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EFFECTS OF ISOFORM-SELECTIVE PHOSPHATIDYLINOSITOL-3 KINASE INHIBITORS ON OSTEOCLASTS

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ABSTRACT

Phosphatidylinositol 3-kinases (PI3K) are key intracellular signaling molecules. Our objective was to determine effects of isoform-selective PI3K inhibitors on osteoclasts. The following inhibitors were investigated (targets in parentheses): wortmannin and LY294002 (pan-p110), PIK75 (α), GDC0941 (α, δ), TGX221 (β), AS252424 (γ), IC87114 (δ) and CAL-120 (δ). Wortmannin, GDC0941, IC87114 and CAL-120 induced dramatic retraction of rat osteoclasts. In contrast, there was no significant retraction in response to vehicle, PIK75, TGX221 or AS252424. Moreover, wortmannin and CAL-120, but not PIK75 or TGX221, disrupted filamentous F-actin belts; and CAL-120 inhibited the formation of sealing zones. In contrast to their selective actions on cytoskeletal organization, PIK75, TGX221 and CAL-120 blocked RANKL-stimulated osteoclast survival. Thus, PI3Kδ appears to play a specific role in regulating osteoclast cytoskeleton. In contrast, multiple PI3K isoforms control osteoclast survival. The PI3Kδ isoform, which has more limited tissue distribution than PI3Kα and PI3Kβ, is an attractive target for anti-resorptive therapeutics.

Keywords — actin ring, antiresorptive therapeutics, apoptosis, cell survival, cytoskeleton, F-actin belt, filamentous actin, osteoclasts, osteoporosis, phosphatidylinositol-3 kinase, PI3K, podosome, RANKL, retraction, sealing zone.

CO-AUTHORSHIP

Chapter two, entitled "Effects of isoform-selective phosphatidylinositol-3 kinase inhibitors on osteoclasts: Actions on cytoskeletal organization and survival", was written by R.P.P. Shugg with suggestions from Drs. S.J. Dixon, F. Jirik, B. Lanutti and S.M. Sims. Dr. B. Lannutti and A. Kashishian (From Gilead Sciences) performed the *in vitro* and cell-based kinase assays shown in Table 2.1. Ashley Thomson helped perform the phalloidin staining and analysis shown in Fig. 2.5. Dr. A. Pereverzev helped perform the rabbit osteoclast EGFP-actin transduction. Dr. N. Tanabe helped performed the survival assay and analysis shown in Fig. 2.9. All other studies were performed by R.P.P. Shugg. The *in vitro* and cell-based kinase assays were performed at Calistoga Pharmaceuticals (Seattle, WA). All other experiments were carried out in the laboratories of Drs. S.J. Dixon and S.M. Sims.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
Arf	ADP-ribosylation factor
Arp 2/3	actin-related protein 2/3
ATP	adenosine 5'-triphosphate
CAN	Canadian Arthritis Network
CIHR	Canadian Institutes of Health Research
Cdc42	cell division cycle 42
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma extra large
BMP	bone morphogenetic protein
Btk	Bruton's tyrosine kinase
c-cbl	Casitas B-lineage lymphoma
DAPI	4',6-diamidino-2-phenylindole
DAP12	DNAX-activating protein of 12 kDa
DC-STAMP	dendritic cell-specific transmembrane protein
DMEM	Dulbecco's modified Eagles medium
DNAPK	DNA-dependent serine/threonine protein kinase
EC_{50}	half maximal effective concentration
ERK1/2	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
F-actin	filamentous actin
fMLP	formyl-methionyl-leucyl-phenylalanine
FcεRI	high-affinity for Fc region of immunoglobulin E
GAP	GTPase-activating proteins
GEF	guanine nucleotide exchange factor
G-actin	globular actin
GM-CSF	granulocyte macrophage colony stimulating factor
GMP	granulocte/macrophage progenitor
GPCR	G protein-coupled receptor

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	half maximal inhibitory concentration
IL-8	interleukin-8
IL-11	interleukin-11
ITAM	immunoreceptor tyrosine-based activation motifs
JNK	Jun N-terminal kinase
LPA	lysophosphatidic acid
M199	medium 199
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MCP-1	monocyte chemotactic protein-1
MPP	multipotent progenitor
MEF	murine embryonic fibroblast
MTT	methylthiazoltetrazolium
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor-κB
OSCAR	osteoclast-associated immunoglobulin-like receptor
OPG	osteoprotegerin
PBMC	peripheral blood mononuclear cells
PDK1	phosphoinositide-dependent kinase-1
PDGF	platelet-derived growth factor
PAF	platelet activating factor
PBS	phosphate-buffered saline
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PI3K	phosphatidylinositol-3 kinase
PtdIns	phosphatidylinositols
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PH	pleckstrin homology
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related protein

RANK	receptor activator of nuclear factor-κB
RANKL	receptor activator of nuclear factor-κB ligand
RGD	arginine, glycine, aspartic
SHIP	Src homology 2-containing inositol-5-phosphatase
S1P	sphingosine-1-phosphate
SD	standard deviation
SEM	standard error of the mean
TGF-β	transforming growth factor-β
TREM2	triggering receptor expressed on myeloid cells-2
TRAF6	tumor necrosis factor receptor-associated factor 6
TNF	tumor necrosis factor
TRAP	tartrate-resistant alkaline phosphatase
VEGF	vascular endothelial growth factor
WASp	Wiskott-Aldrich Syndrome protein
WIP	WASp interacting protein

CHAPTER ONE

Introduction

1.1 Chapter Summary

Osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells) play essential roles in skeletal development, skeletal remodeling and mineral homeostasis. The actions of these two cell types are tightly regulated. Perturbations in the balance between osteoblasts and osteoclasts result in disease states, such as tumor osteolysis (bone loss due to cancer), osteoporosis (excess bone resorption) or periodontitis (chronic inflammation associated with loss of alveolar bone). Class I phosphatidylinositol-3 kinases (PI3K) have critical roles in a variety of cellular processes such as metabolism, differentiation, survival and migration. Recently developed isoform-selective PI3K inhibitors show considerable promise for the treatment of inflammatory disease and cancer, and are making their way into the early phases of clinical trials (Okkenhaug *et al.*, 2002; Durand *et al.*, 2009; Liu *et al.*, 2009; Cleary & Shapiro, 2010; Lannutti *et al.*, 2011). This chapter summarizes background information on osteoclastogenesis and bone remodeling. Furthermore, the findings from previous studies on PI3K isoform expression, signaling pathways and functional roles of will be discussed.

1.2 Bone Physiology

1.2.1 Composition, Structure and Function of Bone

Bone is a specialized connective tissue that functions to protect vital organs, to serve as a reservoir for ions such as calcium and phosphate, and to serve as a site for muscle attachment to support locomotion (Marks & Popoff, 1988). Mechanical stimuli along with local and systemic factors, such as parathyroid hormone (PTH) and calcitonin, act in concert to maintain bone homeostasis (Harada & Rodan, 2003). Bone is one of the only tissues in the body that is composed of both organic and inorganic phases. The inorganic phase of bone is predominantly hydroxyl-apatite [$Ca_{10}(PO_4)_6(OH)_2$], however bone mineral also contains carbonate, fluoride and magnesium referred to, collectively, as crystalline carbonate-substituted apatite (Rey *et al.*, 1991). The inorganic phase of bone provides load bearing strength and rigidity to the bone composite. The organic phase is composed primarily of type I collagen (~90%), and trace amounts of type III and V collagen and other proteins, which provide elasticity and flexibility to bone (Ashhurst *et al.*, 1990). Fiber organization allows the highest density of collagen per unit volume of tissue in preferentially orientated structures called lamellae. The lamellae can be parallel or deposited concentrically surrounding a channel containing blood vessels called a Haversian system. When there is no preferential organization of collagen fibers, this type of bone is called woven bone and occurs during development and fracture healing.

The non-collagenous proteins of the bone extracellular matrix belong to the proteoglycan and glycoprotein classes. The proteoglycans include versican, decorin, biglycan, hyaluronan; whereas, the glycoproteins include osteonectin, osteopontin, bone sialoprotein, and osteocalcin (Robey, 1996). These proteins are highly anionic that have

a high ion-binding capacity and play a role in the fixation of hydroxyl-apatite crystals to collagen (Rees *et al.*, 2001).

The external part of bones is formed by a thick and dense layer of calcified tissue, called the cortex, which encloses the medullary cavity where hematopoietic bone marrow is housed. Toward the ends of long bones, the cortex becomes progressively thinner, and the internal space is filled with a calcified network of trabeculae, called trabecular bone, where the bone marrow within the medullary cavity is continuous. The bone surface at the ends of bones which take part in a joint are covered in a layer of articular cartilage, which acts to separate, lubricate and absorb shock between bones.

Anatomically, there are two types of bone: flat bones and long bones. Flat bones are found in the skull, scapula, and mandible; whereas, the tibia, femur, radius, and humerus are examples of long bones. These two types arise by intramembranous or endochrondral, bone formation, respectively.

During intramembranous ossification, mesenchymal cells proliferate and concentrate within a highly vascularized area of embryonic connective tissue and differentiate into preosteoblasts and osteoblasts (Hall *et al.*, 1995). These cells synthesize the osteoid, a non-mineralized bone matrix composed primarily of type I collagen, and begin to calcify the osteoid in a delayed and irregularly distributed fashion, forming woven bone. During this process, osteoblasts become entrapped within the matrix and are then termed osteocytes (Franz-Odendaal *et al.*, 2006). Later this bone is remodeled and replaced by mature lamellar bone.

During the development of long bones, the process of endochondral ossification is initiated by the differentiation of mesenchymal stem cells into prechondroblasts and

chondroblasts, which secrete a collagenous matrix to form cartilage (Mackie *et al.*, 2008). The chondroblasts that become entrapped within the matrix are then termed chondrocytes and continue to proliferate. These chondrocytes are arranged into four morphologically distinct zones adjacent to one another along the growing bone: 1) resting, 2) flattened proliferating chondrocytes, 3) prehypertrophic chondrocytes and 4) hypertrophic chondrocytes (Mackie *et al.*, 2008). Hypertrophic chondrocytes secrete alkaline phosphatase, which promotes mineralization of the extracellular matrix (Ismail *et al.*, 2004), and angiogenic factors such as vascular endothelial growth factor (VEGF) to promote vascular invasion (Miyamoto & Suda, 2003). Osteoblasts form bone on the remnants of the calcified cartilage. In parallel, hypertrophic chondrocytes begin to undergo apoptosis, and the newly established vessels provide access for osteoclasts, which resorb the mineralized cartilage, creating a medullary cavity for the bone marrow (Miyamoto & Suda, 2003).

All bones within the body are continuously being remodeled by osteoblasts and osteoclasts throughout our lives. It has been proposed that the entire skeleton is replaced every 10 years. The process of resorption is relatively faster than the process of bone formation, and therefore these processes must be kept in balance or skeletal diseases will result (Harada & Rodan, 2003). Many signaling networks have been identified that serve to ensure balanced activity, however some are not well defined.

1.2.2 Regulation of bone formation

There is an established coupling between resorption and formation of bone. Although, the signaling pathway for recruitment of mesenchymal cells and osteoblast precursors is unclear, it may be due to factors released during resorption that lead to

proliferation and migration of osteoblast precursors to the bone surface (Manolagas, 2000). It is known that mesenchymal cells concentrate at areas of bone resorption and differentiate into cells of the osteoblast lineage under the control of cytokines and growth factors such as bone morphogenetic proteins (BMPs) and Wnt ligands (Manolagas, 2000). Osteoblasts at these sites lay down osteoid that subsequently becomes mineralized. Following, the majority of osteoblasts undergo apoptosis, however some remain on the surface, called lining cells, and some further differentiate into osteocytes (Manolagas, 2000).

1.2.3 Osteoclastogenesis

Osteoclasts are derived from hematopoietic stem cells in the bone marrow, which further differentiate into multipotent progenitors (Yin & Li, 2006). Multipotent progenitors are capable of undergoing further differentiation to produce cells of two different lineages - the common lymphoid progenitor (CLP) or common myeloid progenitor (CMP). CLP cells produce T cells, B cells and natural killer cells; whereas CMP cells can differentiate into two further lineages: either the erythrocyte/megakaryocyte lineage or into the granulocyte/macrophage progenitor (GMP) (Yin & Li, 2006). The transcription factor PU.1 is the earliest known marker of osteoclast differentiation and is essential for the commitment of these initial GMP cells into the monocyte/macrophage cells, which are precursors to osteoclasts (Tondravi *et al.*, 1997). Deletion of PU.1 results in severe osteopetrosis in mice due to the absence macrophages and osteoclasts (Tondravi *et al.*, 1997). PU.1 is important because it regulates the expression of the macrophage colony stimulating factor (M-CSF) receptor, c-fms, on early osteoclast precursors and monocytes, which is critical for their survival.

Monocytic cells and osteoclast precursors exit the bone marrow and enter the circulation until they are recruited to the bone surface. Under normal conditions, or at sites of damage and tissue injury, stromal and immune cells release chemoattractant agents that recruit osteoclast precursors to the bone surface. M-CSF, receptor activator of nuclear factor κB ligand (RANKL), sphingosine-1 phosphate (S1P) and VEGF are all known chemoattractants of osteoclast precursor cells (Henriksen *et al.*, 2003; Ishii *et al.*, 2009). The process of osteoclast differentiation is summarized Fig. 1.1.

Osteoclast precursor proliferation and resistance to apoptosis are induced by M-CSF binding to its receptor c-fms (Arai *et al.*, 1999). c-fms is a tyrosine kinase that autophosphorylates itself upon ligand binding, which activates ERK1/2 and phosphatidylinositol-3 kinase (PI3K) pathways that drive the transcription of c-Fos and RANK to promote osteoclastogenesis, and Bcl-2 and Bcl-xl to promote survival (Teitelbaum, 2007).

