was very sensitive to dasatinib treatment and was completely inhibited at 0.5 nM (figure 5.5A; middle panel). Similarly, figure 5.5B (upper panel) shows that phosphorylation of FMSD802VY708F at tyrosine 723 is highly resistant to dasatinib and 60 nM dasatinib is required for complete inhibition of tyrosine 723 whereas *in vitro* kinase activity of FMS D802VY708F was very sensitive to dasatinib with an IC50 value of around 1 nM (see chapter 4, figure 4.2). A mutation of the 'gatekeeper' residue FMS threonine 663 to isoleucine confers resistance to dasatinib (see chapter 4; figures 4.3 and 4.3) and consistently, phosphorylation of tyrosines 723 and 708 is also resistant to dasatinib (figure 5.5C). These results have been repeated at least 3 times.

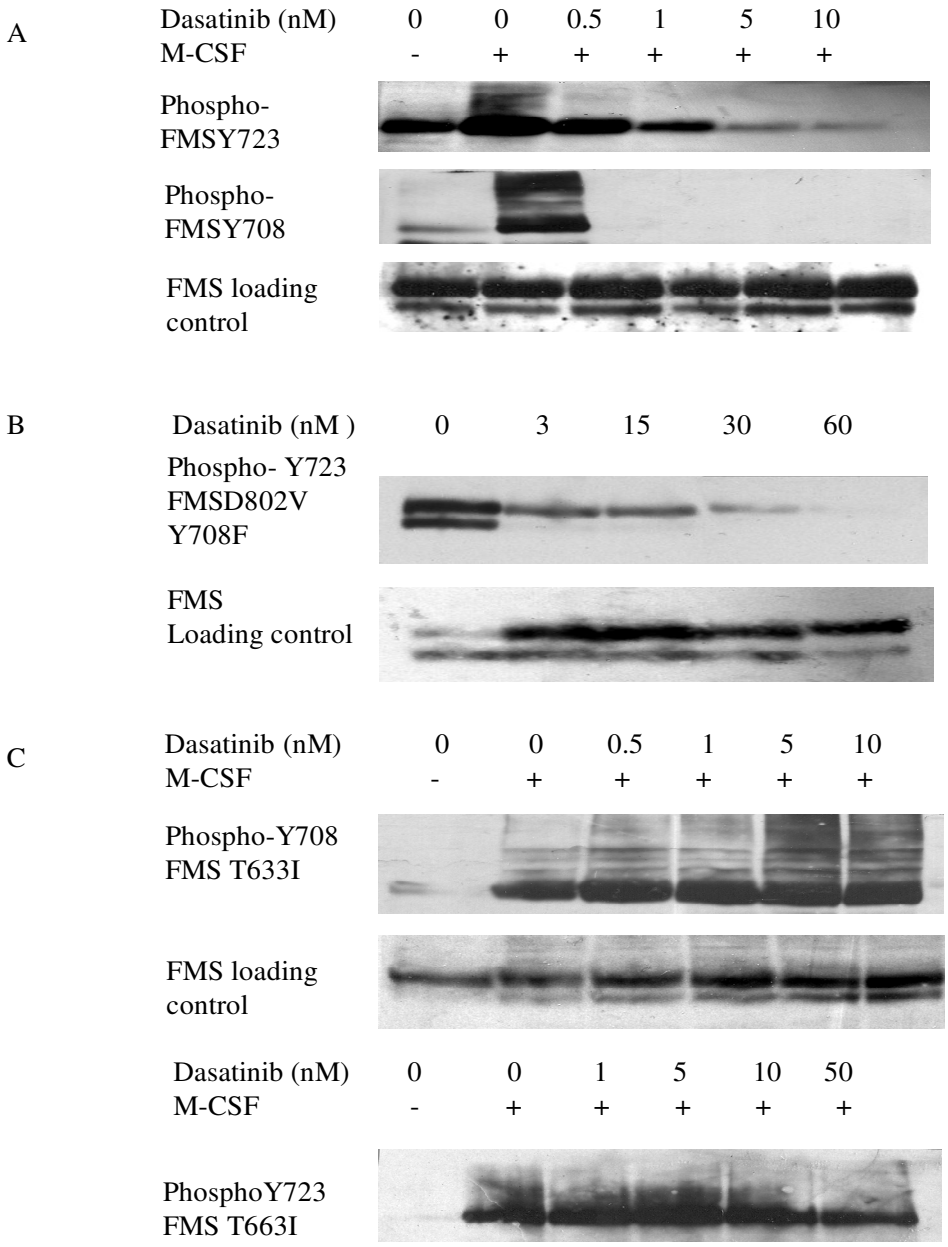


Figure 5.5. Dasatinib inhibits FMS receptor phosphorylation at tyrosine 708 but phosphorylation of tyrosine 723 is less sensitive. Phosphorylation of the FMS mutant T663I is resistant to dasatinib. (A) Rat-2cfms fibroblasts were pretreated with dasatinib for 2 hours and stimulated with M-CSF for 5 mins then total lysates were immunoblotted for either FMS phospho-tyrosine 723 (A; upper panel) or FMS phospho-tyrosine 708 (A; middle panel). Total FMS receptor is shown as a loading control (FMS792 Ab) (A; lower panel). (B) Rat-2 FMS D802VY708F fibroblasts were treated with dasatinib for 2 hours and immunoblotted for either FMS phospho-tyrosine 723 (B; upper panel) or total FMS receptor as a loading control (FMS792 Ab) (B; lower panel). (C) Rat-2 FMS T663I fibroblasts were pretreated with dasatinib for 2 hours and stimulated with M-CSF for 5 mins. Total lysates were immunoblotted for FMS phospho-tyrosine 708 (C; upper panel) or FMS Phospho-723 (C; lower panel). Total FMS receptor is shown as a loading control (FMS 792 Ab) (C; middle panel).

Figure 5.6A compares the effect of imatinib upon the growth of FDC-P1 cells expressing the wild type FMS receptor and two FMS receptor mutants; FMS Y569A, which is a mutation of the juxtamembrane region, and FMS Y569AY809F, which is a double mutation of the juxtamembrane region and the activation loop. This data is courtesy of J. Taylor. The wild type receptor was sensitive to imatinib with an IC50 value of slightly less than 1 μM as expected (Taylor et al., 2006). The juxtamembrane region mutation, FMS Y569A was more sensitive to imatinib than the wild type receptor with an almost total inhibition at 1 μM. This is in accordance with a previous observation that imatinib binding to the KIT receptor is slightly obstructed by the juxtamembrane region (Mol et al., 2004a). This mutation of the FMS juxtamembrane region may therefore shift this region slightly to enable stronger imatinib binding. The double mutant, FMS Y569AY809F has a similar sensitivity to imatinib compared to the wild type receptor. This may be explained by a slight hindrance to imatinib binding due to a shift in the conformation of FMS towards a more activated state due to the second mutation of the activation loop (Mol et al., 2004a). The oncogenic receptor FMS D802V confers resistance to imatinib in this assay, so confirming that the inhibition seen here is due to specific inhibition of the receptor function.

In light of the inhibitory effect of imatinib on these receptor mutants, I have also examined the effect of imatinib on the phosphorylation of FMS Y723 in each of these mutant receptors. The phosphorylation of wild type FMS Y723 is inhibited by imatinib, although total inhibition occurs at slightly higher concentrations than expected (Taylor et al., 2006) (figure 5.6B). The two receptor mutations, FMS Y569A and FMS Y569AY809F are oncogenic mutations and therefore are

constitutively phosphorylated on both the immature and mature form of the receptor (figure 5.6C and D, first lane). Surprisingly, only the phosphorylation of the immature band of FMS Y569A is inhibited by imatinib, and the phosphorylation of the mature band appears to be resistant to imatinib (figure 5.6C), and this resistance is in contrast to the cell proliferation data shown in figure 5.6A. Furthermore, the double mutant receptor, FMS Y569AY809F appears to be fully resistant to imatinib, also in contrast to the cell proliferation data shown (compare figure 5.6A and 5.6D).