The primary driver of osteoclastogenesis is receptor activator of nuclear factor κB ligand (RANKL) (Roodman, 1996; Suda *et al.*, 2001). RANKL is a membrane-bound or soluble protein produced by bone marrow stromal cells, osteoblasts and T-cells. As a response to RANKL binding to its receptor, activator of nuclear factor κB (RANK), post-mitotic, committed osteoclast precursors fuse to yield large multinucleated cells. Bone resorption is completely abolished in *RANK*$^{-/-}$ mice due to lack of osteoclasts (Lacey *et al.*, 1998). RANK is related to the tumor necrosis factor (TNF) receptor and has a long cytoplasmic tail that complexes with signaling molecules, such as TNF receptor-associated factor 6 (TRAF6) and activates NF-κB, Jun N-terminal kinase (JNK), mitogen-activated protein kinases (MAPK) p38 and Ca^{2+}/calmodulin pathways that

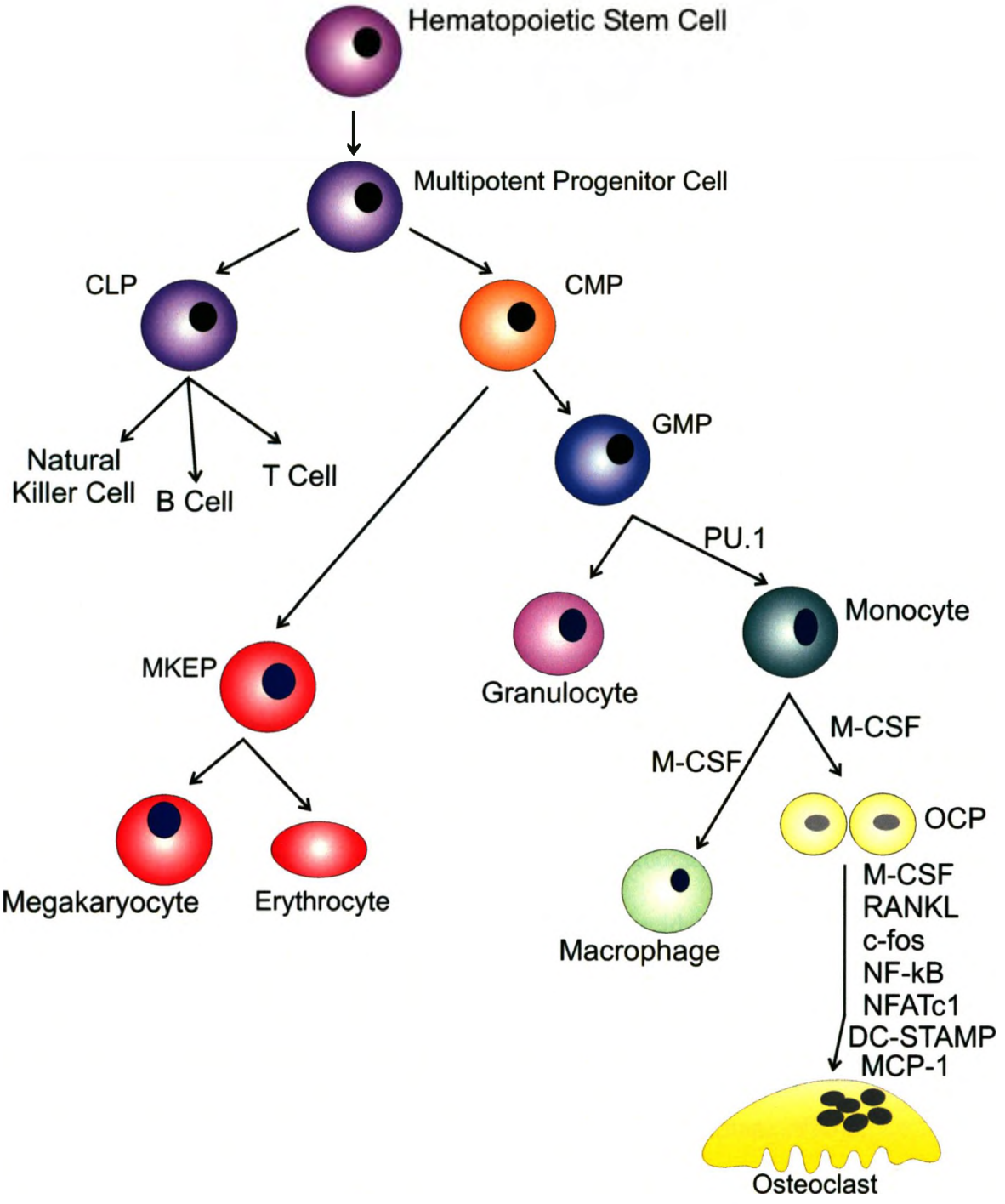

Figure 1.1 Schematic diagram illustrating osteoclastogenesis from hematopoietic stem cells. Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; M-CSF, macrophage colony stimulating factor; OCP, osteoclast precursor; RANKL, receptor activator of nuclear factor κ B ligand; NF-κB, nuclear factor kappa B; NFAT, nuclear factor of activated T cells; DC-STAMP, dendritic cell-specific transmembrane protein; MCP-1, monocyte chemotactic protein 1; MKEP, megakaryocyte erythroid progenitor.

leads to activation of the transcription factor NFATc1 (Asagiri & Takayanagi, 2007). NFATc1 is the primary driver of expression of many genes required for osteoclast function and regulation, including cathepsin K, β3 integrin, TRAF6 and the calcitonin receptor (Ikeda *et al.*, 2004). The transcription factor c-Fos drives transcription of NFATc1. Notably, *c-Fos*$^{-/-}$ mice lack osteoclasts, but not macrophages, and exhibit severe osteopetrosis (Grigoriadis *et al.*, 1994). NFATc1 deletion is embryonic lethal, however cultured embryonic stem cells from *NFATc1*$^{-/-}$ mice do not undergo differentiation into osteoclasts, indicating NFATc1 is required for osteoclastogenesis (Asagiri *et al.*, 2005).

The last and unique step of osteoclastogenesis is the fusion of osteoclast precursors to become large mature multinucleated osteoclasts. The seven transmembrane spanning receptor, dendritic cell-specific transmembrane protein (DC-STAMP) (Yagi *et al.*, 2006) and the monocyte chemotactic protein-1 (MCP-1) have been shown to promote the fusion of osteoclast precursors into multinucleated osteoclasts (Kim *et al.*, 2005). The exact process and ligand for DC-STAMP has yet to be elucidated.

Other factors that stimulate osteoclast formation include interleukin-11 (IL-11), parathyroid hormone-related protein (PTHrP) and prostaglandin E_2, which appear to act primarily by inducing RANKL expression by osteoblasts (Roodman, 1996).

1.2.4 Osteoclast Attachment

Osteoclast precursors are recruited to the bone surface from blood vessels or marrow where they attach and fuse together to form multinucleated cells that develop machinery to resorb bone. Osteoclasts attach to bone via integrins including collagen integrin receptors called α2β1 and αvβ1, and a vitronectin receptor called αvβ3 (Duong *et al.*, 2000). β3 knockout mice have dysfunctional osteoclasts and decreased bone

resorption suggesting that αvβ3 is the most essential integrin for osteoclast attachment (McHugh *et al.*, 2000). The αvβ3 integrins interact with proteins expressing a sequence of arginine, glycine and aspartic acid residues (RGD), such as vitronectin, fibronectin, bone sialoprotein and osteopontin, thereby activating a signaling cascade to prepare the osteoclast for bone resorption (Teitelbaum, 2007).

Osteoclasts can adhere to several substrates on which they form distinct filamentous actin (F-actin) containing structures, called podosomes. Podosomes are small punctate adhesion structures, each consisting of a core of F-actin and actin-associated proteins surrounded by integrins and integrin-associated proteins (Saltel *et al.*, 2008). Actin is one of the most abundant intracellular proteins that comprise the microfilament system for cell movement. Actin exists as an individual subunit monomer known as globular actin (G-actin) and as long filamentous chains of monomers, called F-actin. F-actin and G-actin are dynamically remodelled to allow cell movement and morphological changes. Osteoclasts exhibit two different actin cytoskeletal organizations according to the substrate they attach. *In vitro*, on non-mineralized substrates, such as glass or plastic, they form clusters of podosomes, or a band of podosomes at the periphery of the cell, called F-actin belts. On mineralized substrates, such as bone or calcium phosphate matrices, podosomal units condense, forming a ring in the interior of the cell, called F-actin rings or sealing zone (Saltel *et al.*, 2008). In osteoclasts, the formation of podosome clusters and rings is microtubule independent; whereas the podosome belts depend on stabilized acetylated microtubules (Destaing *et al.*, 2003). It has been observed that osteoclasts degrade bone only within the area defined by F-actin super structures (Badowski *et al.*, 2008) and that podosomes are essential for extracellular

matrix degradation and osteoclast migration (Mizutani *et al.*, 2002; Linder & Aepfelbacher, 2003; Calle *et al.*, 2006).

1.2.5 Bone Resorption

Once osteoclasts adhere to the bone, they form a sealing zone. The sealing zone has been shown to be associated with actin-related protein (Arp) 2/3 complex, Wiskott-Aldrich Syndrome protein (WASp) and WASp interacting protein (WIP), cortactin, formin, paxillin, c-src, Pyk2 (proline-rich tyrosine kinase 2), c-cbl, myosin II and colocalised αvβ3 integrins (Chabadel *et al.*, 2007; Saltel *et al.*, 2008). Osteoclasts unable to form this seal are unable to resorb bone (Duong & Rodan, 1998; Mulari *et al.*, 2003a). The sealing zone gives rise to an enclosed microenvironment between the osteoclast and bone surface called the resorption lacuna. The plasma membrane within the compartment is extensively folded, creating a characteristic feature of the osteoclast, termed the ruffled border. Within it, the ruffled border of the osteoclast increases surface area for the transport of products for the degradation of inorganic and organic phases. Systematically, H^+ ions (generated by cytosolic carbonic anhydrase II) via vacuolar ATPases and passive movement of Cl^- ions via chloride channels are released into the resorption lacuna to dissolve the inorganic phase, thereby releasing calcium and phosphate (Zaidi *et al.*, 2003). Normal function of both the ClC7 chloride channels and vacuolar ATPases are absolutely critical for the breakdown of the mineral (Zaidi *et al.*, 2003). The pH within the resorption lacuna is approximately 4.5-5 (Teitelbaum & Ross, 2003). To degrade the organic phase (type I collagen), osteoclasts release metalloproteinases and lysosomal enzymes, such as cathepsin K, which require an acidic pH to be functional (Zaidi *et al.*, 2003). Mutation in the cathepsin K gene results in

dysfunctional osteoclasts and osteopetrosis (Zaidi *et al.*, 2003). Digested fragments of matrix proteins are transcytosed from apical to basolateral membranes which is proposed to occur at the center of the sealing zone (Salo *et al.*, 1997; Mulari *et al.*, 2003). The process of osteoclast resorption is summarized in Fig. 1.2. After a cycle of resorption, the osteoclast either undergoes apoptosis or moves to a new resorption site.

1.2.5 Regulation of Osteoclasts

Hormones, growth factors and cytokines regulate bone mass directly or indirectly, through their actions on osteoclasts or osteoblasts. These factors can influence differentiation of precursors, life span of mature cells, among many others effects. This section will review some of the important factors that affect osteoclasts and bone mass.

Estrogen – estrogen is a sex steroid produced primarily by a developing follicle in the ovaries and plays important role in female reproduction. Estrogen is carried in the blood by albumin, and easily passes through the cell membrane of target cells where it binds to a nuclear receptor (estrogen receptor α or β) to promote secondary sex characteristics (Mauvais-Jarvis, 2010; Heldring *et al.*, 2007). Estrogen and its analogs exert a protective effect on the skeleton, which is most apparent by the decrease in bone density in woman following menopause (Heldring *et al.*, 2007). Estrogen inhibits the production of RANKL, and increases the production of osteoprotegerin (OPG - a decoy receptor which prevents RANKL from binding RANK and stimulating osteoclasts) by osteoblasts, which express both receptors, α and β (Bord *et al.*, 2003). Also, estrogen also can bind directly to osteoclasts through estrogen receptor α to induce apoptosis (Nakamura *et al.*, 2007).

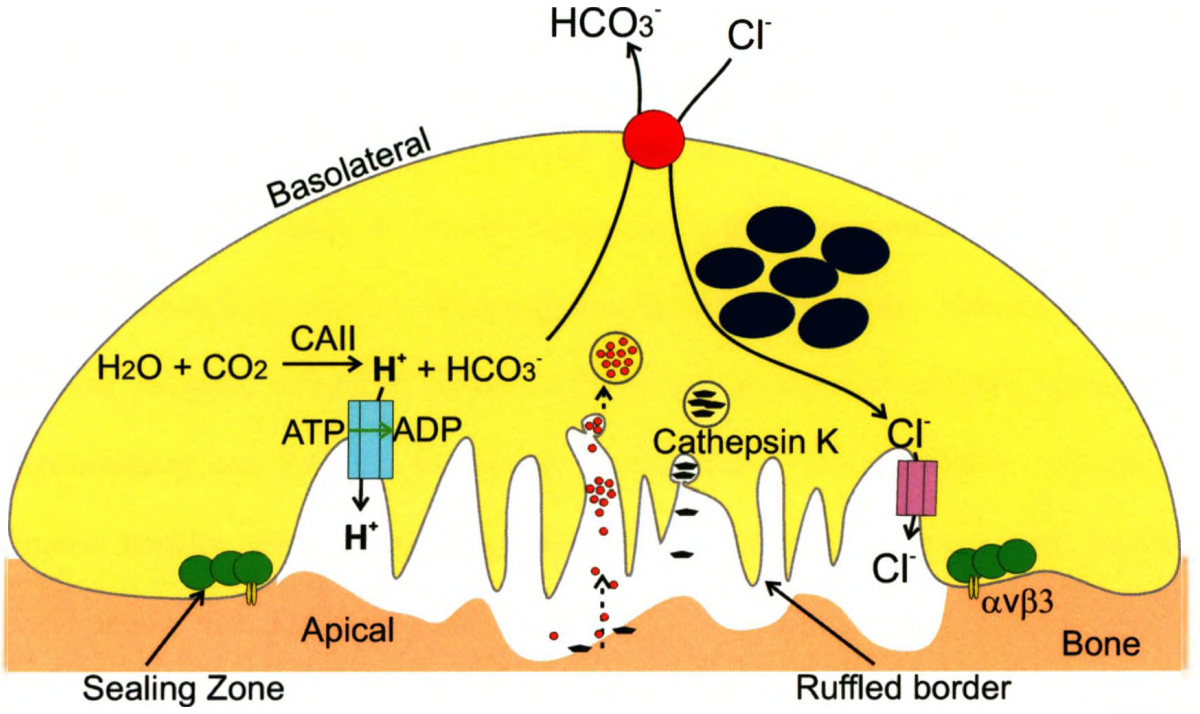

Figure 1.2 Schematic representation of osteoclast resorption on bone. Osteoclasts adhere to bone via αvβ3 integrins and form a sealing zone consisting of F-actin and other integrin binding proteins. Protons (H^+) are generated by the activity of carbonic anhydrase II (CAII) and are transported to the resorption lacuna through V-type H^+ ATPases residing in the ruffled border close to sealing zone. Electrical neutrality is maintained by passively exchanging chloride ions (Cl^-) for bicarbonate (HCO_3^-) at the basolateral membrane. Cl^- moving into the cell is transported into the resorption lacuna passively through the anion channel ClC7. Lysosomal enzymes, such as cathepsin K, are secreted into the resorption lacuna via vesicles to digest type I collagen and other organic tissue. Organic fragments are then removed by transcytosis.

Vitamin D – 1, 25 $(OH)_2$ vitamin D_3 synthesis requires ultraviolet light to convert precursor 7-dehydrocholesterol to previtamin D_3 in the epidermis (Bouillon *et al.*, 1995). Vitamin D_3 is then transported and converted in to its biologically active form $1,25(OH)_2D_3$ in the liver and kidney. The major role of vitamin D in the body is to regulate Ca^{2+} and PO_4^{3-} metabolism through bone, the kidney and intestine. Vitamin D maintains blood Ca^{2+} concentrations by absorption in the gastrointestinal tract, release of calcium stored in bone, and by enhancing reuptake in renal tubules. Vitamin D binds to its nuclear receptor, vitamin D receptor (VDR) and up regulates alkaline phosphatase (mineralization) and RANKL expression in osteoblasts (Holick, 2006). Vitamin D promotes healthy mineralization, growth and remodeling of bone, and prevents hypocalcaemia and osteoporosis (Holick, 2007). Vitamin D deficiency can lead to rickets in children and osteomalacia in adults (Holick, 2007).

Parathyroid hormone (PTH) – PTH is secreted by the parathyroid gland in response to low blood concentrations of calcium. Interestingly, PTH stimulates bone formation when administrated intermittently but causes severe bone loss with continuous administration. Intermittent dosing promotes healthy mineralization, growth and remodeling of bone; whereas continuous dosing increases bone resorption by enhancing RANKL production by osteoblasts (Malluche *et al.*, 2006).

Calcitonin – Calcitonin is released by parafollicular cells in the thyroid gland in response to elevated blood concentrations of calcium. Mature osteoclasts express calcitonin receptors which are G protein coupled-receptors that bind the 32-amino acid peptide hormone, calcitonin, leading to inhibition of resorption by osteoclasts (Warshafsky *et al.*, 1985; Zaidi *et al.*, 2002; Karsdal *et al.*, 2006).

Transforming Growth Factor-β (TGF-β) – TGF-β has a fundamental role in the control of bone resorption. TGF-β binds to its serine/threonine kinase receptor on osteoclast precursors and enhances osteoclastogenesis and bone resorption liberating more TGF-β from the bone matrix (Zaidi *et al.*, 2003; Fox & Lovibond, 2005; Casimiro *et al.*, 2009). TGF-β also acts on osteoblasts, reducing the availability of the osteoclast differentiation factor, RANKL and thereby indirectly limits further osteoclast formation (Fox & Lovibond, 2005).

1.3 The Vicious Cycle Hypothesis

Interaction between tumor cells and osteoclasts cause not only osteoclast activation and subsequent bone loss, but also induce aggressive tumor cell proliferation. Bone metastases are classified as osteolytic when a decrease in bone density occurs via increased bone resorption (Mundy, 2002). Osteolysis results in increases in extracellular Ca^{2+} and release of bone-derived growth factors including TGF-β and insulin-like growth factor 1 (IGF-1) (Casimiro *et al.*, 2009). These growth factors bind to receptors on tumor cells to promote proliferation and production of PTHrP, IL-8 and IL-11 (Mundy, 2002; Casimiro *et al.*, 2009). Production of PTHrP and IL-11 by tumor cells activates osteoblasts to produce RANKL and downregulate OPG. There is evidence that an independent RANKL-mediated pathway is activated by IL-8 to stimulate osteoclast development (Bendre *et al.*, 2005). In this way a 'vicious cycle' is set up between the tumor cells and bone (Fig. 1.3). In support of this mechanism, preventing osteoclast

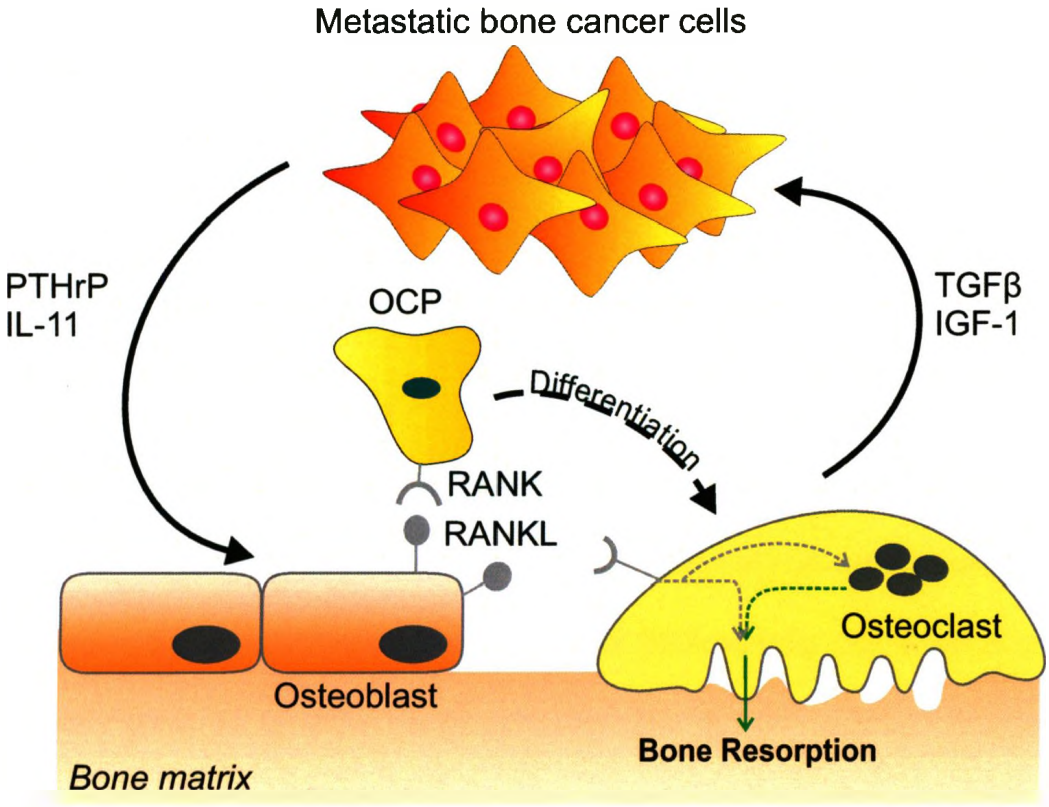

Figure 1.3 Schematic representation of the 'vicious cycle' between bone cells and tumour cells in osteolytic metastases. Tumour cells invade bone and release parathyroid hormone-related protein (PTHrP) and interleukin-11 (IL-11) which stimulate osteoblast receptor activator of nuclear κB ligand (RANKL) expression which increases differentiation of osteoclast precursors (OCP) and increases activity of mature osteoclasts. Resorbed bone releases transforming growth factor ß (TGF-ß) and insulin-like growth factor 1 (IGF-1), thereby stimulating tumour-cell proliferation and further PTHrP and IL-11 release, which, in turn, causes more bone resorption (reviewed in Mundy, 2002).

activation with current therapies such as bisphosphonates or blockade of RANKL decreases both the osteolytic effects of bone metastases and tumor growth (Mundy, 2002). Blockade of tumor cell growth and osteoclast resorption by isoform-selective PI3K inhibitors could be a useful therapeutic for bone metastasis.

1.4 Phosphatidylinositol-3 Kinase (PI3K)

1.4.1 PI3K Signaling Pathways

PI3K is an evolutionarily conserved signaling pathway. PI3K was first noted as a key player in cancer research in the 1980s with the discovery of transforming activity of viral oncogenes being functionally associated with PI3K activity (Whitman *et al.*, 1985). Further study went on to show PI3Ks phosphorylate the D3 hydroxyl position of the inositol ring of phosphatidylinositides when stimulated by activated platelet-derived growth factor receptors and certain oncogenes in the protein-tyrosine kinase family (Kaplan *et al.*, 1987; Auger *et al.*, 1989; Varticovski *et al.*, 1989). Through further study, it was discovered that PI3Ks are heterodimers with separate regulatory and catalytic subunits, and isoforms which can be grouped into three classes based on domain organization, primary structure and substrate specificity (Vivanco & Sawyers, 2002; Kong & Yamori, 2007; Berndt *et al.*, 2010).

Class I PI3Ks are the most studied and are clearly implicated in human cancers, and will therefore be the focus of our study. Class I PI3Ks are heterodimeric enzymes consisting of a regulatory subunit in complex with a 110-kDa catalytic subunit which phosphorylate phosphatidylinositols (PtdIns) in the 3' hydroxyl position. Receptor tyrosine kinases and G protein-coupled receptor signaling pathways activate PI3K, which

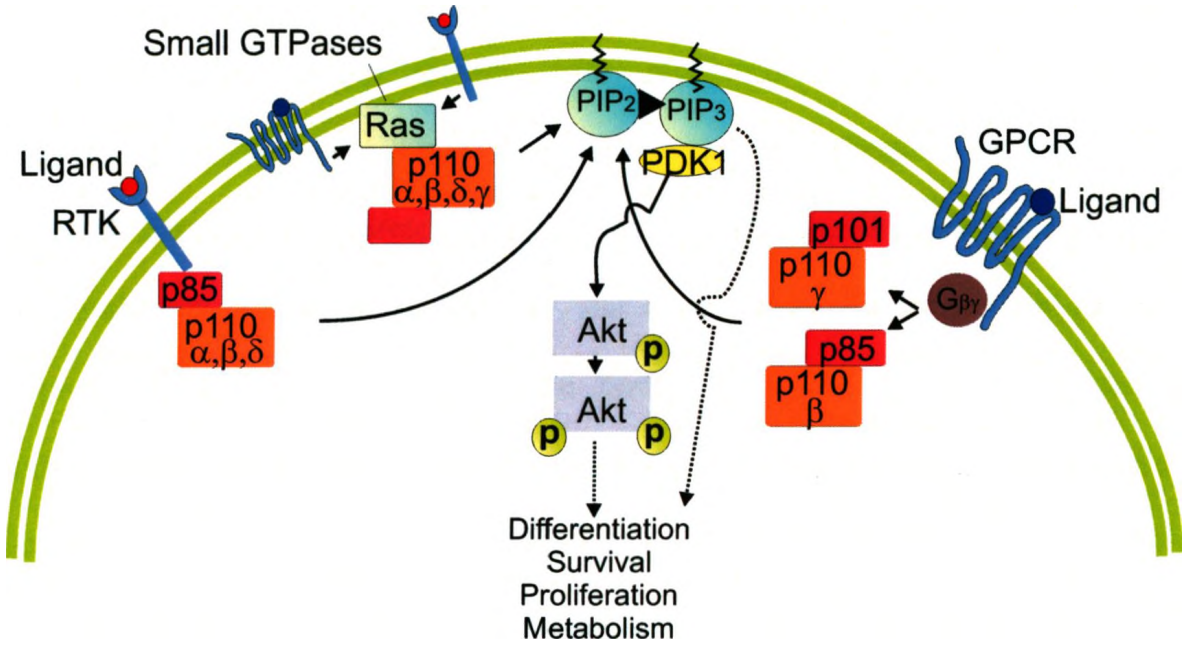

Figure 1.4 Schematic representation of the Class IA and IB PI3K-Akt axis. Receptor tyrosine kinases (RTK) and G protein-coupled receptor (GPCR) signaling pathways activate PI3K (orange - catalytic subunit, red - regulatory subunit) which phosphorylates the primary substrate phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3). RTKs and GPCRs can also activate small GTPases such as Ras, which then activate Class I PI3Ks (middle). Class IA PI3Ks include p110 subunits α, β and δ and are activated by RTKs and associated binding proteins. Class IB PI3Ks include p110 subunit γ and β are activated by Gβγ proteins by GPCRs. Availability of PIP_3 recruits phosphatidylinositol-dependent kinase 1 (PDK1) and Akt to the membrane, where PDK1 phosphorylates Akt, which in turn activates many downstream effectors. Formation of PIP_3 also activates downstream effectors through other associated proteins. Schematic modified from Vanhaesebroeck, et al. 2010.

phosphorylates the primary substrate PtdIns 4,5-bisphosphate (also called PIP_2) to PtdIns 3,4,5-trisphosphate (also called PIP_3). PIP_3 is mainly found on the plasma membrane, although these lipids might also be present in endosomal compartments and even in the nucleus (Vanhaesebroeck *et al.*, 2010). PIP_3 induces downstream effects through adapter proteins such as protein kinases with pleckstrin homology (PH) domains (phosphoinositide-dependent kinase-1 (PDK1), Akt, and Bruton's tyrosine kinase (BTK)), GTPase-activating proteins (GAP) for GTPases of the Rho, Ras and Arf families (Vanhaesebroeck *et al.*, 2005). Notably, Akt is generally linked with PI3K activation and phosphorylation of both Thr308 and Ser473 are required for its full activation (Vanhaesebroeck *et al.*, 2005). Cellular responses to PI3K stimulation are diverse and include, proliferation, cell cycle progression, migration and survival (Vivanco & Sawyers, 2002; Kong & Yamori, 2007; Papakonstanti *et al.*, 2008; Liao & Hung). Class I PI3K includes the following catalytic p110 isoforms: α, β, γ and δ. Class I PI3Ks are further divided into class IA and IB, due to differences in regulatory subunits and activation (Vanhaesebroeck *et al.*, 2005). Class IA includes isoforms p110 α, β and δ and regulatory subunits p85α, p85β, p55γ (encoded by the same gene) and p55α, p55β, p50α (alternative splice units of p85α) and signals downstream of receptor-tyrosine kinases (Vivanco & Sawyers, 2002; Ward *et al.*, 2003). In contrast, class IB includes catalytic subunit p110γ and regulatory subunit p101, and is activated by βγ subunits downstream of G protein-coupled receptors (Vivanco & Sawyers, 2002). Also, recently p110β has been shown to be activated by Gβγ proteins by GPCRs (Guillermet-Guibert *et al.*, 2008). The class IA and IB PI3K-Akt pathway is summarized in Fig. 1.4.