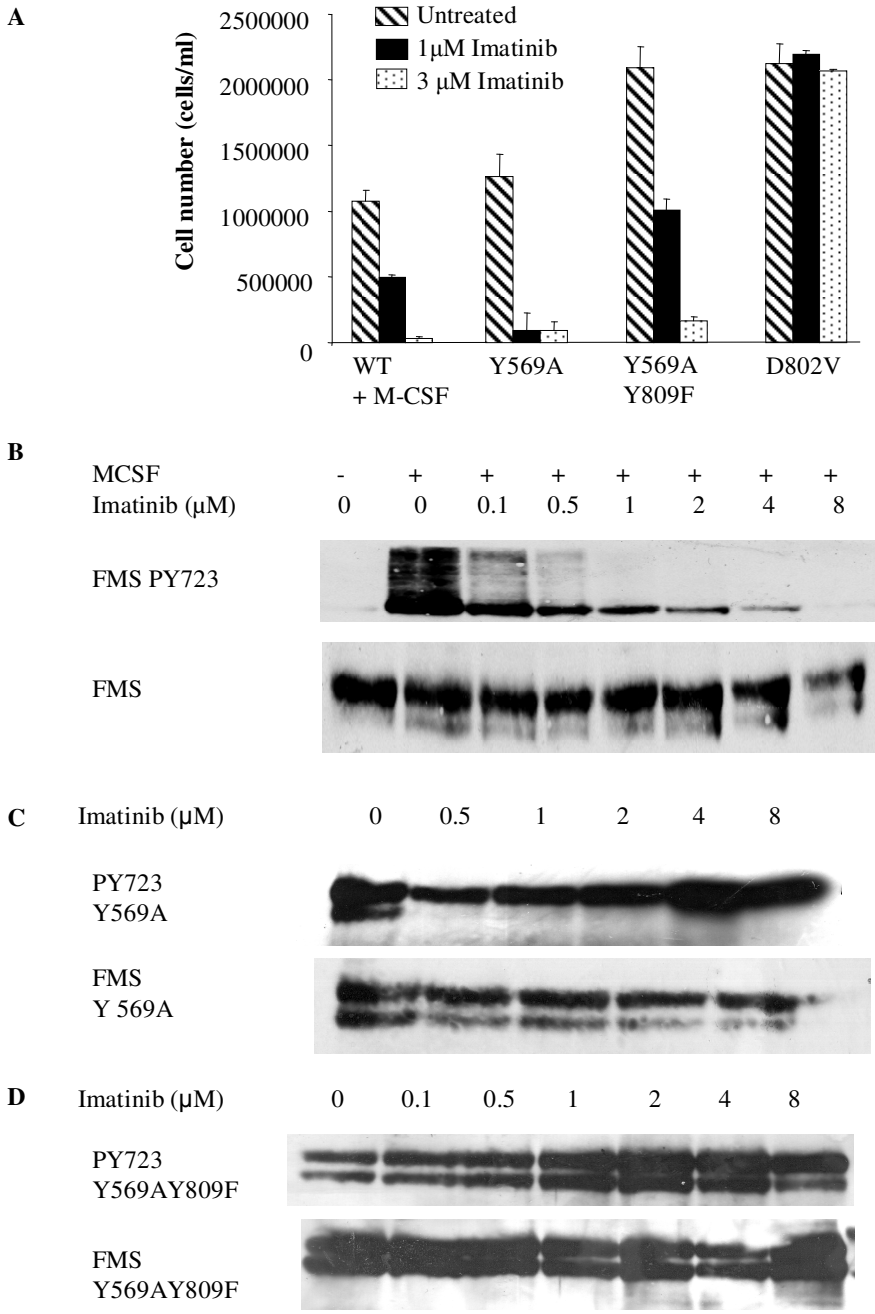


Figure 5.6. Imatinib inhibits FMS, FMS Y569A and FMS Y569AY809F induced cell proliferation but their phosphorylation on tyrosine 723 is less sensitive. (A) FDC-P1 cells expressing the wildtype or mutant forms of FMS were plated at $5x10^5$ cells/ml in medium free of IL-3 with $5x10^5$ U/ml M-CSF and with 1 μM or 3 μM imatinib. The cells were counted after a week of growth and triplicate assays were averaged. (B-D) Rat-2 fibroblasts expressing either wild type FMS (B), FMS Y569A (C) or FMS Y569A Y809F were pre-treated with the indicated concentrations of imatinib for 2 hours and stimulated with 5000 U/ml M-CSF for 5 minutes if appropriate. Cell lysates were immunoblotted for either FMS phospho-tyrosine 723 (B-D; upper panel) or FMS (Ab 792)(B-D; lower panel). (A) Courtesy of J. Taylor.

5.2.2.2 Receptor downregulation

Another interest in developing these inhibitors is that they provide new opportunities to study general mechanisms of FMS activation and downregulation. Receptor downregulation is usually considered to be a product of receptor internalization and receptor degradation. For most receptors, this process is rapidly enhanced by ligand binding, therefore ensuring that the ligand only stimulates a transient signal to the cell. The FMS receptor downregulates constitutively in the absence of ligand binding with a half life of approximately 1.5-3 hours depending on cell type (Rettenmier et al., 1987b). Upon ligand binding, the receptor internalises more rapidly and is either degraded or recycled (Manger et al., 1984).

It has been reported that tyrosine kinase activity is required for this second, ligand dependent internalisation. The main evidence for this is a kinase inactive mutant K616A. This mutant receptor internalizes constitutively at the same rate as the wild type receptor stimulated by M-CSF and is not degraded (Carlberg et al., 1991). K616 is a lysine residue in the ß3 strand and is highly conserved amongst kinases and a mutation to alanine is widely reported to inhibit downregulation and is considered to be evidence that receptor tyrosine kinase activity is required for ligand induced downregulation. This lysine mutation has been made in many other kinases including the EGF receptor (K721A) (Honegger et al., 1987), the PDGF receptor (K634A) (Sorkin et al., 1991), the HGF receptor (K589A) (Jeffers et al., 1997) and the insulin receptor (K1018A) (Russell et al., 1987).

In contrast to this, Uden et al (1998) have described various FMS kinase inactive receptors which are not impaired for degradation upon M-CSF stimulation. The

mutant FMS receptor FMS E633H is kinase inactive and is not ubiquitinated in response to M-CSF (N.Dibb unpublished) but does downregulate in a similar manner to the wild type receptor (Uden et al., 1999). This indicates that the accelerated downregulation which is stimulated by M-CSF is induced by structural changes and is independent of intrinsic kinase activity. The FMS K616A mutant receptor has presumably suffered a structural change which has resulted in a receptor which is unable to undertake the changes in conformation necessary for both kinase activity and downregulation. In support of this, there is a family of atypical receptor tyrosine kinases which are unable to autophosphorylate but are capable of activating downstream signalling molecules such as MAPK upon ligand binding. These receptors include Mep (EphB6) (Gurniak and Berg, 1996), Colon carcinoma kinase-4 (Cck4) (Mossie et al., 1995) and H-Ryk (Katso et al., 1999). Further, a mutation of the conserved lysine residue mentioned previously (K616 in FMS) to alanine (K334A in H-Ryk) inactivates these receptors, leaving them unable to signal, supporting the hypothesis that this mutation impairs structural changes in the receptor (Katso et al., 1999).

I have investigated the effect of the kinase inhibitors identified here on the downregulation of the FMS receptor. When FMS is stimulated with M-CSF it downregulates at an accelerated rate and this is shown in figure 5.7A as a decrease in amount of the higher molecular weight mature receptor compared to the smaller immature from of the receptor together with an overall decrease in total amount of receptor (compare first and second lane, figure 5.7A) (Uden et al., 1999; Taylor et al., 2006). This effect on downregulation is inhibited by imatinib at concentrations similar to those required to inhibit other aspects of FMS activity (see chapter 3).

Dasatinib also inhibited M-CSF induced FMS downregulation in both Rat-2 cfms fibroblasts (figure 5.7B) and in primary macrophage cultures (figure 5.7C).

The mutant FMS receptor, FMS D802V is very unstable and downregulates constitutively in the absence of M-CSF at such an accelerated rate that little of the receptor reaches the cell surface (Morley et al., 1999). As a result the receptor is difficult to see by western blotting unless the receptor has suffered a second mutation of Tyr 708 to Phe. This mutant partially stabilizes the immature form of the receptor but the mature form of the receptor appears to be degraded at a similar speed to the wild type receptor when stimulated by M-CSF (Morley et al., 1999). Figure 5.7D shows that the ratio of mature FMS receptor to immature receptor is low in Rat-2 D802VY708 cells (figure 5.6D; lane1) and the mature form of the receptor was stabilized at low nanomolar concentrations of dasatinib (figure 5.7D) indicating some inhibition of mature receptor turnover.

When the more unstable FMS D802V is overexpressed it can be seen by western blotting more easily, this is shown in figure 5.7E (lane 1) in the cell line FMS D802V clone 8. This unstable mutant receptor was also further stabilized by dasatinib at low nanomolar concentrations (figure 5.7E). This was confirmed in figure 5.7F with the Rat-2 FMS D802V cell line which does not over express this receptor and is therefore difficult to see by western blotting (5.7F, lane 1). This receptor was not stabilized by nilotinib (figure 5.7F; lanes 2, 3) or tandutinib (figure 5.7F; lanes 4,5) but was slightly stabilized by dasatinib (figure 5.7F; lanes 6,7) at 1 nM and 5 nM. The FMS receptor with the mutation T663I is not stabilized by dasatinib using a

similar assay (figure 5.7G), confirming that this receptor mutation confers resistance to dasatinib and that the FMS receptor stabilization seen by dasatinib is due to a direct effect on the receptor.