Attenuation of Class I PI3K signaling pathways is achieved by dephosphorylation of the PI3K lipid products by a phosphatase known as phosphatase and tensin homolog deleted on chromosome ten (PTEN) and Src homology 2-containing inositol-5-phosphatase (SHIP). PTEN has both protein and lipid phosphatase activity. As a lipid phosphatase, PTEN removes the 3' phosphate of PtdIns 3,4,5-trisphosphate to make PtdIns 4,5-bisphosphate. PTEN is considered a tumor suppressor. On the other hand, SHIP phosphatases also act as negative regulators of PI3K activity, converting PtdIns 3,4,5-trisphosphate to PtdIns 3,5-bisphosphate. There are two *SHIP* genes encoding SHIP-1 and SHIP-2. SHIP-2 is widely expressed; conversely, SHIP-1 is exclusive to hematopoietic cells including osteoclasts (Takeshita *et al.*, 2002; Rauh *et al.*, 2004; Zhou *et al.*, 2006).

1.4.2 PI3K p110 Isoform Function

The availability of two pharmacological pan-p110 PI3K inhibitors, wortmannin (discovered and isolated from the fungi *Penicillium wortmanni*) and LY294002 (reversible, morpholino derivative of the quercetin flavonoid; structurally unrelated to wortmannin) have contributed greatly to our understanding of the biological roles of PI3K and their effector proteins (Ward *et al.*, 2003; Maira *et al.*, 2009). Although, wortmannin and LY294002 have been shown to have therapeutic potential, such as decreased cancer growth *in vitro* and *in vivo*, both compounds are toxic and show severe side effects (Cheng *et al.*, 2005; Liu *et al.*, 2009; Maira *et al.*, 2009; Teranishi *et al.*, 2009). Neither wortmannin nor LY294002 exhibits any degree of selectivity for individual p110 isoforms (Engelman, 2009). PI3K inhibitors work by binding to the ATP-binding site on class I PI3Ks (Liu *et al.*, 2009). Massive efforts have been put forth

by academic and private laboratories to develop isoform-selective PI3K inhibitors as therapeutics. Huge advances have been made by crystallography of PI3Ks and their chemical scaffolds. Three dimensional structures of isoforms have been crucial in structure-based drug design approaches to identify new molecular entities to improve specificity and potency (Maira *et al.*, 2009) and therefore, decrease potential side effects.

There is growing evidence for distinct biological roles of specific PI3K isoforms. Research using gene knockouts in mice, as well as new pharmacological inhibitors have started to address the issues on PI3K isoform overlap and non-redundant functions, and have revealed important roles for specific PI3K isoforms in immunity, metabolism and cardiac function. Distinct roles of PI3K isoforms in different cell types could be explained by their relative expression levels. For example, MDA-MB-231 breast cancer cells express low levels of p110α and high levels of p110δ which influence stimulus-induced cytoskeleton changes (Sawyer *et al.*, 2003). Consistent with this hypothesis, endothelial cells express low levels of p110δ and moderate levels of p110α to regulate cell migration (Graupera *et al.*, 2008; Papakonstanti *et al.*, 2008). Generally, p110α and p110β are found to be ubiquitously expressed, whereas p110δ expression is low in most cells, but highly enriched in hematopoietic stem cells (Papakonstanti *et al.*, 2008). In knock out studies of p110α, it was determined that homozygous mutations led to embryonic lethality; whereas mice bearing heterozygous mutations were viable, but displayed defective responsiveness to hormones such as, insulin and leptin that led to reduced somatic growth, hyperinsulinaemia, glucose intolerance, hyperphagia and increased adiposity (Knight *et al.*, 2006; Papakonstanti *et al.*, 2008; Kim *et al.*, 2009). Further, important links have been made between p110β and cell survival in thrombosis

and prostate tumor growth (Bradley *et al.*, 2008; Jia *et al.*, 2008); p110δ and the inflammatory response (proliferation and function of B cells and T cells); and p110γ and fibroblast and B cell differentiation and proliferation (Graupera *et al.*, 2008; Beer-Hammer *et al.*, 2010). However, at present, there is little evidence about the functions of PI3K isoforms in osteoclasts (summarized below).

1.4.3 The Role of PI3K in Cancer

The PI3K pathway is one of the most commonly activated signaling pathways in human cancer and it has been estimated that at least 50% of all cancer types are related to deregulation of this signaling pathway (Jia *et al.*, 2008; Falasca, 2011). Activation of this pathway can be brought about in various ways that include, a) activating point mutations in the p110α isoform or over expression of non-mutated p110 α, β, γ and δ isoforms (Knight *et al.*, 2006; Jia *et al.*, 2008), b) activated Ras oncogenes that allosterically stimulate PI3K p110 isoforms (Kang *et al.*, 2006), c) loss of expression of PTEN, a phosphatase responsible for negative regulation of PIP_3 formation (Vivanco & Sawyers, 2002; Knight *et al.*, 2006), or d) overexpression or constitutive activation of oncogenic receptor tyrosine kinases (Lurje & Lenz, 2009).

1.4.4 The Role of PI3K in Bone

PI3K has been shown to be a critical downstream player in osteoclasts, mediating its effects through at least three cell-surface receptors: M-CSF-activated c-fms, RANKL-activated RANK, and the extracellular matrix receptor αvβ3. Wortmannin in osteoclasts, disrupts actin ring formation, ruffled border formation, chemotaxis and reduces pit formation during resorption (Hall *et al.*, 1995; Nakamura *et al.*, 1995; Pilkington *et al.*, 1998). The effector actions of PI3K in osteoclasts are diverse, influencing survival and

activity, mediating cytoskeletal remodeling and motility, and regulating attachment structures. Less is known about the roles of PI3K in osteoblasts, however evidence suggests a role for PI3K in osteoblast differentiation and survival (Golden & Insogna, 2004).

1.4.5 PI3K p110 Isoforms in Osteoclasts

A recent study by Kang and colleagues reports that the PI3K catalytic isoform p110γ plays an important role in bone homeostasis (Kang *et al.*, 2010). They report that genetic inactivation of the *p110γ* gene leads to an increase in bone mass, likely due to impairment in osteoclastogenesis. Decreased osteoclast formation was accompanied by downregulated osteoclast gene expression, including such genes as osteoclast-associated immunoglobulin-like receptor (OSCAR), DC-STAMP, and NFATc1. PI3K p110γ is activated by βγ subunits downstream of GPCRs (Vivanco & Sawyers, 2002). For example, lysophosphatidic acid (LPA) activates GPCRs in osteoclasts to increase survival (Lapierre *et al.*, 2010). Interestingly, p110γ deficiency impaired M-CSF-induced Akt phosphorylation and therefore, the authors concluded that p110γ is likely to mediate its effects on osteoclastogenesis through chemokine receptor signaling which cooperates with M-CSF-induced signaling pathway. They suggest that GPCR-mediated PI3K activation (likely through chemokine receptors) provides a biologically significant supplementation to the M-CSF-mediated protection from apoptosis (Kang *et al.*, 2010).

There is also evidence for a role of p110α in bone provided by Grey and colleagues. In their study, they assessed the effects of pharmacological inhibitors on p110 α, β and δ in the RAW 264.7 monocyte-macrophage-like cell line. They determined that the isoform-selective inhibitor PIK75 (p110α inhibitor) decreased resorptive activity by

osteoclast-like cells and concentration-dependently decreased osteoclastogenesis from undifferentiated RAW 264.7 cells, with no effect of p110β and δ inhibitors (Grey *et al.*, 2010). Thus further investigation into the roles of PI3K isoforms is required.

1.4.5.1 PI3K Activation by M-CSF

M-CSF is a growth factor that binds to its tyrosine kinase receptor, c-fms which activates PI3K to promote proliferation, motility and survival (Varticovski *et al.*, 1989). PI3K is a known partner with cytoplasmic tyrosine kinase c-src and Ras in mediating the effects of activated c-fms (Grey *et al.*, 2000; Fukuda *et al.*, 2005; Sakai *et al.*, 2006). Notably, osteoclasts express the highest concentration of c-fms receptor of any cell type in the hematopoietic cell linage (Varticovski *et al.*, 1989). A growing body of evidence shows that PI3K plays an integral role in mediation of M-SCF-induced pseudopod ruffling and motility in osteoclasts (Pilkington *et al.*, 1998; Palacio & Felix, 2001; Fukuda *et al.*, 2005). Antibody blockade of PI3K p110 subunit isoforms in macrophages has revealed distinct downstream responses to M-CSF that are mediated by specific PI3K isoforms (Vanhaesebroeck *et al.*, 1999). In particular, p110β and p110δ isoforms are required for c-fms-mediated cell migration and cytoskeletal reorganization, while the p110α isoform has a role in c-fms-mediated DNA synthesis (Vanhaesebroeck *et al.*, 1999).

1.4.5.2 PI3K Activation by RANKL

Evidence indicates an important role for PI3K downstream from activated RANK, in which c-src acts as a mediator connecting RANK to PI3K/Akt signaling in osteoclasts (Wada *et al.*, 2006). When the expression of c-src is suppressed, the number of multinucleated cells formed is significantly reduced *in vitro* (Kumagai *et al.*, 2004).

Also, Sugatani and colleagues have shown that PTEN overexpression in RAW 264.7 osteoclast precursors blocks RANKL-induced osteoclast differentiation, as well as Akt activation (Sugatani *et al.*, 2003). This suggests that PI3K is an important participant in RANK-mediated differentiation. In parallel, the importance of SHIP1 in osteoclastogenesis has been shown *in vitro* and *in vivo*. *In vitro* cultures showed increased osteoclastogenesis and increased numbers of hyper-resorptive osteoclasts in SHIP1-deficient cells as a result of hypersensitivity to stimulation with RANKL and M-CSF (Takeshita *et al.*, 2002). Particularly, SHIP1 deficient mice have osteoporosis (Takeshita *et al.*, 2002).

The activation and fusion of osteoclasts require the adaptor molecule DNAX-activating protein of 12 kDa (DAP12), which contains immunoreceptor tyrosine-based activation motifs (ITAM). The receptor, known as triggering receptor expressed on myeloid cells-2 (TREM2), is the main DAP12-associated receptor in osteoclasts. *In vitro* experiments have shown that deficiency in DAP12 or TREM2 leads to impaired osteoclast development and the formation of mononuclear osteoclasts. It has been shown that this complex is downstream of RANK, converging on PI3K (Peng *et al.*, 2010).

1.4.5.3 PI3K Activation by integrins

Osteoclasts adhere to the bone surface via αvβ3 integrins, which engage the organization of dynamic attachment structures. These multi-molecular structures are called podosomes, comprised of actin, vinculin, gelsolin, α-actinin, talin, and other scaffolding molecules (Golden & Insogna, 2004; Teitelbaum, 2007; Novack & Teitelbaum, 2008). PI3K has been shown to be coimmunoprecipitated with αvβ3 integrin from osteoclasts (Lakkakorpi *et al.*, 1997). Interaction of gelsolin and c-src with PI3K

occurs directly through the SH2 domains on the p85 subunit of PI3K (Chellaiah & Hruska, 1996; Chellaiah *et al.*, 1998; Grey *et al.*, 2000). Association of the actin-binding protein gelsolin with PI3K is essential for actin filament formation in osteoclasts. Gelsolin-deficient osteoclasts are hypomotile and fail to produce podosomes (Chellaiah *et al.*, 1998). c-src and PI3K function at the point where adhesion signals converge, transmitting the signals for proper actin cytoskeletal organization that is required for resorption activity.

1.5 Rationale, Objectives and Hypothesis of Research

1.5.1 Rationale

Currently, investigators have begun to elucidate effects of isoform-selective PI3K inhibitors on varying cell types. Some inhibitors have shown considerable promise for the treatment of cancer and are making their way into the early phases of clinical trials (Lannutti *et al.*, 2011). There is evidence that the PI3K pathway affects a number of osteoclast functions, including differentiation (Mandal *et al.*, 2009), survival (Munugalavadla *et al.*, 2008), chemotaxis (Pilkington *et al.*, 1998, 2001), cytoskeletal organization (Lakkakorpi *et al.*, 1997) and bone resorption (Nakamura *et al.*, 1995; Nakamura *et al.*, 1997; Palacio & Felix, 2001; Fukuda *et al.*, 2005). Generally, PI3K p110α and p110β are ubiquitously expressed, whereas p110δ expression is low in most cells, but highly enriched in cells of the hematopoietic lineage (Papakonstanti *et al.*, 2008). In macrophages, DNA synthesis is inhibited by antibodies to p110α specifically, whereas actin reorganization and migration are inhibited by antibodies against p110β and p110δ, specifically (Vanhaesebroeck *et al.*, 1999). These findings support the hypothesis

that specific PI3K isoforms have distinct signaling roles. In osteoclasts, it is unknown whether the isoforms are functionally redundant or have distinct roles. There is possibility that isoform-selective PI3K inhibitors can serve not only as therapeutics for bone diseases, but also as a tool for identifying roles of different PI3K isoforms in osteoclasts. Currently, little is known about the effects of isoform-selective PI3K inhibitors on osteoclasts.

1.5.2 Objectives

We are focusing on the PI3K Class I p110 isoforms α, β, γ and δ, with the goal of identifying inhibitors useful for suppressing osteoclast function.

1. To characterize the effects of isoform-selective PI3K inhibitors on the viability of monocyte-macrophage-like cells.
2. To investigate the effects of isoform-selective PI3K inhibitors on osteoclast morphology and motility.
3. To determine the effect of isoform-selective PI3K inhibitors on cytoskeletal organization in osteoclasts plated on varying substrata.
4. To evaluate the effect of isoform-selective PI3K inhibitors on osteoclast survival.

1.5.3 Hypothesis

It is hypothesized that a PI3K isoform will be identified that selectively alters osteoclast cytoskeletal function and survival.