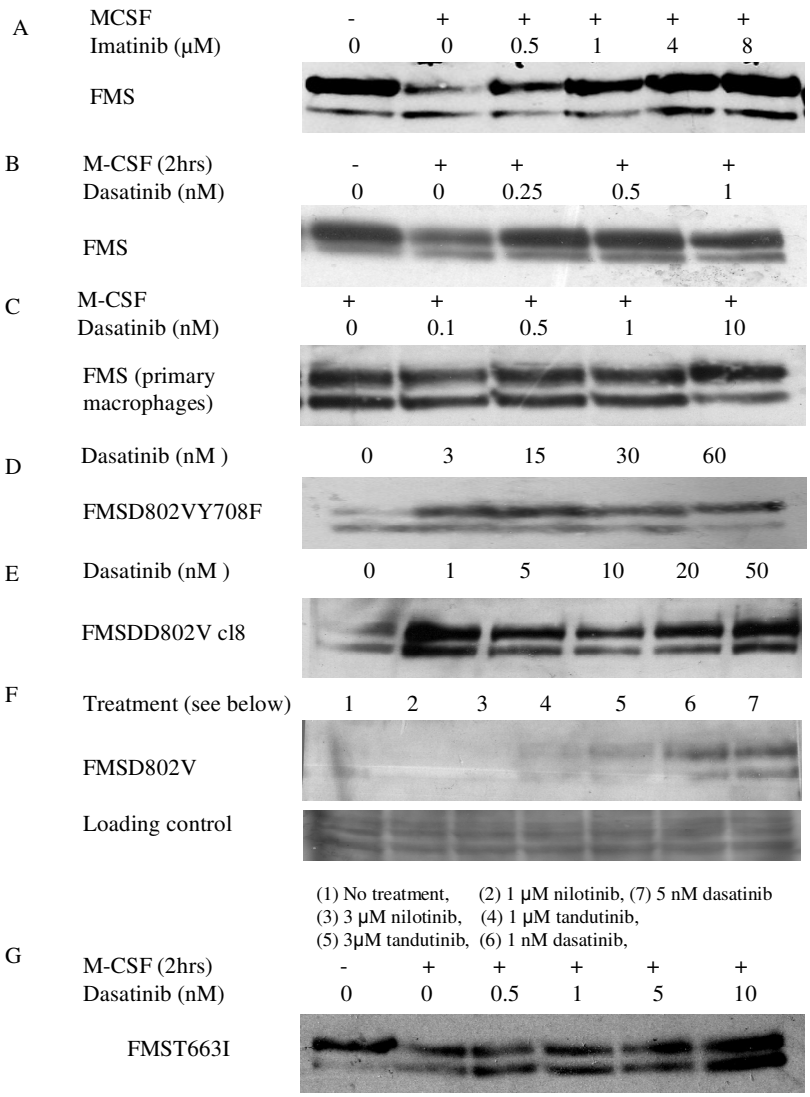


Figure 5.7. Imatinib and Dasatinib inhibit M-CSF induced downregulation of the wild type receptor in rat-2cfms fibroblast cells and in primary macrophages. Dasatinib also stabilises the mutant receptors D802VY708F and D802V. The FMS T663I mutation is not stabilized by dasatinib. (A) Bac1.2F5 macrophages were starved of M-CSF for 24 hours, then pretreated with imatinib for 1 hour before being re-stimulated with 5000 U/ml M-CSF for 1 hour. (B) Rat-2 cfms fibroblasts were pretreated with the indicated concentrations of dasatinib for 1 hour and stimulated with M-CSF for 1 hour. (C) Primary mature macrophages from ovarian cancer ascites were cultured in M-CSF and treated with dasatinib for 2 hours. (D,E) Rat-2 FMSD802VY708F fibroblasts (D) or Rat-2 FMS D802V cl8 fibroblasts (E) were treated with dasatinib for 2 hours. (F) Rat-2 FMS D802V cells were treated with the indicated concentrations of either nilotinib, tandutinib or dasatinib for 2 hours. (G) Rat-2 FMS T663I cells were treated with the indicated concentrations of dasatinib for 1 hour and then stimulated with M-CSF for 1 hour. All lysates were immunoblotted with an antibody against the C-terminus of the FMS receptor (Ab792). Ponseau stained nitrocellulose membrane shows even loading in (F).

Figure 5.8 shows that the Src kinase inhibitor SU6656 (figure 5.8A) and the pan-kinase inhibitor staurosporine (figure 5.8E) also target the FMS receptor. FMS receptor phosphorylation was inhibited by SU6656 with an IC50 value of approximately 1 μM (figure 5.8A) and in accordance with figure 5.7, SU6656 also inhibits M-CSF induced receptor degradation at similar concentrations to those required to inhibit other aspects of receptor activity (figure 5.8B). SU6656 also targets FMS D802VY708F with a similar IC50 to the wild type receptor (figure 5.8C; upper panel) and accordingly stabilises the mature form of the mutant receptor (figure 5.8C; lower panel). In confirmation of this, SU6656 inhibits the accelerated degradation of the particularly unstable FMSD802V receptor and stabilizes both the mature and immature receptor (figure 5.8D). Staurosporine was also an effective inhibitor of FMS phosphorylation with an IC50 value of around 20 nM (figure 5.8E) and figure 5.8F shows that staurosporine inhibited the accelerated FMS turnover induced by M-CSF at similar concentrations to those that were required to inhibit phosphorylation (figure 5.8E).

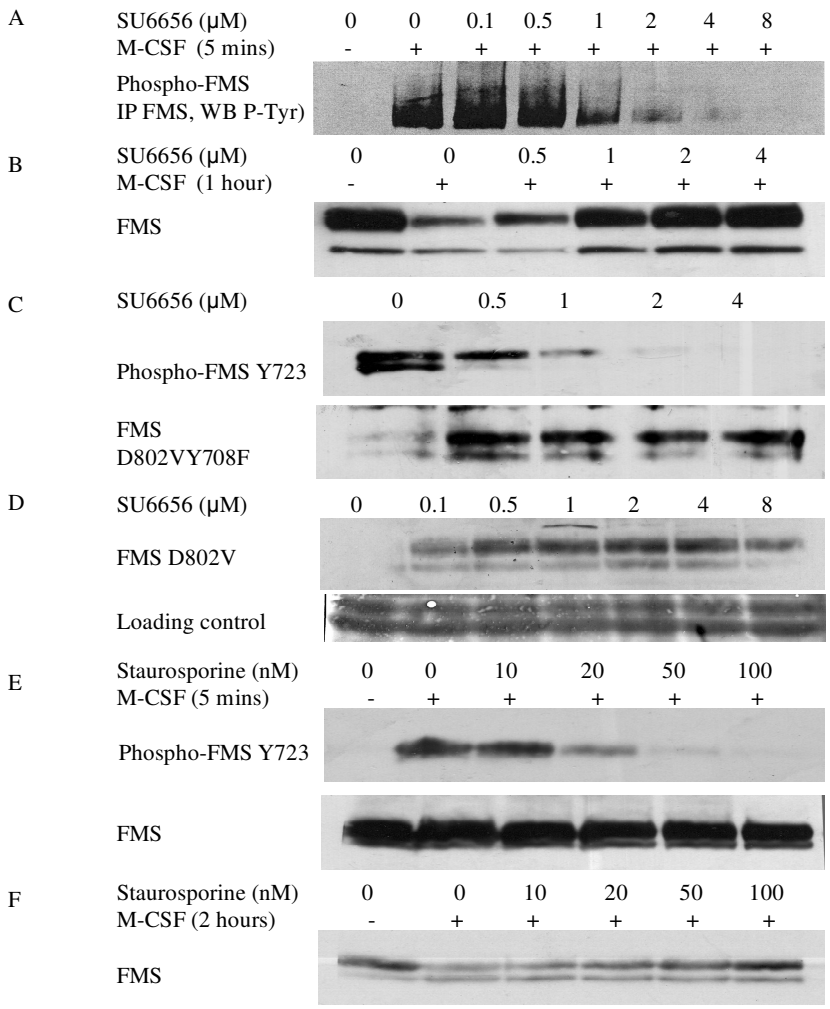


Figure 5.8. The Src kinase inhibitor SU6656 and the pan-kinase inhibitor staurosporine also target the FMS receptor and inhibit both phosphorylation and receptor degradation of FMS and FMS D802V. (A) Rat-2 cfms fibroblasts were treated with the indicted concentrations of SU6656 and stimulated with 5000 U/ml M-CSF for 5 mins. Immunoprecipitated receptor was then immunoblotted with an antibody against total phosphorylated tyrosine (PY99). (B) Rat-2 cfms fibroblasts were pretreated with the indicated concentrations of SU6656 for 1 hour then stimulated with M-CSF for 1 hour and immunoblotted for total FMS receptor. (C) Rat-2FMS D802VY708F cells are treated with SU6656 for 2 hours and immunoblotted for either FMS phospho-723 (C, upper panel) or total FMS receptor (C, lower panel). (D) Rat-2 D802V cells were treated for 2 hours with SU5565 and immunoblotted for total FMS receptor (D, upper panel), D; lower panel shows total protein loading on nitrocellulose membrane. (E) Rat-2 cfms cells were pre-treated with the indicated concentrations of staurosporine for 2 hours and stimulated with M-CSF for 5 minutes before being immunoblotted for FMS phospho-tyrosine 723 (E, upper panel) or for total FMS receptor (E, lower panel). (F) Rat-2 cfms fibroblasts were pretreated with various concentrations of staurosporine for 1 hour and stimulated with M-CSF for 1 hour then immunoblotted with an antibody FMS antibody Ab 792. (A) Courtesy of J. Taylor.

Figure 5.9A shows immunofluorescence staining of FMS in N9 microglia cells. In these cells, FMS had a clear cell surface localisation, creating a halo around the cell. Figure 5.9B shows that M-CSF stimulation caused a movement of the receptor into internal vesicles. A similar effect has been reported previously for the KIT receptor (Jahn et al., 2002). This can also be observed in Rat-2 cfms fibroblasts which express the wild type FMS receptor (figure 5.9 C,D). The FMS K616A kinase inactive mutant receptor did not appear to localise into equivalent vesicles upon stimulation with M-CSF and had an evenly distributed cell surface appearance with little vesicles localisation both before (figure 5.9E) and after M-CSF stimulation (figure 5.9F). This is in accordance with a previous report and confirms that this mutant receptor does not internalise in response to M-CSF (Carlberg et al., 1991). I also investigated a second kinase inactive receptor, FMS E633H (Uden et al., 1999). In contrast to FMS K616A, this mutant receptor appeared similar to the wild type receptor and the majority of cell surface receptor moved to internal vesicles with M-CSF stimulation for 15 minutes (figure 5.9H). This is in accordance with Uden et al (1999) and shows that this kinase inactive receptor internalises in response to M-CSF. Figure 5.9I,J shows that the FMS staining with this antibody does not cross react with any other cell component in Rat-2 fibroblasts.