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CHAPTER TWO

Effects of isoform-selective phosphatidylinositol-3 kinase inhibitors on osteoclasts: Actions on cytoskeletal organization and survival

2.1 Chapter Summary

Phosphatidylinositol 3-kinases (PI3K) participate in numerous signaling pathways, and control a number of distinct biological functions. Studies using pan-PI3K inhibitors suggest roles for PI3K in osteoclasts, but little is known about the function of specific PI3K p110 isoforms in these cells. Our objective was to determine effects of isoform-selective PI3K inhibitors on osteoclasts. The following inhibitors were investigated (targets in parentheses): wortmannin and LY294002 (pan-p110), PIK75 (α), GDC0941 (α, δ), TGX221 (β), AS252424 (γ) and IC87114 (δ). In addition, we characterized the novel PI3K inhibitor CAL-120 and found it was highly selective for PI3Kδ. Only high concentrations of PIK75 and LY294002 reduced the viability of monocyte-macrophage-like cells. Osteoclasts were isolated from the long bones of neonatal rats and rabbits. Wortmannin, GDC0941, IC87114 and CAL-120 induced dramatic retraction of osteoclasts within 15-20 min to 65-75% of initial area. In contrast, there was no significant retraction in response to vehicle, PIK75, TGX221 or AS252424. Moreover, wortmannin and CAL-120, but not PIK75 or TGX221, disrupted filamentous F-actin belts; and CAL-120 inhibited the formation of sealing zones in osteoclasts on resorbable substrates. In contrast to their selective actions on cytoskeletal organization, PIK75, TGX221 and CAL-120 blocked the stimulatory effects of RANKL on osteoclast survival. These data are consistent with a specific role for PI3Kδ in regulating the osteoclast cytoskeleton. In contrast, multiple PI3K isoforms contribute to the control of osteoclast survival. Thus, the PI3Kδ isoform, which has more limited tissue distribution than PI3Kα and PI3Kβ, is an attractive target for novel anti-resorptive therapeutics.

2.2 Introduction

Bone is remodeled by the coupled process of breakdown of old bone by osteoclasts and formation of new bone by osteoblasts (Novack & Teitelbaum, 2008). Maintenance of bone integrity is dependent on the coordinated activity of osteoclasts and osteoblasts, with perturbations to this balance causing skeletal disease (Manolagas, 2000). Osteoclasts and osteoclast precursors receive signals from adjacent cells, soluble mediators and the extracellular matrix to regulate changes in differentiation, survival, resorptive activity and recruitment to bone (Teitelbaum, 2007).

Class I phosphatidylinositol-3 kinases (PI3K) have critical roles in a variety of cellular processes such as metabolism, differentiation, survival and migration (Vivanco & Sawyers, 2002). PI3Ks are further divided into class IA and IB, all of which are heterodimeric enzymes consisting of a regulatory subunit in complex with a 110kDa catalytic subunit that phosphorylates the primary substrate phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Vanhaesebroeck *et al.*, 2005). PIP_3 is the active form, and mediates downstream effects through adapter proteins such as protein kinases with pleckstrin homology domains and GTPase-activating proteins (Vivanco & Sawyers, 2002; Liu *et al.*, 2009). Class IA includes catalytic isoforms p110 α (PI3Kα), β (PI3Kβ), and δ (PI3Kδ) and regulatory subunits p85α or β, p55α or γ, p50α and signals downstream of tyrosine-receptor kinases (TRK). In contrast, Class IB includes catalytic subunit p110γ (PI3Kγ) and regulatory subunits p101 and p84, and signals downstream of G protein-coupled receptors (GPCR) (Vivanco & Sawyers, 2002; Vanhaesebroeck *et al.*, 2005). Recent data indicate that most class I

PI3K subunits might be activated directly (e.g. Gβγ protein activating PI3Kγ and PI3Kβ (Guillermet-Guibert *et al.*, 2008)) or indirectly (e.g. Ras) (Vanhaesebroeck *et al.*, 2010).

Genetic manipulation experiments, as well as utilizing new pharmacological inhibitors have allowed researchers to address issues on PI3K isoform overlap and non-redundant functions, and have revealed important roles for specific PI3K isoforms in immunity, metabolism and cardiac function. Some examples include, PI3Kα in insulin signaling and oncogenesis, PI3Kβ in thrombosis, and PI3Kδ and PI3Kγ in immune function and inflammation (Vanhaesebroeck *et al.*, 2005; Engelman, 2009; Liu *et al.*, 2009). Generally, PI3Kα and PI3Kβ are thought to be ubiquitously expressed, whereas PI3Kδ and PI3Kγ expression is low in most cells, but high in cells of hematopoietic origin (Chantry *et al.*, 1997; Kok *et al.*, 2009). Recently developed isoform-selective PI3K inhibitors show promise for the treatment of inflammatory disease (Marone *et al.*, 2008; Durand *et al.*, 2009) and cancer (Cleary & Shapiro, 2010), and are making their way into the early phases of clinical trials (Liu *et al.*, 2009; Lannutti *et al.*, 2011).

In osteoclasts PI3K affects survival, resorptive activity, cytoskeletal organization and motility (Hall *et al.*, 1995; Pilkington *et al.*, 1998, Nakamura, 1995; Golden & Insogna, 2004; Chellaiah, 2006). Investigations on PI3K isoforms in macrophages demonstrate that PI3Kδ is important in cell migration and vesicle trafficking (Papakonstanti *et al.*, 2008; Low *et al.*, 2010). In addition, investigations in osteoclasts demonstrate that PI3Kγ modulates osteoclastogenesis (Kang *et al.*, 2010). Despite our current understanding, relatively little evidence is available on the functions of PI3K isoforms in osteoclasts, thereby providing a rationale for ongoing evaluation and possible therapeutic development.

2.3 Materials and Methods

2.3.1 Materials

Medium 199 (M199, Earles, 12340) buffered with 25 mM HEPES and 26 mM HCO_3^-, HCO_3^- -free M199 (Hanks, 12350) buffered with 25mM HEPES, heat-inactivated fetal bovine serum (FBS, 12483), and antibiotic solution (penicillin 10,000 units/ml; streptomycin 10,000 µg/ml; and amphotericin B 25 µg/ml, 15240) were purchased from Invitrogen (Burlington, Canada). Dulbecco's modified Eagles medium (DMEM, D7777) with 4500 mg/L glucose, L-glutamine, and sodium pyruvate, without sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) (crystallized) was from ICN Biomedicals. Mounting medium (Vecta-Shield) was from Vector Laboratories (Burlingame, CA,). Recombinant Mouse RANKL was purchased from R&D systems (Minneapolis, MN). GDC0941 bismesylate (1377), TGX221 (1417), AS252424 (1424) and PIK75 (1334) were purchased from Axon Med Chem (Groningen, Holland). IC87114 and CAL-120 were kind gifts from Gilead Sciences (Forest City, CA). Wortmannin (681675) and LY292004 (440202) were purchased from Calbiochem (Darmstadt, Germany). Stock solutions of PI3K inhibitors were prepared in dimethyl sulfoxide (DMSO) from Sigma-Aldrich. Vehicle controls were DMSO in all cases.

2.3.2 Osteoclast Isolation

Osteoclasts were isolated from the long bones of newborn Wistar rats or New Zealand White rabbits, as described in previous publications (Naemsch *et al.*, 2001; Pereverzev *et al.*, 2008). All procedures were approved by Council on Animal Care of The University of Western Ontario and were in accordance with the guidelines of the Canadian Council on Animal Care. Briefly, long bones are dissected free of soft tissue

and minced with a scalpel in HCO_3^- buffered M199 supplemented with 15% FBS and 1% antibiotic solution. The resulting cells are suspended by repeated passage through a glass pipette and plated on FBS-coated 12-mm glass cover slips, MatTek glass bottom culture dishes (MatTek Corporation, Ashland, MA), or calcium phosphate-coated discs (BD BioCoat™ Osteologic™ Discs, BD Biosciences, Bedford, MA). Freshly isolated osteoclasts were incubated at 37°C in 5% CO_2 for 1 h, washed gently with PBS to remove non-adherent cells and incubated in medium. Osteoclasts are identified as having three or more nuclei, positive staining for tartrate-resistant phosphatase (TRAP), retracting in response to calcitonin, and the ability to resorb mineralized substrates.

2.3.3 *In vitro* Kinase Profiling

Biochemical *in vitro* lipid kinase assays were analyzed using the SelectScreen® biochemical kinase assay service (Invitrogen Ltd.). A stock solution of CAL-120 was prepared in DMSO at a concentration of 10 mM. Ten-point kinase inhibitory activities were measured over a concentration range (5 nM to 10^4 nM) with ATP at a concentration consistent with each enzyme's K_m.

2.3.4 PI3K Isoform-Selective Cell-Based Assays

Murine embryonic fibroblast (MEFs, American Type Culture Collection (ATCC), Manassas, VA) were used for the analysis of PI3Kα and PI3Kβ signaling. Cells were transferred to serum-free medium for two hours and then stimulated with platelet-derived growth factor (PDGF) (10 ng/mL; Cell Signaling, Danvers, MA) or LPA (10 μM; Echelon, Salt Lake City, UT) for 10 min at 37°C to activate PI3Kα and PI3Kβ, respectively. After washing once in cold phosphate-buffered saline (PBS), the cell pellet was resuspended in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol,

1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM $NaVO_4$, 1 μg/mL leupeptin, and 1 μg/mL aprotinin) for 15 minutes on ice. Whole-cell lysates were obtained by centrifugation at 14000*g* for 15 minutes at 4°C, and the soluble protein was analyzed by Western blotting for Akt and pAkt levels.

For the analysis of PI3Kδ and PI3Kγ signaling, basophil activation was measured in isolated peripheral blood mononuclear cells (PBMC) or whole blood. Blood samples were obtained after written informed consent obtained in accordance with the Declaration of Helsinki and with local institutionally approved protocols. Basophil activation was measured using the Flow2 CAST kit according to the manufacturer's standardized methods (Buhlman Laboratories AG, Switzerland). Briefly, PI3Kδ was activated with anti-FcεRI and PI3Kγ was activated with formyl-methionyl-leucyl-phenylalanine (fMLP) in the absence or presence of increasing concentrations of CAL-120. To monitor the basophil cell population and cellular activation, anti-CD63-FITC and anti-CCR3-PE antibodies were added to each sample. Cells were fixed and analyzed on a FC500MPL flow cytometer.

2.3.5 Assessment of Cell Viability

The virally transformed murine monocyte-macrophage-like cell line RAW 264.7 was obtained from the ATCC (Manassas, VA). The effect of inhibitors on RAW 264.7 cell survival was evaluated using the methylthiazoltetrazolium (MTT) assay. RAW 264.7 cells were seeded in Falcon flat bottom 96-well plates (353072) at a density of 2.5 – 3 x 10^4 cells/cm^2 in 100 μL DMEM medium with 10% FBS and 1% anitbiotic. After seeding, the cells were allowed to attach for 24 h then exposed to control or treatment for 24 h at

varying concentrations. After incubation at 37°C in 5% CO_2, MTT substrate (Roche Applied Science, Mississauga, ON) was added at a final concentration of 0.5 mg/mL for 4 h. Following 4 h incubation, 100 µL solubilization solution was added to each well to dissolve the formazan crystals and samples were analyzed after 24 h overnight. Spectrophotometrical absorbance of the samples were taken using an ELISA reader by Tecan (Mannedorf, Switzerland) using a wavelength of 550 nm and a reference wave length of 700 nm.

2.3.6 Assessment of Osteoclast Morphology

To perform time-lapse recordings culture medium was removed and replaced with a HEPES-buffered M199 medium (HCO_3^--free) supplemented with 15% FBS and 1% antibiotic solution. Dishes were placed in a heated stage and maintained at ~35°C. Osteoclasts were observed using a Nikon Eclipse TE300 phase contrast microscope and images were captured using Image Master 5 Software (Photon Technology International). For data analysis, the periphery of each osteoclast was traced periodically to quantify the planar area using Image Master Software. Planar area is expressed as a percentage of the average initial area before time 0. Initial planar area was 5440 ± 3200 μm^2 (mean ± S.D., $n = 90$ osteoclasts).

2.3.7 Assessment of Osteoclast F-actin Organization

Osteoclasts were plated on FBS-coated glass cover slips or BD BioCoat™ Osteologic™ cover slips and fixed with 4% paraformaldehyde with 2% sucrose in PBS for 10 min at room temperature, washed three times with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Following three washes with PBS, cells were incubated in 1% - 3% bovine serum albumin for 1 h at room temperature,

stained for filamentous actin (F-actin) using 66 nM Alexa Fluor® 488 phalloidin for 20 min at room temperature in the dark, and washed three times with PBS. Following the wash, osteoclasts were visualized for fluorescence. The number of osteoclasts portraying a complete F-actin belt or sealing zone present under each condition were counted along with to the total number of osteoclasts (per 12 mm coverslip). Osteoclasts observed to have a F-actin belt or sealing zone to encompass 75% or more of the cell perimeter were counted as 'complete,' and anything below 75% was counted as 'incomplete'. Images under each condition were captured with Zeiss AxioVision 4.8 imaging software using a Zeiss Observer Z1 microscope (Chester, VA) or Zeiss LSM 5.0 software using a Zeiss LSM 510 META confocal microscope.

2.3.8 Assessment of Osteoclast Survival

Rat osteoclasts were isolated and plated on FBS coated coverslips and incubated at 37°C in 5% CO_2 for 1 h. Coverslips were then washed gently with PBS to remove non-adherent cells and incubated for an additional 0.5-1 h in HCO_3^--buffered M199 supplemented with 15% FBS and 1% antibiotic solution. Osteoclasts were then counted using phase-contrast microscopy as described previously (Korcok *et al.*, 2005). Cultures were incubated for an additional 15-18 h at 37°C in 5% CO_2. Following incubation, the number of osteoclasts per coverslips was counted, and survival was expressed as the percentage of the initial osteoclast number on the same coverslip. Number of osteoclasts per coverslip was 75 ± 25 (mean ± S.D., $n = 90$ coverslips).

2.3.9 Statistical Analysis

Results are presented as means ± S.E.M. Differences between two groups were evaluated using a Student's t-test and differences among two or more groups were

evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test or two-way ANOVA followed by Bonferroni's multiple comparisons test, respectively. Differences were accepted as statistically significant at $p < 0.05$.