Figure 5.9

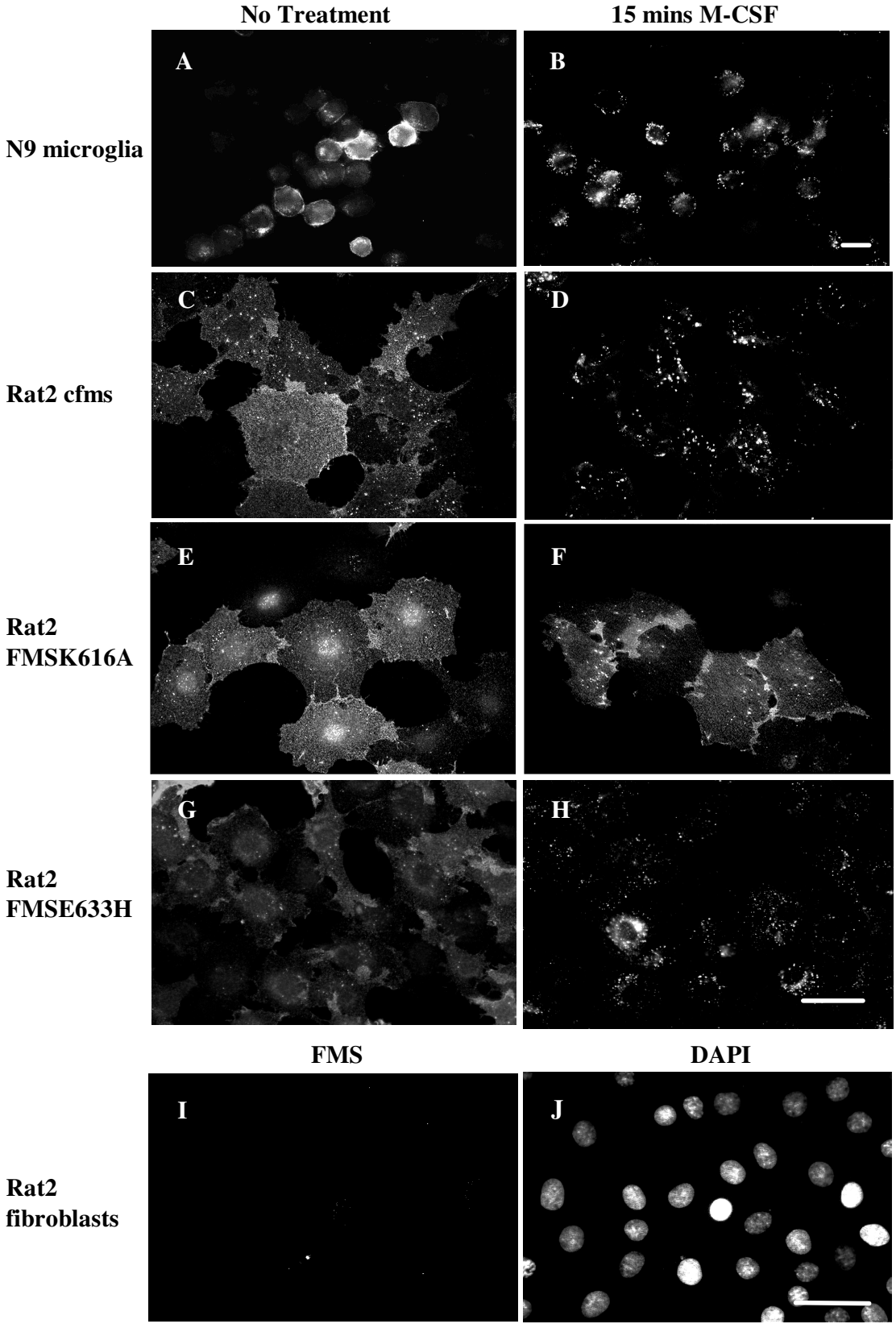

No Treatment
15 mins M-CSF
A
B
N9 microglia
C
D
Rat2 cfms
E
F
Rat2
FMSK616A
G
H
Rat2
FMSE633H
FMS
DAPI
I
J
Rat2
fibroblasts

Figure 5.9 The wild type FMS receptor and the kinase inactive FMS receptor FMS E633H , but not the kinase inactive receptor FMS K616A downregulate into intracellular vesicles in response to M-CSF. (A,B) N9 microglia cells treated with or without M-CSF for 15 mins. Cell were probed with a FMS antibody specific to the N-terminus of the receptor (AFS-98) and visualised with an alexa flour 488 conjugated secondary antibody. (C-H) Rat-2 fibroblasts expressing either wild type FMS (C-D) or the FMS mutants K616A (E-F) or FMS E633H (G-H) were stimulated with M-CSF for 15 minutes (D,F,H) or were not treated (C,E,G). Cells were fixed and probed with an antibody specific to the FMS receptor N-terminus (3-4A4) and visualised with an alexa flour 488 conjugated secondary antibody. (G) Rat-2 fibroblasts immunostained for the FMS receptor (negative control) (H) DAPI staining of G. Ruler = 50 μm.

It has been demonstrated previously that the lysosome inhibitor methylamine inhibits the constitutive degradation of FMS but does not have an apparent effect on the accelerated, ligand inducible degradation, indicating that FMS downregulates down two distinct pathways (N. Dibb, unpublished) (Seglen et al., 1979). This effect on the constitutive pathway is confirmed in figure 5.10A which shows that methylamine caused a build up of receptor as assayed by western blotting. Figure 5.10 confirms this data by immunofluorescence staining of FMS in Rat-2 cfms fibroblasts and shows that the constitutive degradation of both the wild type receptor (figure 5.10; B-D) and the kinase inactive mutant receptor FMS E633H (figure 5.10; E-G) are inhibited by methylamine. Both the mutant and wild type receptor aggregated in large vesicles close to the centre of the cell, with the most striking accumulation after 4 hours of methylamine treatment at 10 mM (figure 5.10 D,G).

Figure 5.10

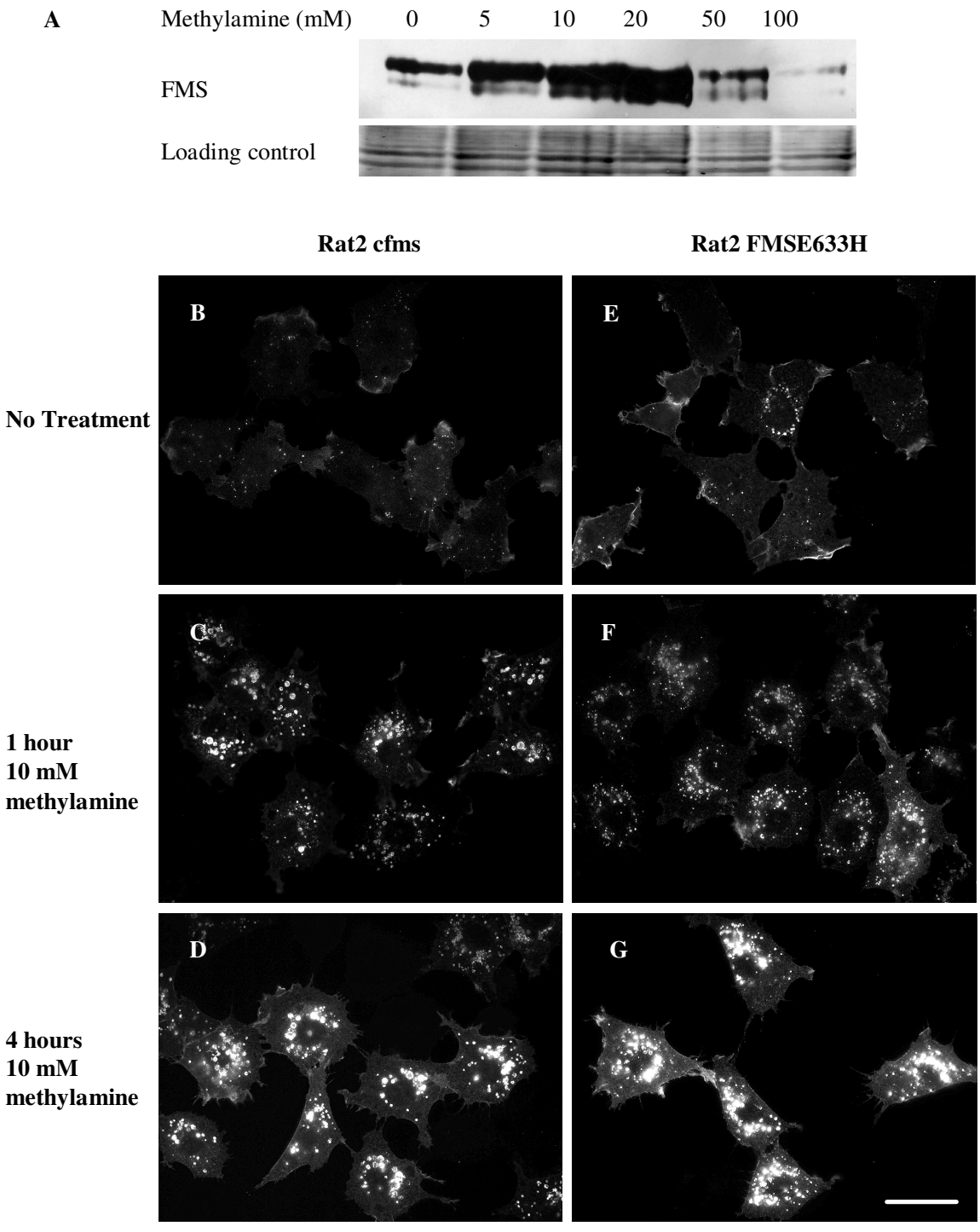

A
Methylamine (mM)
0
5
10
20
50
100
FMS
Loading control
Rat2 cfms
Rat2 FMSE633H
No Treatment
1 hour
10 mM
methylamine
4 hours
10 mM
methylamine
B
E
C
F
D
G

Figure 5.10. The wild type FMS receptor and the kinase inactive receptor FMS E633H both aggregate into intracellular vesicles when incubated with the lysosome inhibitor methylamine. (A) Methylamine was incubated with Rat-2cfms cells at the indicated concentrations for 3 hours and immunoblotted for the FMS receptor using an antibody against the c-terminus (Ab 792). (B-G) 10 mM methylamine was added to either Rat-2 cfms (B-D) or Rat-2 E633H (E-G) fibroblasts and incubated at 37°C for 0 hours (B,E) 1hour (C, F) or 4 hours (D,G). Cells were then fixed and probed with an antibody specific for the N-terminus of the FMS receptor (3-4A4). An Alexa-Fluor 488 secondary antibody was used for visualisation. Ruler = 50 μM