2.4 Results

2.4.1 CAL-120 is a Potent and Selective Inhibitor of PI3Kδ

CAL-120 ((S)-2-(1-(9H-purin-6-ylamino)ethyl)-6-fluoro-3-phenylquinazolin-4(3H)-one) was characterized using *in vitro* and cell-based kinase assays. Other compounds used in the study were characterized in previous studies (i.e. PIK75 (Knight *et al.*, 2006), TGX221 (Jackson *et al.*, 2005), AS252424 (Pomel *et al.*, 2006), IC87114 (Sadhu *et al.*, 2003), GDC0941 (Folkes *et al.*, 2008), Wortmannin and LY294002 (Marone *et al.*, 2008)). We demonstrate that CAL-120 was 25- to 300-fold more selective for PI3Kδ relative to other PI3K class I enzymes (IC_{50}: PI3Kα 357 nM; PI3Kβ 153 nM; and PI3Kγ 47 nM; PI3Kδ 0.8 nM). CAL-120 was also 10^3-fold more selective against PI3Kδ than against related kinases, such as CIIβ, hVPS34, DNAPK, and mTOR. No other activity was observed against a panel of >340 diverse kinases (Table 2.1A).

EC_{50} values were determined using *in vitro* cell-based assays as described previously (Lannutti *et al.*, 2011). In fibroblasts, the PDGF receptor signals through PI3Kα and the G protein–coupled receptor for LPA signals through PI3Kβ (Jia *et al.*, 2008). CAL-120 reduced PDGF-induced pAkt by only 25% at 10 μM, whereas CAL-120 reduced LPA-induced pAkt by 50% at 1.2 μM (Table 2.1B). Expression of PI3Kγ and PI3Kδ is largely restricted to cells of hematopoietic origin, including basophils. In basophils, FcεRI signals through PI3Kδ, whereas fMLP signals through PI3Kγ via G protein–

A

Class I PI3Ks				Class II PI3K	Class III PI3K	Related kinases			
p110α	p110β	p110γ	p110δ	CIIβ	hVPS34	PIP5Kα	PIP5Kβ	DNAPK	mTOR
357	153	47	0.8	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$

*All values shown are IC_{50} values (nM).

B

PI3K Isoforms	PI3Kα	PI3Kβ	PI3Kγ	PI3Kδ
Cell Type	Primary Fibroblast	Primary Fibroblast	Primary Basophil	Primary Basophil
Stimulus	PDGF-induced pAkt	LPA-induced pAkt	fMLP receptor CD63 expression	Anti-FcεRI-induced CD63 expression
EC_{50} (nM)	>20,000	1,200	2,345	4.9

*In serum-containing medium, values show 2-fold increase

Table 2.1. CAL-120 is a potent and selective inhibitor of PI3Kδ. A, CAL-120 *in vitro* activity profiles (IC_{50} values) against recombinant enzymes of PI3K Class I, II, and III and other related kinases. CAL-120 was dissolved in dimethyl sulfoxide at a stock concentration of 10 mM and 10-point kinase inhibitory activities were measured with ATP at a concentration consistent with the K_m of each enzyme. B, Potency of CAL-120 in cell-based assays evaluating the activity of specific PI3K Class I isoforms (EC_{50} values). For the analysis of PI3Kα and PI3Kβ signaling, murine embryonic fibroblasts were incubated for 1 h with several concentrations of CAL-120 followed by stimulation with PDGF or LPA. Soluble protein was analyzed by Western blotting for Akt and $pAkt^{473}$ levels. For the analysis of PI3Kγ and PI3Kδ signaling, basophil activation was measured in isolated peripheral blood mononuclear cell or whole blood. PI3Kγ was activated with formyl-methionyl-leucyl-phenylalanine and PI3Kδ was activated with anti-FCεRI. To determine the basophil cell population and to monitor cellular activation, anti-CCR3-PE and anti-CD63-FITC antibodies were added and each sample was analyzed on a FC500MPL flow cytometer, respectively. *In vitro* kinase studies $n = 4$ independent experiments and for isoform-selective cell-based assays $n = 8$ independent experiments. Experiments were carried out at Calistoga Pharmaceutical in Seattle, Washington by Dr. B. Lannutti and A. Kashishian.

coupled receptors with either stimulus leading to expression of CD63 (Laffargue *et al.*, 2002; Ali *et al.*, 2004). CAL-120 suppressed FcεRI PI3Kδ-mediated CD63 expression with an EC_{50} of 4.9 nM, and fMLP PI3Kγ-mediated CD63 expression with an EC_{50} of 2345 μM (Figure 1B). Thus, CAL-120 had 240- to 4000-fold selectivity for PI3Kδ over the other class I PI3K isoforms and virtually no effect on other kinases.

2.4.2 Effect of Isoform-Selective PI3K Inhibitors on the Viability of Monocyte-Macrophage-like Cells

RAW 264.7 cells are a leukemic murine monocyte/macrophage cell line that, when treated with RANKL (100 ng/ml) for 4 days, produce multinucleated, TRAP-positive osteoclast-like cells (Armstrong *et al.*, 2009). To assess possible toxicity, we incubated RAW 264.7 cells with inhibitors at varying concentrations for 24 h, after which viability was assessed using an MTT assay. We compared control cultures to those in the presence of isoform-selective PI3K inhibitors. The inhibitors were: wortmannin (pan-p110), LY294002 (pan-p110), GDC0941 (α, β and δ inhibitor), PIK75 (α), TGX221 (β), AS252424 (γ), IC87114 (δ) and CAL-120 (δ). Toxic effects were only observed for PIK75, at concentrations of 1 and 10 μM, and LY294002, at concentration of 100 μM ($p < 0.05$) (Fig. 2.1). Since toxic effects were observed only for PIK75 and LY294002 at high concentrations it was concluded that lower concentrations with all PI3K inhibitors were not effecting the cell viability.

2.4.3 Inhibition of PI3Kδ Induces Osteoclast Retraction

Conventional PI3K inhibitors such wortmannin and LY294002 have been shown to induce osteoclast retraction and inhibit motility (Pilkington *et al.*, 1998). However, these conventional inhibitors are not selective for particular PI3K p110 isoforms,

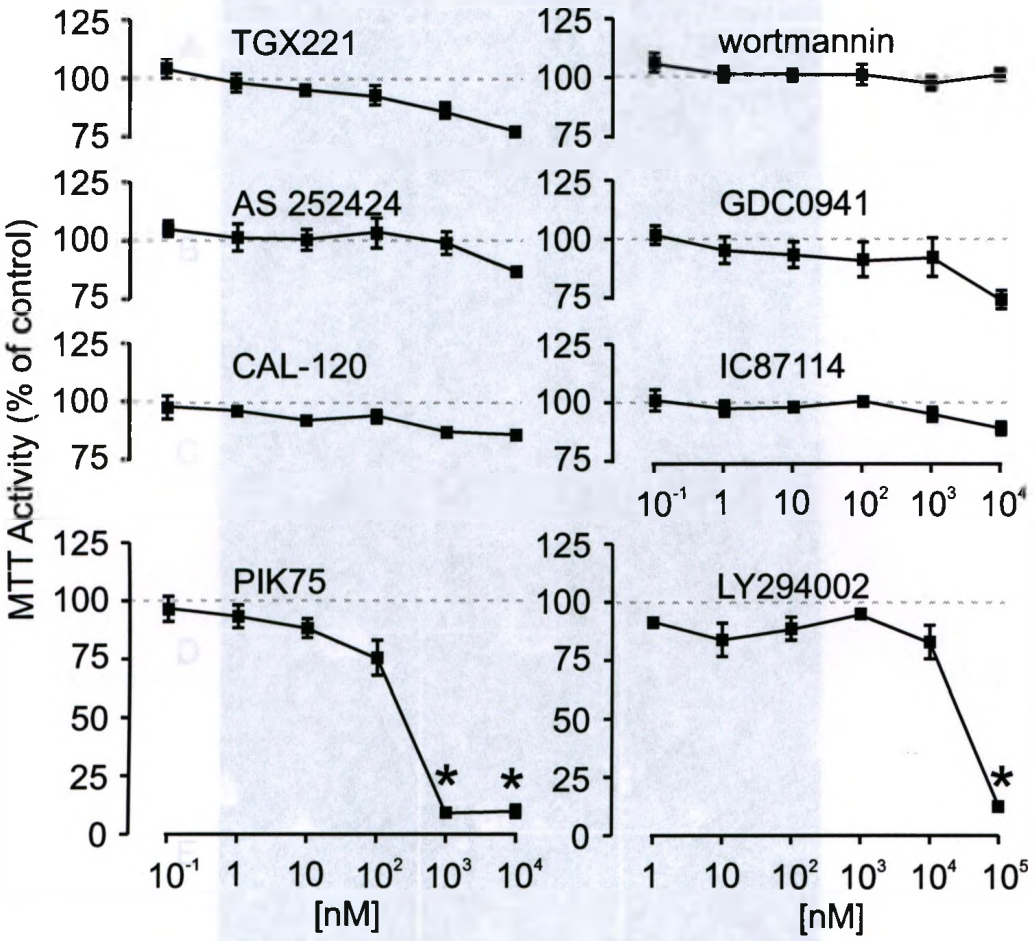


Fig. 2.1. Effects of PI3K isoform-selective inhibitors on viability of RAW 264.7 monocyte-macrophage-like cells. RAW-264.7 cells were treated with inhibitors (100 pM to 10 μM, or 1 nM to 100 μM for LY294002) or vehicle for 24 h, after which viability was assessed using an MTT assay. Data are expressed as a percentage of values for vehicle-treated control samples. Dashed lines indicate 100%. Viability was diminished only by PIK75 at concentrations of 1 and 10 μM and LY294002 at 100 μM. Data are means ± S.E.M., $n = 4$ independent experiments, except for IC87114 and LY294002 where $n = 3$. Differences were assessed using one-way ANOVA and Tukey's multiple comparisons test. * indicates $p < 0.05$ compared to vehicle.

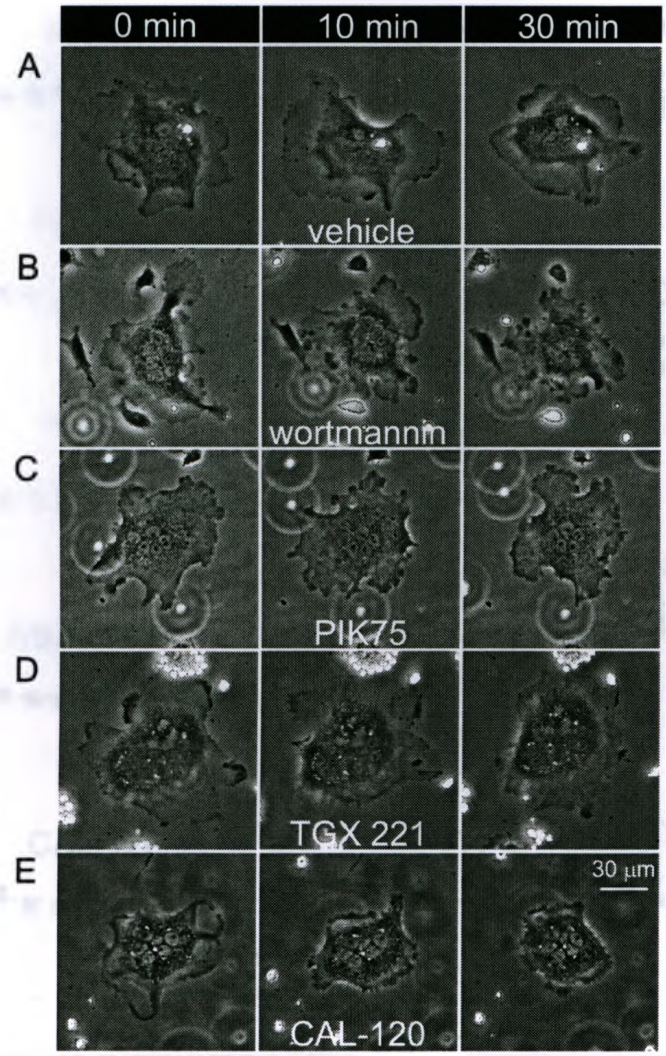


Fig. 2.2. Wortmannin and CAL-120 induce osteoclast retraction. Rat osteoclasts were bathed in HEPES-buffered M119 medium with 15% FBS and antibiotics and imaged using time-lapse phase-contrast microscopy. A-E illustrates 5 different osteoclasts, each illustrated at 3 times. Images labeled time 0 min illustrate the cells immediately prior to addition of vehicle, wortmannin (1 μM), PIK75 (1 μM), TGX221 (1 μM), or CAL-120 (1 μM) to the bath. Prior to treatment, osteoclast lamellipodia were well spread and motile. Wortmannin and CAL-120 induced prompt retraction of lamellipodia, which was sustained for at least 30 min. Images are representative of the responses of 9-15 osteoclasts from 4-8 independent preparations. Scale bar represents 30 μm for all panels. Images displayed were gamma adjusted for clarity of the periphery. Supplemental Videos 1 and 2 illustrate the responses of cells in panels A and E, respectively.

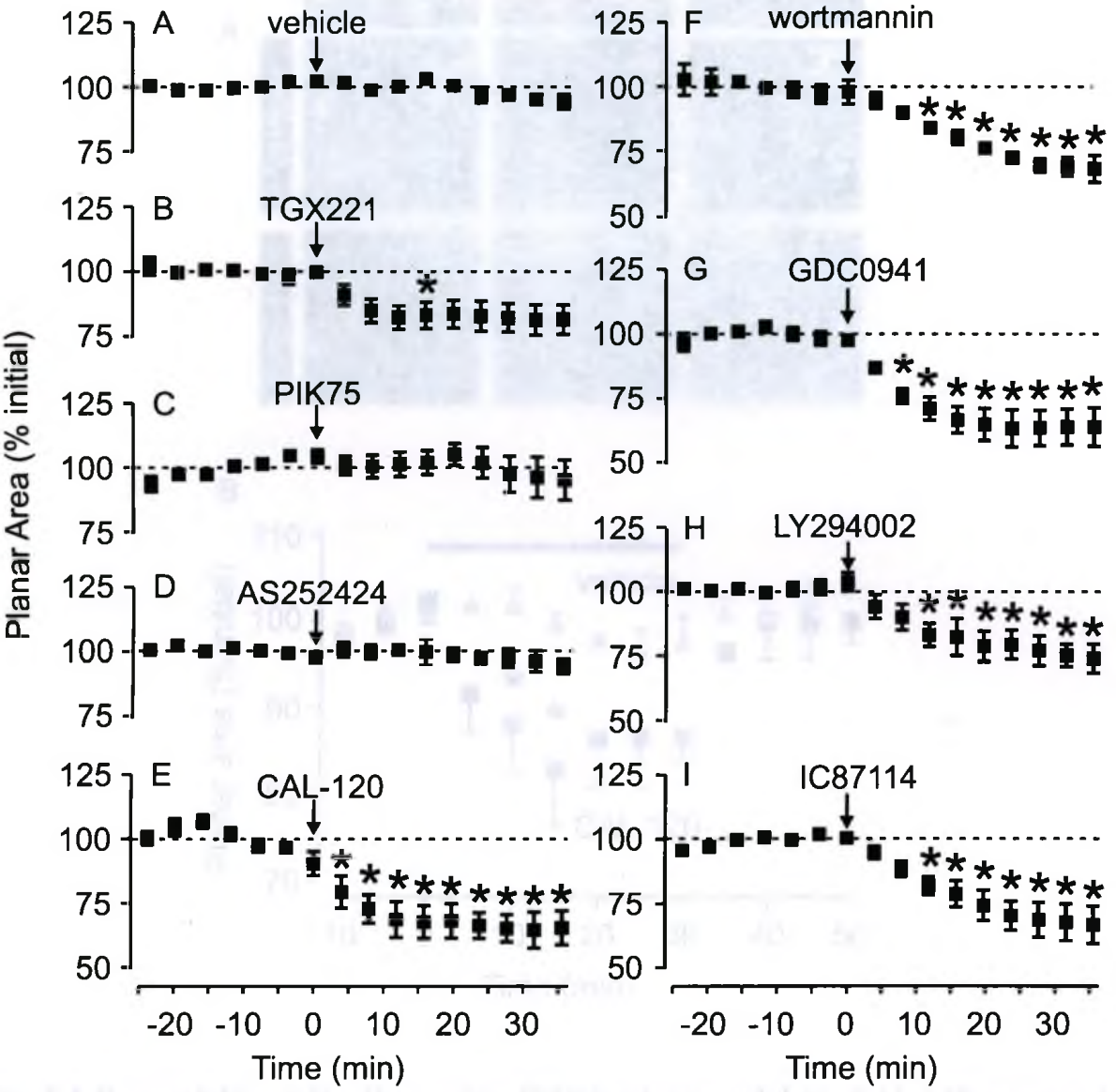


Fig. 2.3. PI3Kδ is important in osteoclast lamellipodia spreading. Rat osteoclasts were imaged by time-lapse phase-contrast microscopy and treated with the indicated test substance at time 0, as described in the legend to Fig. 3. Image analysis software was used to calculate the planar area of osteoclasts at 4-min intervals. Data are expressed as a percentage of the mean initial area before time 0 (from -24 to 0 min) and are means ± S.E.M. A, There was no marked change in osteoclast area in vehicle-treated cells (n = 4 independent preparations, a total of 9 osteoclasts). B-D, The following inhibitors had no net effect on osteoclast area: TGX221 (1 μM, n = 8 independent preparations, a total of 10 osteoclasts), PIK75 (1 μM, n = 5 independent preparations, a total of 10 osteoclasts), and As252424 (1 μM, n = 5 independent preparations, a total of 9 osteoclasts). E-I, In contrast, the following inhibitors caused significant, sustained decreases in osteoclast planar area: CAL-120 (1 μM, n = 5 independent preparations, a total of 15 osteoclasts), wortmannin (1 μM, n = 4 independent preparations, a total of 8 osteoclasts), GDC0941 (1 μM, n = 5 independent preparations, a total of 8 osteoclasts), LY294002 (50 μM, n = 3 independent preparations, a total of 7 osteoclasts) and IC87114 (5 μM, n = 4 independent preparations, a total of 12 osteoclasts). Differences were assessed using a two-way ANOVA and Bonferroni's multiple comparisons test. * indicates $p < 0.05$ compared to vehicle at the corresponding times.

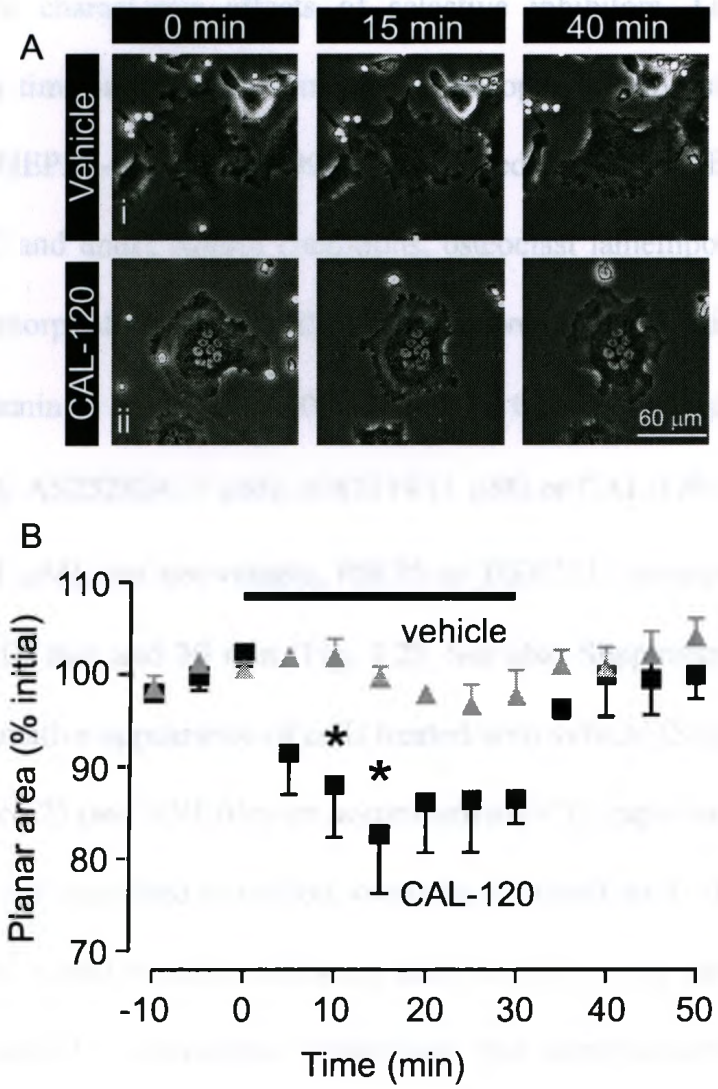


Fig. 2.4. Reversibility of the effects of the PI3Kδ-selective inhibitor CAL-120 on osteoclast retraction. Rat osteoclasts were imaged by time-lapse phase-contrast microscopy and exposed to vehicle or CAL120 (1 µM) from 0 to 30 min, as indicated by the horizontal bar above the data in B. A illustrates 2 different osteoclasts, each shown at 3 times. Ai, lamellipodia remain well spread in vehicle-treated osteoclast. Aii, CAL-120 induces retraction of lamellipodia (evident at 15 min). Following wash out of CAL-120 at 30 min, lamellipodia respread (evident at 40 min). B, Planar area of osteoclasts was calculated periodically and expressed as a percentage of the mean initial area before time 0 (from -10 to 0 min). Data are means ± S.E.M. There was no marked change in osteoclast area in vehicle-treated cells ($n = 3$ independent preparations, a total of 8 osteoclasts). In contrast, planar area exhibited a significant decrease in the presence of CAL120 and returned promptly to control values following its wash out ($n = 3$ independent preparations, a total of 5 osteoclasts). Differences were assessed using a two-way ANOVA and Bonferroni's multiple comparisons test. * indicates $p < 0.05$ compared to vehicle at the corresponding times.

prompting us to characterize effects of selective inhibitors. Live osteoclasts were monitored using time lapse phase-contrast microscopy. Freshly isolated rat osteoclasts were bathed in HEPES-buffered M199 supplemented with 15% FBS and 1% antibiotic solution at 35°C and under control conditions, osteoclast lamellipodia were well spread (0 min). Basal morphology and motility were recorded for 25 min before addition of vehicle, wortmannin (1 μM), LY294002 (50 μM), GDC0941 (1 μM), PIK75 (1 μM), TGX221 (1 μM), AS252424 (1 μM), IC87114 (1 μM) or CAL-120 (1 μM). Wortmannin and CAL-120 (1 μM), but not vehicle, PIK75 or TGX221, caused prompt retraction of lamellipodia at 10 min and 30 min (Fig. 2.2). See also Supplemental Videos 1 and 2, showing representative appearance of cells treated with vehicle (Suppl Video 1) or CAL-120 (Suppl. Video 2) (see AVI files on accompanying CD, captions are in Appendix B). Despite marked and sustained retraction, osteoclasts treated with GDC0941, IC87114 or CAL-120 still remained motile, indicating that the cells were not quiescent as when retraction is induced by wortmannin. Osteoclasts that were considered motile exhibited pseudopod ruffling, while osteoclasts considered being quiescent had no pseudopod ruffling.

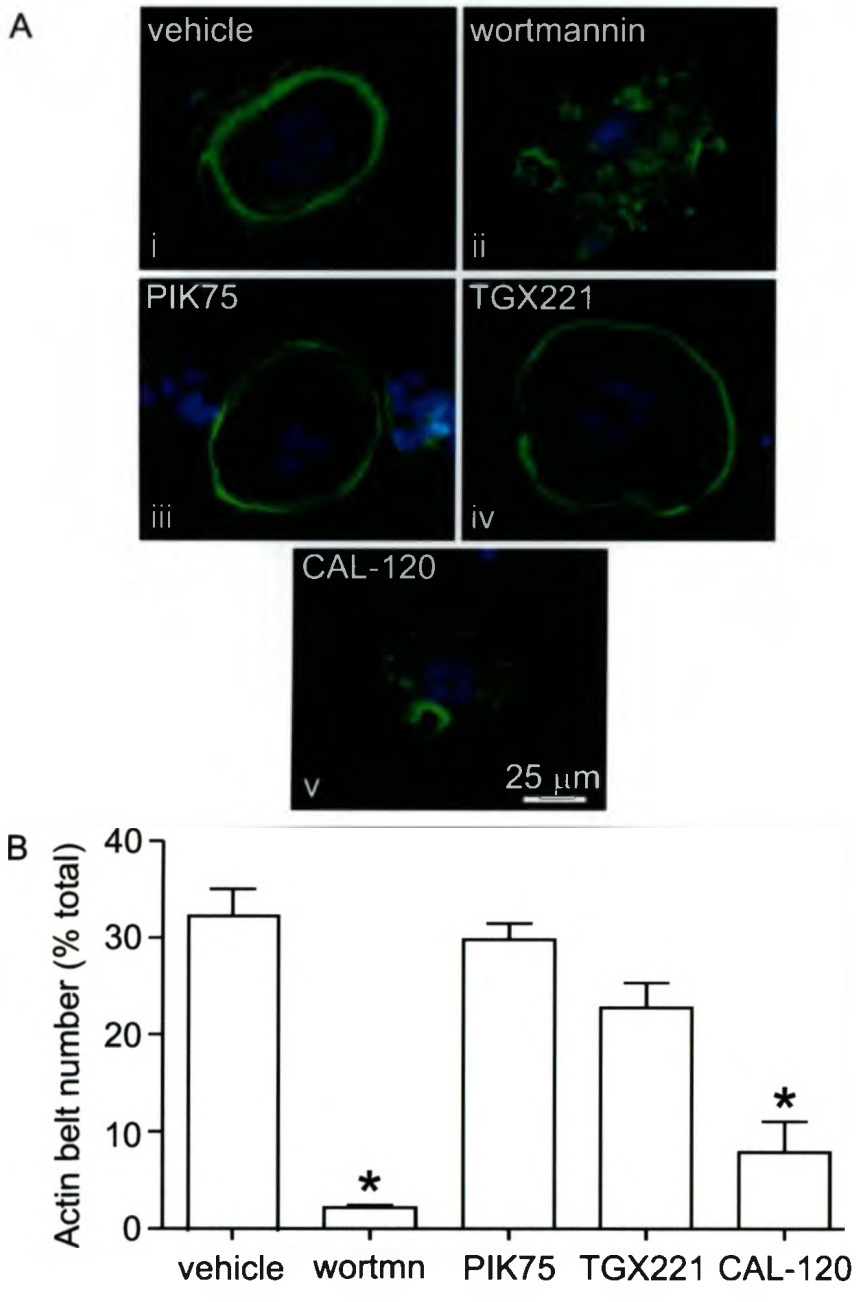
To quantify the change in morphology, planar area of osteoclasts were measured periodically over 60 min, with area expressed as a percentage of the mean area for each cell before treatment. Within 10-15 min, GDC0941, LY294002, wortmannin, IC87114 and CAL-120 (Fig. 2.3E-I) induced significant retraction to 65-75% of initial area. In contrast, there was no significant response to TGX221, PIK75, AS252424 at 1 μM or vehicle (Fig. 2.3A-E), consistent with the involvement of a specific PI3K isoform in regulating the osteoclast cytoskeleton.

Some PI3K inhibitors are irreversible such as wortmannin (Wymann *et al.*, 1996), so we examined the reversibility of the PI3Kδ selective inhibitor, CAL-120 (Fig. 2.4A). Planar area of rat osteoclasts was measured periodically over 60 min, with area expressed as a percentage of the mean area for each cell before treatment. Following a control period of 10 min, vehicle, or CAL-120 (1 μM) was applied to the bath at 0 min and washed out at 30 min (Fig. 2.4B). Vehicle caused no change in osteoclast planar area before or after wash (Fig. 2.4Ai). In contrast, lamellipodia retracted in the presence of CAL-120 with apparent re-spreading following wash (Fig. 2.4Aii). CAL-120 caused significant retraction of osteoclasts at 10 min and 15 min, followed by a return to baseline after wash out. This demonstrates that the retraction is reversible and reveals a distinct feature of CAL-120. It should be noted that these studies involved lengthy time-lapse recordings of single osteoclasts precluding us from carrying out full concentration-dependence studies.