I next investigated the effect of methylamine on the ligand inducible pathway, with the aim to deduce whether the wild type and mutant receptor follow similar downregulation pathways. Figure 5.11 A,B confirm that untreated Rat-2 cfms cells had a dispersed, cell surface appearance (figure 5.11A) and that M-CSF induced aggregation of the FMS receptor into internal vesicles (figure 5.11B). Figure 5.11C, and 5.12C confirms that when the cells are pretreated with methylamine, internal FMS containing vesicles appear in the centre of the cell, although dispersed, cell surface receptor is also visible (figure 5.11C, 5.12C). Stimulation with M-CSF causes the more dispersed FMS receptor on the cell surface to disappear, indicating that methylamine does not inhibit the internalisation of the receptor from the surface (figure 5.11D, 5.12D). The FMS E633H receptor appears to downregulate in a similar, methylamine insensitive way in response to M-CSF stimulation (figure 5.11; E-H, 5.12; E-H) indicating that both the wild type receptor and FMSE633H downregulate in response to M-CSF via similar pathways. Figure 5.12 shows more detailed, higher magnification photographs of figure 5.11

Figure 5.11

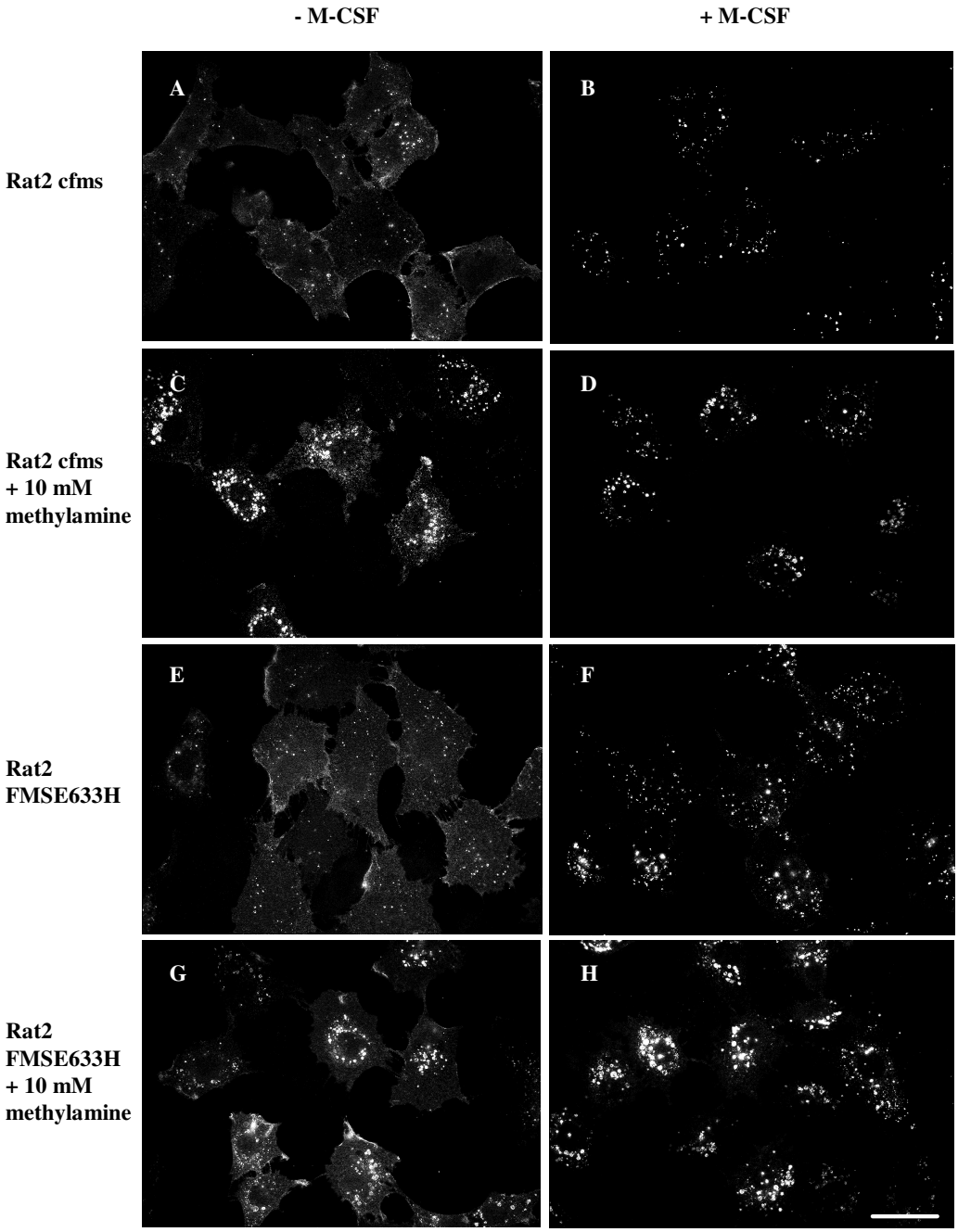


Figure 5.11. M-CSF induced aggregation of the FMS receptor into internal vesicles and this is not inhibited by 10 mM methylamine . Rat-2 cfms (A-D) or Rat-2 FMS E633H cells (E-H) were treated with 10 mM methylamine for 3 hours before stimulation with M-CSF for 15 minutes (B, D, F, H) and were then fixed and probed with an antibody specific for the N-terminus of the FMS receptor (3-4A4) An Alexa-Fluor 488 secondary antibody was used for visualisation. Ruler = 50 μM

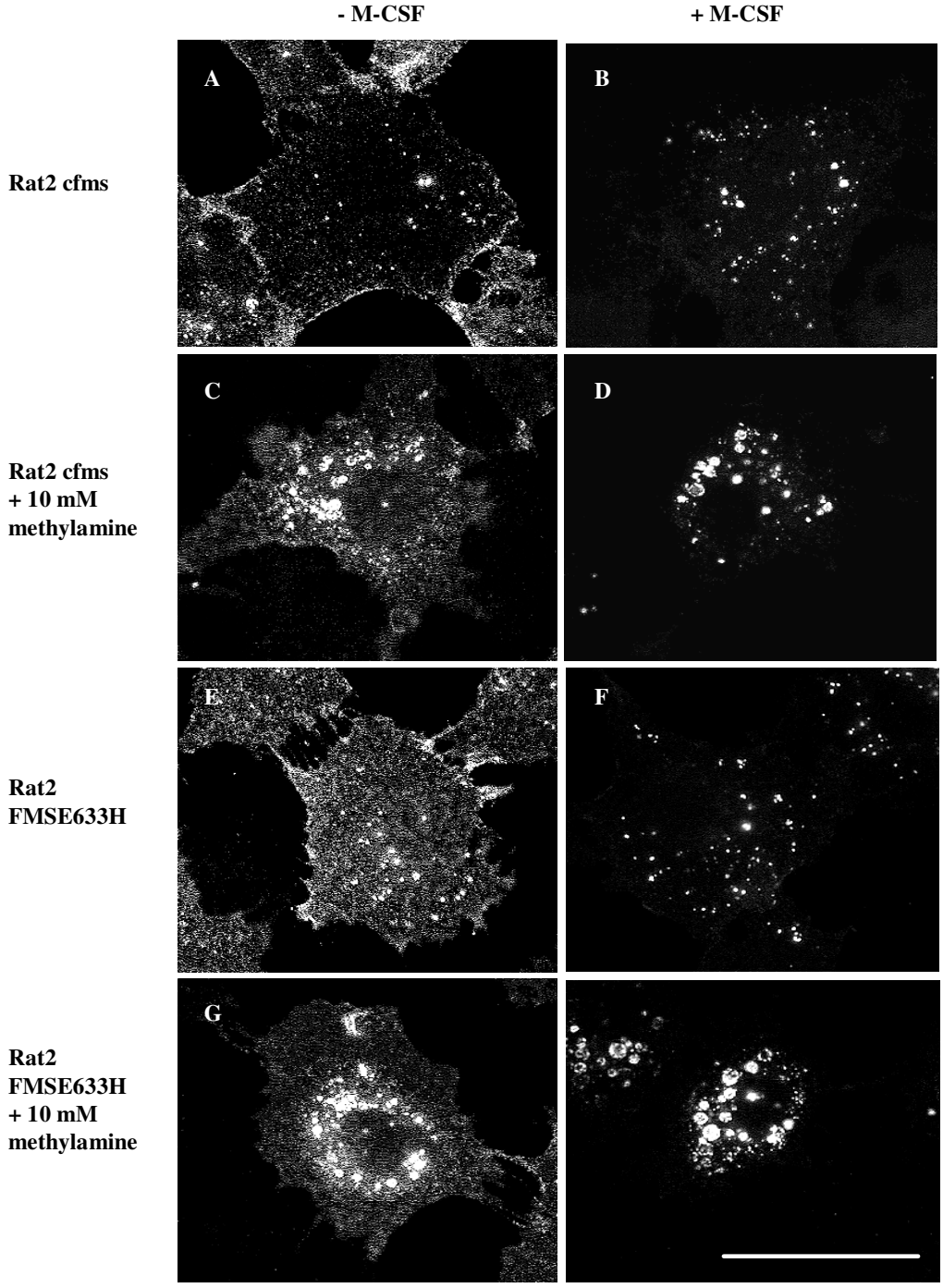


Figure 5.12. Higher magnification view of figure 11. Rat-2 cfms (A-D) or Rat-2 FMS E633H cells (E-H) were treated with 10 mM methylamine for 3 hours before stimulation with M-CSF for 15 minutes (B, D, F, H) and were then fixed and probed with an antibody specific for the N-terminus of the FMS receptor (3-4A4) An Alexa-Fluor 488 secondary antibody was used for visualisation. Ruler = 50 µM

Figure 5.13A confirms that the kinase inactive receptor FMSE633H is not phosphorylated on tyrosine 723 as expected (Uden et al., 1999) and figure 5.13B (lanes 1 and 2) confirms that this receptor is degraded upon stimulation with M-CSF in Rat-2 FMSE633H cells. This receptor degradation was inhibited effectively by both dasatinib (5.13B; lanes 3-6) and imatinib (5.13C). This provides further evidence that neither kinase activity, nor ubiquitination are necessary for degradation in this receptor.

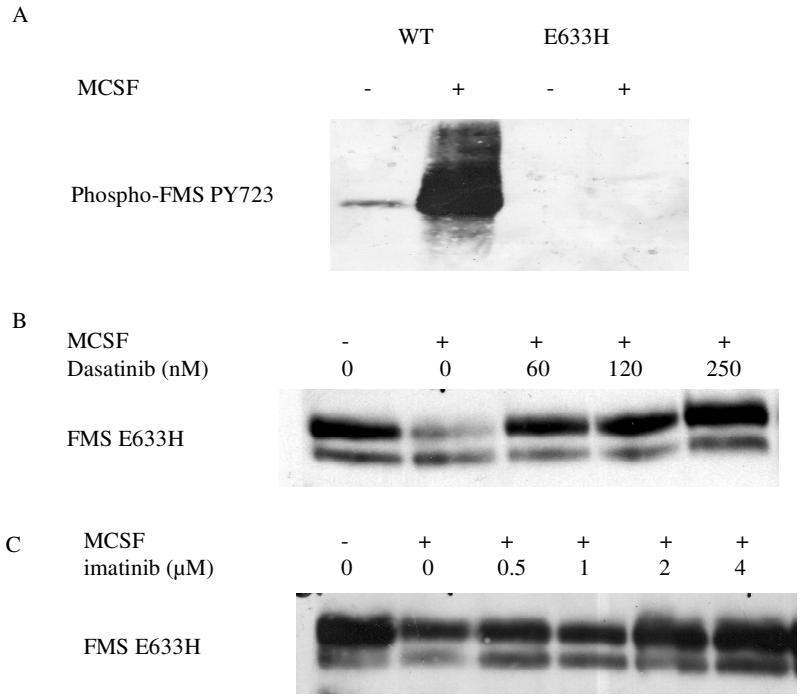


Figure 5.13. Both dasatinib and imatinib inhibit M-CSF induced degradation of the kinase inactive mutant receptor FMS E633H. (A) Rat-2 cfms and Rat-2 FMSE633H cells were stimulated with M-CSF for 5 minutes , lysed and western blotted with an antibody specific to FMS phosphotyrosine 723. (B,C) Rat-2 FMSE633H cells were pretreated with either dasatinib (C) or imatinib (D) for 1 hour then stimulated with M-CSF for 1 hour. Cell lysates were immunoblotted for total FMS receptor.

5.3 Discussion

5.3.1 Tandutinib

I found tandutinib to be a potent inhibitor of FMS receptor phosphorylation, signalling and FMS induced fibroblast transformation (figures 5.1-5.2). In support of this, the FMS mutation D802V confers resistance to tandutinib (figure 5.1 and 5.2B-D). Tandutinib also inhibits *in vitro* osteoclast and macrophage colony formation at similar concentrations to FMS inhibition (figure 5.3). This opens new possibilities for treating diseases with tandutinib where cell types which are dependent on FMS signalling are implicated.

The concentration of tandutinib required to inhibit the FMS receptor, macrophage colony formation and osteoclastogenesis *in vitro* is less than the plasma trough levels of around 2 micromolar observed in patients who are treated with tandutinib (DeAngelo et al., 2006). This raises the possibility of some inhibitory effect of tandutinib upon these processes; however it should be noted that the inhibitory effects of tandutinib and imatinib upon FMS are similar *in vitro* (see chapter 3) and that imatinib is very well tolerated by patients and is a well established treatment and so may be a better choice as a therapeutic FMS inhibitor. Nevertheless, tandutinib also has the potential to be developed as an inhibitor of the wide range of diseases that are known to be dependent upon FMS expression.

5.3.2 FMS kinase inhibitors as research tools

5.3.2.1 Anomalous FMS phosphorylation

Tyrosine kinase inhibitors of FMS are useful research tools and have previously been unavailable to the lack of specificity for the FMS receptor of many of the pan kinase inhibitors that are available. I have shown in figures 5.4 - 5.6 that in the presence of the various FMS kinase inhibitors established here, at least one FMS phosphorylation site remains phosphorylated when other aspects of receptor activity are inhibited, including *in vitro* kinase activity. What is more surprising is that different kinase inhibitors allow different tyrosine residues to be phosphorylated. For example, figure 5.4A shows that phosphorylation of FMS tyrosine 723 is extremely sensitive to nilotinib, whereas figure 5.4B shows that the same tyrosine is resistant to tandutinib and conversely, phosphorylation of FMS tyrosine 708 is resistant to nilotinib but sensitive to tandutinib. This seems to be evidence that at least one other kinase is able to phosphorylate FMS tyrosines 723 and 708, although it is unclear whether this second kinase is necessary for phosphorylation of these residues.

Both tyrosines 708 and 723 were previously thought to be FMS autophosphorylation sites although these experiments were carried out *in vitro* and the authors acknowledge that these results are not necessarily relevant *in vivo* (van der Geer and Hunter, 1990; Tapley et al., 1990; Reedijk et al., 1992). Tyrosine 723 is thought to bind to the p85 subunit of phosphatidylinositol 3'-kinase (PI3 Kinase) once it has been phosphorylated, allowing downstream signalling to AKT (Reedijk et al., 1992). In my experiments, tyrosine 723 is phosphorylated in the presence of tandutinib and dasatinib, but downstream phosphorylation of AKT is inhibited along with FMS

kinase activity (Chapter 4, figure 4.3). It would be useful to know whether p85 still binds to FMS when these inhibitors are bound.

At present it is not clear what the physiological relevance of these findings are and they need to be tested further. These results do show however that the phosphorylation state of the receptor is not necessarily an appropriate marker of receptor activity in this type of inhibitor assay.

5.3.2.2 FMS downregulation

I have also shown preliminary results that various FMS inhibitors, including imatinib can inhibit M-CSF inducible FMS degradation. Imatinib is thought to bind to only the inactive form of the FMS and KIT receptors and as a result, mutant receptors which are permanently in an active conformation confer resistance to imatinib (Mol et al., 2004a; Mol et al., 2004b). It is therefore expected that imatinib would inhibit downregulation of the wild type receptor as an active conformation is thought to be required to trigger accelerated, M-CSF induced downregulation of FMS (Uden et al., 1999). The FMSE633H mutant receptor is kinase inactive and downregulates in response to M-CSF and this is evidence the accelerated degradation is due to a change in structure of FMS upon M-CSF stimulation and not due to FMS kinase activity. It therefore seems possible that there could be a kinase inhibitor that binds to the active form of the receptor which would not inhibit M-CSF induced downregulation. Our structural model of dasatinib binding to FMS predicts that dasatinib may bind to the active conformation of FMS (Chapter 4, figure 4.4). This is supported by dasatinib inhibition of FMSD802V and FMSD802VY708F (Chapter 4, figure 4.1 and 4.2) as these particular mutant are thought to force the kinase domain into a permanently

activated conformation (Mol et al., 2004a; Mol et al., 2004b). It was therefore surprising that dasatinib inhibited M-CSF stimulated FMS downregulation (figure 5.7B) and that it also stabilised both FMS D802VY708F (figure 5.7D) and FMS D802V (figure 5.7E,F). FMS T633I confers resistance to dasatinib, and as expected dasatinib does not stabilise this receptor confirming that stabilisation of the wild type receptor is due to a direct effect of the inhibitor on the receptor (figure 5.7.G). A second FMS and FMSD802V kinase inhibitor, SU6656 (figure 5.8A) confirms these results because it stabilised M-CSF induced degradation of the wild type receptor (figure 5.8B) and also stabilised FMSD802V and FMSD802VY708F (figure 5.8 C,D). These results may indicate that that the kinase inhibitors themselves inhibit a structural change which is required for downregulation, such as dimerization.

The main evidence available that FMS kinase activity is not required for downregulation are the various kinase inactive FMS mutant receptors, including FMSE633H which still downregulate in response to M-CSF (Uden et al., 1999). I confirmed that FMSE633H internalises in response to M-CSF (figure 5.9A-H) in a similar, methylamine independent way to the wild type receptor (figure 5.10-5.12) and also confirmed that FMSK616A does not downregulate in this assay as reported previously (5.9E-F) (Carlberg et al., 1991). I have also shown that both the wild type receptor and FMSE633H are both constitutively downregulated by pathways which are sensitive to the lysosome inhibitor methylamine (figure 5.10).

Interestingly, both imatinib and dasatinib inhibit the degradation of FMSE633H in response to M-CSF (figure 5.14B,C). This indicates that imatinib and dasatinib inhibit the M-CSF inducible downregulation of the wild type receptor and the

FMSE633H receptor by inducing or inhibiting a change in receptor structure, and is further evidence that kinase activity is not required for FMS downregulation.

5.3.3 Conclusion

FMS kinase inhibitors are useful research tools to study the general mechanisms of FMS activity and structure and here I have used imatinib and dasatinib to further establish that kinase activity and ubiquitination are not necessary for downregulation of this receptor and that downregualtion is likely to be controlled by changes in receptor structure. I have also shown that other kinases are able to phosphorylate FMS on two residues in the kinase insert region, although the physiological relevance of this finding is unclear. This needs to be developed further but could lead to the identification of secondary drug targets for signalling by the PDGF receptor family.

CHAPTER 6. GENERAL DISCUSSION

The FMS receptor is a member of the PDGF receptor family of receptor tyrosine kinases and is mainly expressed on cells of the monocyte lineage where it is important for differentiation, activation and survival of macrophages, osteoclasts and microglia (Cecchini et al., 1994; Michaelson et al., 1996; Wiktor-Jedrzejczak et al., 1982; Wegiel et al., 1998). FMS is therefore a potential target for treatment of a wide variety of diseases in which these cell types are implicated, including inflammatory diseases, tumour metastasis and osteolytic bone cancer (Dewar et al., 2006; Murray et al., 2003; Ando et al., 2006; Lin et al., 2002; Nowicki et al., 1996; Ohno et al., 2008). Before this project began, there were no available small molecule inhibitors of the FMS receptor and my initial aim was to identify some such inhibitors and investigate their use both as potential therapeutic agents and as tools to study FMS receptor activity *in vitro*.

6.1 FMS receptor kinase inhibitors

There has recently been much success in developing small molecule inhibitors which specifically target tyrosine kinases. Many of these inhibitors have FDA approval and are routinely used to treat their intended malignancy (see introduction, table 1.2). These inhibitors are however not always totally specific for the kinase which they were initially designed to inhibit. Because many of these kinase inhibitors are tolerated extremely well by patients, this strongly indicates that these inhibitors may be useful in treating diseases where other kinases are implicated if these secondary targets are identified and are targeted at therapeutically relevant concentrations.

I therefore screened various clinically relevant kinase inhibitors which would be expected to also target the FMS receptor and identified four inhibitors which target FMS at clinically relevant concentrations, these are imatinib, nilotinib, tandutinib and dasatinib (Brownlow et al., 2008a; Brownlow et al., 2008b; Taylor et al., 2006).

Osteoclasts are causally implicated in osteolytic bone cancer, osteoarthritis and osteoporosis. I therefore investigated whether the inhibitors which I had identified had the potential to inhibit osteoclastogenesis. I have shown that in fact all of these inhibitors prevent osteoclast formation from normal mobilised peripheral blood at similar concentrations to those at which they target the FMS receptor. This is with the exception of nilotinib which was a much more potent inhibitor of osteoclastogenesis than of FMS indicating that nilotinib targets another kinase at low concentrations which is important for osteoclastogenesis (see table 6.1 for summary of results). Since the start of this study, bone thickening has been reported in patients who have been taking relatively high doses of imatinib for long periods, supporting the potential of imatinib to inhibit osteoclast formation *in vivo* and the potential of FMS inhibitors to target osteoclasts (Berman et al., 2006; Joensuu and Reichardt, 2006; Fitter et al., 2007).

Table 6.1. Summary of kinase inhibitor IC50 values.

Inhibitor	IC50 FMS	Plasma levels	IC50 Macrophage colony formation	IC50 Osteoclastogenesis	IC50 tumour associated macrophages	IC50 spontaneous, tumour associated Osteoclasts
Imatinib	600 nM	1.5 -5 μM (Druker et al., 2001).	500 nM	650 nM	600 nM	< 1000 nM
Nilotinib	250 nM	1.7 – 3.6 μM (Weisberg et al, 2006)	500 nM	50 nM	Not tested	Not tested
Tandutinib	400 nM	2 μM (DeAngelo et al., 2006)	500 nM	400 nM	Not tested	Not tested
Dasatinib	0.5 nM	7.4 nM (unpublished, figure 3.11)	0.1-1 nM	0.1-.0.5 nM	0.1-0.5 nM	1 nM

I have also shown that dasatinib, but not imatinib inhibits spontaneous osteoclastogenesis from ovarian cancer ascites samples (table 4.2). These osteoclasts were slightly different to normal osteoclasts as they were produced without the requirement for recombinant M-CSF and RANKL. The reasons for this are unclear but it is presumably due to the presence of another growth factor or cytokine which they have been exposed to *in vivo* or that is being released from another cell type in this culture system. This finding may not be physiologically relevant as metastasis to the bone is reported extremely rarely in ovarian cancer patients (Kingston et al., 2001). It would be interesting to further develop these results and also to apply them to an *in vitro* model of bone metastasis from breast cancer, which is much more common than bone metastasis from ovarian cancer (Akhtari et al., 2007; Roodman, 2001). Figure 4.8 shows preliminary results that osteoclasts developed from breast cancer pleural effusion fluid in the absence of recombinant M-CSF and RANKL and that these osteoclasts were sensitive to both imatinib and dasatinib, but I was unable to repeat these results due to a lack of material available during the course of my project.

Macrophages are thought to be important inflammatory cells of the tumour microenvironment and are heavily implicated in the progression of various solid tumours including ovarian cancer and breast cancer (Condeelis and Pollard, 2006; Pollard, 2004; Allavena et al., 2007; Luo et al., 2006). These tumour associated macrophages are thought to assume a wound healing, M2 macrophage type phenotype in the tumour microenvironment in response to the many immunosuppressive cytokines and growth factors. (Hagemann et al., 2006; Mantovani et al., 2002). Importantly, M-CSF is often found at high levels in this environment in both ovarian

cancer and breast cancer, and is a strong prognostic indicator of poor outcome (Kacinski, 1997; Kacinski, 1995). M-CSF recruits large numbers of macrophages and these cells are thought to support tumour growth by stimulating angiogenesis and also to support tumour metastasis (Allavena et al., 2007). I therefore investigated whether these inhibitors have the potential to target macrophages. I have shown that all four of the inhibitors reported here target murine macrophage colony formation and that both imatinib and dasatinib inhibit tumour associated macrophage production from ovarian cancer ascites fluid *in vitro* (figures 3.5, 4.6, 5.3)

The most interesting of these inhibitors was dasatinib as it is such a potent inhibitor of FMS with an IC50 value of 0.5 nM (figure 4.1). The plasma levels of dasatinib achieved in patients has not yet been reported so I designed an *in vitro* assay to estimate the plasma levels achieved in patients who are taking dasatinib to treat CML (figure 4.11). The average patient plasma level for dasatinib was estimated to be 7.4 nM (as calculated from figures 4.10 and 4.11). The therapeutic potential of each of these four inhibitors can now be compared based on the difference between the plasma levels and the concentration of inhibitor required to inhibit FMS, assuming that the bigger the difference between the two values, the more likely the inhibitor is to effectively inhibit FMS *in vivo*.

Table 6.1 summarises the IC50 values for all of the assays discussed here. It seems that dasatinib is the most potent inhibitor of FMS as the plasma concentration is approximately 14 times greater than the concentrations required to inhibit FMS, compared to around 5 times for imatinib, 11 times for nilotinib and 5 times for tandutinib. Nilotinib may however be a more potent inhibitor of osteoclastogenesis as

normal osteoclasts were extremely sensitive to nilotinib in these assays with an IC50 value of 50 nM which is around 56 times more potent than the plasma levels of 1.7-3.6 which are reported (table 6.1) (Weisberg et al., 2006).

The macrophages produced in culture are highly dependent on M-CSF stimulation as no macrophages were produced in the absence of exogenous M-CSF. This is a slightly artificial system because, although high concentrations of M-CSF have been consistently reported in the tumour microenvironment of breast cancer and ovarian cancer, there may also be other cytokines and growth factors which could support macrophage survival or differentiation in the absence of FMS signalling (Burke et al., 1996; Kacinski, 1997; Smith et al., 1995; Kacinski, 1995). These other cytokines may also polarise the macrophages to display a different phenotype in the absence of FMS signalling. It is therefore unclear what the outcome of inhibiting the FMS receptor would be *in vivo*.

This problem could be addressed by developing a more realistic model of the tumour microenvironment. One way to do this is to use an animal model of ovarian cancer to further test these inhibitors. This is an expensive and time consuming route and it is currently unclear whether any of the mouse models of ovarian cancer which are currently available fully represent a host-tumour interaction which can be translated to humans (Foulkes, 2002). Another solution would be to attempt to develop an *in vitro* culture system that contains a more comparable mixture of growth factors and cytokines to the normal tumour microenvironment and to test these inhibitors on the macrophages produced in this system. This could be achieved by either creating a co-culture system with primary tumour cells and macrophages or more simply, and

perhaps more realistically, to supplement the growth media of macrophages in culture with ascites solution periodically.

6.2 Inhibitors as research tools

6.2.1. Phosphorylation of the kinase insert region

The identification of new FMS inhibitors has proven useful in investigating some aspects of FMS activity *in vitro*. It has also yielded some surprising results, in particular the observation that phosphorylation of tyrosines 723 and 708 of the kinase insert region are differently affected by each of the four kinase inhibitors that I investigated at concentrations at which the receptor kinase activity is inhibited. This data is summarised in table 6.2.

Table 6.2. Summary of phosphorylation site data (from figures 5.4-5.6).

Inhibitor	Inhibition of tyrosine 723	Inhibition of tyrosine 708
Nilotinib	Yes	No
Tandutinib	No	Yes
Imatinib	Less sensitive	Not tested
Dasatinib	Less sensitive	No

This indicates that at least one other kinase is able to phosphorylate the FMS receptor when bound to the kinase inhibitors (Table 6.2). What is more surprising is that different inhibitors (ie nilotinib and tandutinib) have different effects on the phosphorylation of the kinase insert region.

One possible hypothesis which may explain these surprising results is that these kinase inhibitors could be stabilizing the kinase in several semi-activated structural states. It is already known that the crystal structure of imatinib bound to the KIT receptor does not perfectly align with either the crystal structure of the active or inactive form of the receptor (figure 5.15) and that the structure of KIT bound to imatinib is intermediate between the active and inactive receptor (Mol et al., 2004; figure 5.16). This could mean that the anomalous phosphorylation of the FMS receptor seen in the presence of inhibitors happens because the inhibitors stabilise a partially active structure of FMS in which tyrosines 708 or 723 are accessible for phosphorylation by another kinase(s).

It can be seen from the crystal structure of the KIT receptor that the juxtamembrane region of KIT is phosphorylated *in trans* by another receptor monomer as two of the tyrosines (tyrosines 568 and 570) in this region were phosphorylated in the crystal structure and one of these residues (tyrosine 568) was caught in the act of becoming phosphorylated (Mol et al., 2003). In this structure none of the other tyrosines were phosphorylated, which implies that phosphorylation of the juxtamembrane region may precede the phosphorylation of the other tyrosines including those in the activation loop and the kinase insert region. This is supported by various experiments carried out by the Stanley group who show that the phosphorylation of different tyrosines of the FMS receptor occurs sequentially rather than simultaneously (Li and Stanley, 1991; Yeung and Stanley, 2003). It is therefore possible that the trans phosphorylation of the juxtamembrane region stabilises the active structure and this stabilisation is required for access to the kinase insert region by another kinase. These kinase inhibitors may therefore stabilise the kinase in various semi-activated states

allowing differential access to the kinase insert region depending in the structure of the inhibitor. This could be tested by looking for association of binding proteins with the kinase insert region in the presence of these inhibitors as it would be predicted that these sites should be exposed for binding only in the presence of these kinase inhibitors or M-CSF.

Figure 6.1

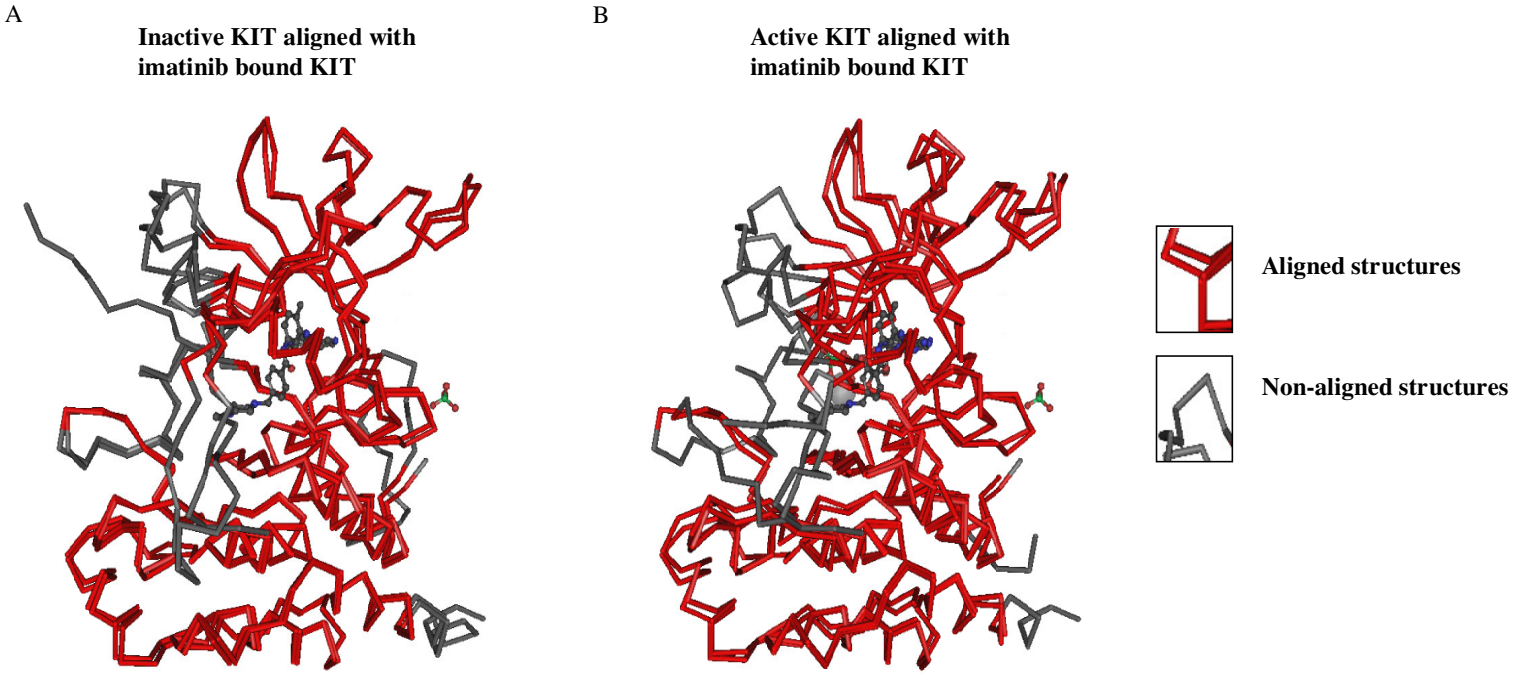


Figure 6.1 The crystal structure of imatinib bound to the KIT receptor does not fully align with either the receptor in its active or inactive form. The following receptor structures were aligned from NCBI using the CN3D program: inactive KIT receptor (1t45) and imatinib bound KIT receptor (1t46) (A), active KIT receptor and imatinib bound KIT receptor 1t46 (B). Red colouring shows closely aligned structures and grey colouring shows non-aligned structures.

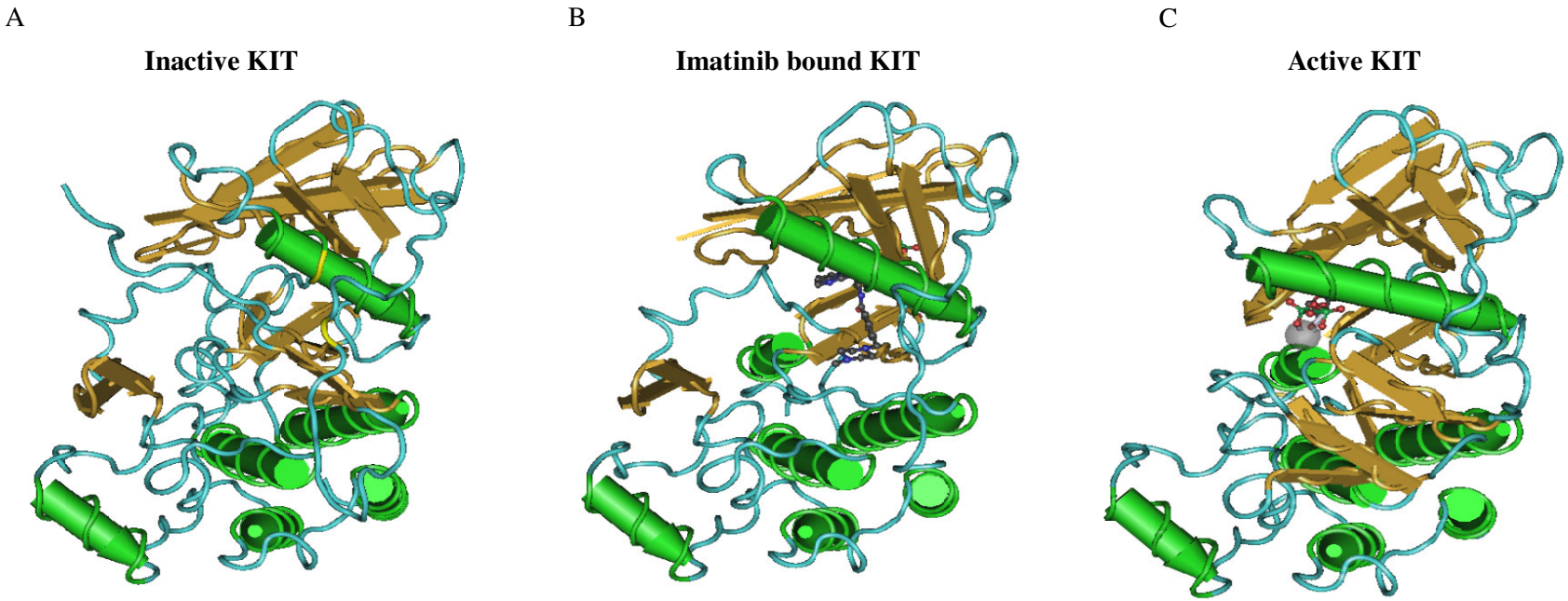


Figure 6.2 The structure of imatinib bound to the KIT receptor may be an intermediate step between the active and inactive receptor states. The following receptor structures were retrieved from NCBI using the CN3D program: inactive KTI receptor 1t45 (A), imatinib bound to the KIT receptor 1t46 (B) and the active KIT receptor 1PKG(C) and orientated to equivalent positions by aligning the 3 structures before recording their images individually.

6.2.2. FMS receptor downregulation

During the course of this project I have created an immunofluorescence assay to study downregulation of the wild type FMS receptor and various mutant receptors and I have used this assay to confirm the previous finding that the FMSE633H kinase inactive mutant receptor downregulates in response to M-CSF (figure 5.9). It would now be useful to characterize carefully the endocytosis pathways of the wild type receptor and the kinase inactive receptors in order to fully establish whether the two receptors are being trafficked through similar pathways. I have also shown that various kinase inhibitors of the FMS receptor inhibit the downregulation of both the wild type receptor and the kinase inactive FMS E633H receptor (figure 5.7, 5.8, 5.13). This was an interesting finding as it gave further evidence that it is a structural change in the receptor which is required for downregulation rather than kinase activation. This could be further characterised by using dimerisation assays to check whether the kinase inhibitors used here prevent dimerisation. It would also be interesting to further investigate whether there are any kinase inhibitors which inhibit kinase activity but allow downregulation of the receptor in response to M-CSF and to elucidate whether our downregulation results can be applied to the whole of the PDGF receptor family.

6.3. Conclusions

I have established that imatinib, tandutinib, nilotinib and dasatinib are inhibitors of the FMS receptor as tested in various biochemical and cell based assays. All of these inhibitors prevented differentiation of human macrophages and osteoclasts *in vitro* and dasatinib and imatinib both inhibited production of tumour associated

macrophages from ovarian cancer ascites. Dasatinib is the most potent of these inhibitors and is present in patient serum at biologically active concentrations which would be expected to target FMS *in vivo*. Together, these kinase inhibitors provide a strong platform for the development of agents which specifically target the FMS receptor and could be an important progression in the targeting of the tumour microenvironment.

I have also provided evidence that the FMS receptor is phosphorylated on the kinase insert region by at least one other kinase in the absence of receptor kinase activity, this project could be developed further to identify the kinase responsible. I have also confirmed previous findings that the kinase inactive receptor FMSE633H downregulates in response to M-CSF and have used the kinase inhibitors identified in this project to support a structural explanation for receptor downregulation.

CHAPTER 7. REFERENCES

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