2.4.4 Inhibition of PI3Kδ Disrupts Actin Organization

We next investigated whether retraction of lamellipodia was associated with changes in the organization of the actin cytoskeleton. *In vitro*, on non-mineralized substrates, such as glass or plastic, osteoclasts form clusters podosomes, or a band of podosomes at the periphery of the cell, called F-actin belts. On mineralized substrates, such as bone or calcium phosphate matrices, podosomal units condense, forming a ring in the interior of the cell, called a sealing zone (Saltel *et al.*, 2008). To examine the disruption of F-actin belts and sealing zones, we quantified the proportion of cells with complete or disrupted F-actin structures. The effects of selected class IA PI3K isoform inhibitors were examined. PIK75, TGX221, CAL-120 or wortmannin were applied to

Fig. 2.5. Effects of PI3K inhibitors on actin belt organization in osteoclasts. Rat osteoclasts were plated on FBS-coated glass coverslips in HCO_3^--buffered M199 with FBS and antibiotics. Samples were treated with vehicle or inhibitor for 10 min and then fixed. F-actin was labeled using Alexa Fluor 488-conjugated phalloidin (green), nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue), and cells were examined by fluorescence microscopy (40x objective, Zeiss Axio Observer Z1). Inhibitors were wortmannin (wortmn), PIK75, TGX221 and CAL-120 (all 1 μM). Ai, Image shows a single untreated rat osteoclast exhibiting a prominent F-actin belt at the cell periphery. Aiii and iv, F-actin belts were also observed in osteoclasts treated with PIK75, TGX221. Aii and v, in contrast, osteoclasts treated with wortmannin or CAL-120 for 10 min displayed a disorganized pattern of F-actin staining with clusters of punctate structures. B, We quantified the number of osteoclasts exhibiting actin belts (encompassing at least 75% of the cell periphery). Osteoclasts treated with wortmannin or CAL-120 displayed significantly fewer actin beltsthan vehicle-treated cells, whereas PIK75 and TGX221 had no significant effect. Data are the percentage of osteoclasts exhibiting actin belts and are means ± S.E.M., $n = 3$ independent experiments with a total of 1537 osteoclasts. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. * indicates $p < 0.05$ compared to vehicle.


A
vehicle
i
wortmannin
ii
PIK75
iii
TGX221
iv
CAL-120
v
25 μm
B
Actin belt number (% total)
40
30
20
10
0
*
*
vehicle
wortmn
PIK75
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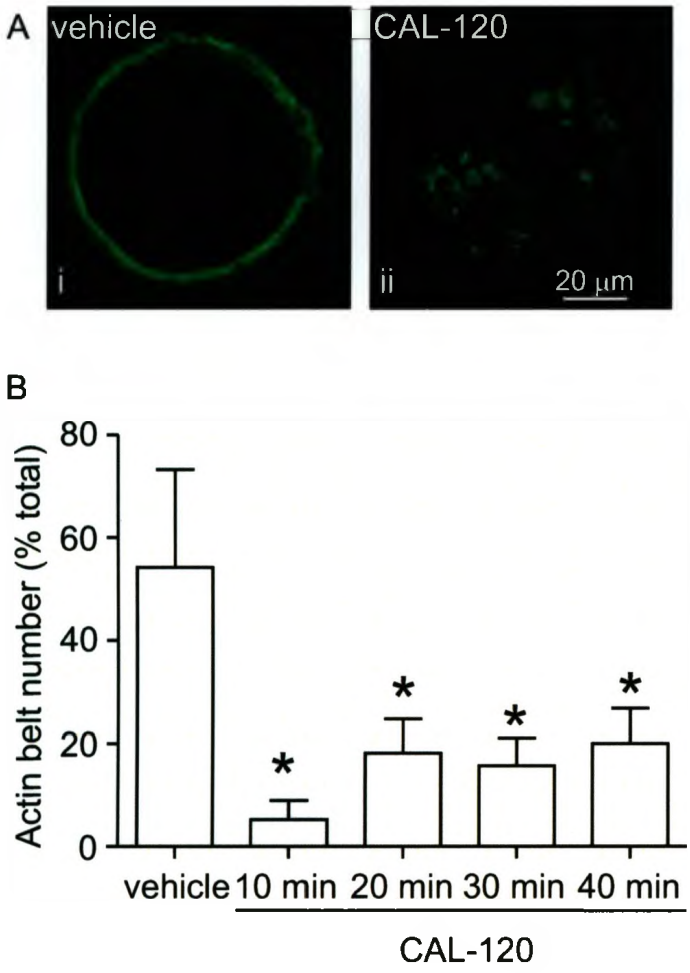

Fig. 2.6. Time course of PI3Kδ-selective inhibitor CAL-120 disruption of F-actin belt. Rat osteoclasts were plated on FBS-coated glass coverslips in HCO_3^--buffered M199 with FBS and antibiotics. Samples treated with vehicle (for 40 min) or CAL-120 (1 μM, for the times indicated) and then fixed. F-actin was labeled using Alexa Fluor 488-conjugated phalloidin (green). A, Representative images of actin belt organization were obtained using a Zeiss LSM 510 META confocal microscope (63x objective). Osteoclast treated with vehicle only for 40 min shows prominent F-actin belt at the cell periphery. In contrast, osteoclast treated with CAL-120 for 20 min shows disorganized clusters of punctate F-actin containing structures. B, The percentage of osteoclasts exhibiting actin belts under each condition was quantified using a Zeiss Axio Observer Z1 microscope (40x objective). Osteoclasts treated with CAL-120 for up to 40 min displayed significantly fewer actin belts than vehicle-treated cells. Data are the percentage of osteoclasts exhibiting actin belts and are means ± S.E.M., n = 4 independent experiments with a total of 1546 osteoclasts. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. * indicates $p < 0.05$ compared to vehicle.

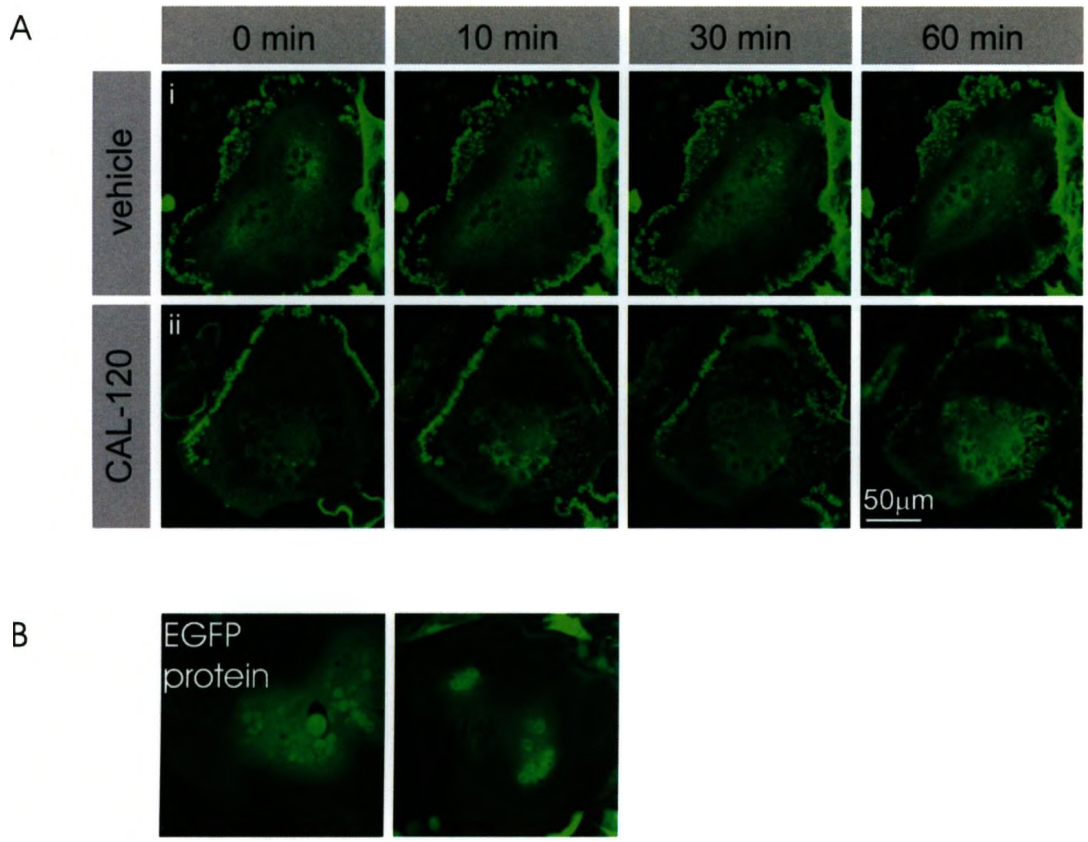

Fig. 2.7. Live cell imaging of the effects of CAL-120 on F-actin belt dynamics in rabbit osteoclast expressing actin-EGFP. To directly observe the effects of the PI3Kδ-selective inhibitor CAL-120 on F-actin belts rabbit osteoclasts were plated on FBS-coated MatTek glass-bottom culture dishes and transduced with adenoviruses expressing actin-EGFP fusion or EGFP proteins. Cells were then bathed in HEPES-buffered M119 medium with 15% FBS at ~26°C and imaged using confocal microscopy (40x objective, Zeiss LSM 510 META confocal microscope). Images labeled 0 min illustrate the appearance of osteoclasts immediately prior to addition of vehicle or CAL-120 (1 μM) to the bath. Ai, F-actin belt remained intact in vehicle-treated osteoclast. Aii, CAL-120 induced gradual disappearance of the peripheral actin structures and an increase in actin fluorescence in the central region of the osteoclast. Images are representative of a total of 4 osteoclasts from 2 independent preparations. B, Control samples transduced with EGFP protein alone showed uniform distribution of fluorescence. See Supplemental Videos 4 & 5 of the responses illustrated in panels Ai and Aii.

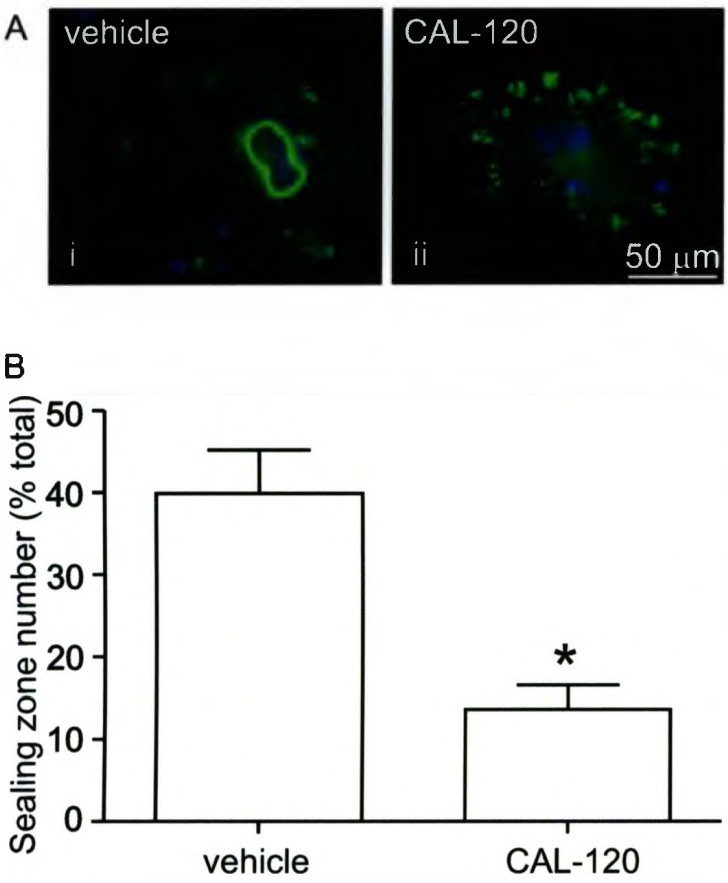

Fig. 2.8. Effect of PI3Kδ-selective inhibitor CAL-120 on sealing zone formation in osteoclasts. Rat osteoclasts were plated on resorbable calcium phosphate-coated discs in HCO_3^--buffered M199 with FBS and antibiotics. Samples were treated with vehicle or CAL-120 (1 μM) for 10 min and then fixed. F-actin was labeled using Alexa Fluor 488-conjugated phalloidin (green), nuclei were stained with DAPI (blue), and cells were examined by fluorescence microscopy (40x objective, Zeiss Axio Observer.Z1). Ai, Untreated osteoclast exhibits a prominent F-actin-rich sealing zone, characteristic of active osteoclasts on resorbable substrates. Note that sealing zones are more centrally located than the peripheral actin belts illustrated in Figs. 6 & 7. Aii, osteoclasts treated with CAL-120 exhibited fewer sealing zones and displayed clusters of F-actin containing structures. B, We quantified the number of osteoclasts exhibiting sealing zones. Osteoclasts treated CAL120 displayed significantly fewer actin belts than vehicle-treated cells. Data are the percentage of osteoclasts exhibiting sealing zones and are means ± S.E.M., $n = 3$ independent experiments with a total of 592 osteoclasts. Data were analyzed by unpaired Student's *t*-test. * indicates $p < 0.05$ compared to vehicle.

osteoclasts for 10 min then cells were analyzed using fluorescence microscopy by an individual blinded to the treatments (Fig. 2.5A). Wortmannin and CAL-120 caused a significant reduction in the incidence of F-actin belts in comparison to vehicle (Fig. 2.5B), whereas TGX221 and PIK75 had no significant effect (Fig. 2.5B). To determine whether disruption of F-actin belts is sustained in the continued presence of CAL-120, osteoclasts were treated with vehicle or CAL-120. CAL-120 treatment resulted in disruption of the cytoskeletal organization compared to vehicle treated (Fig. 2.6A). In the continued presence of CAL-120, osteoclast F-actin cytoskeletal disruption was maintained (Fig. 2.6B). To complement these studies we examined the effect of CAL-120 on F-actin belts in live cells using rabbit osteoclasts virally transduced with EGFP-actin. When viewed using confocal microscopy, vehicle treatment caused no change in F-actin belt organization (Fig. 2.7Ai). In contrast, CAL-120 gradually induced disruption of F-actin belt organization starting within 10 min (Fig. 2.7Aii). See also Supplemental Videos 3 and 4, showing representative appearance of cells treated with vehicle (Suppl. Video 3) or CAL-120 (Suppl. Video 4) (see AVI files on accompanying CD, captions are in Appendix B).

To examine sealing zones, rat osteoclasts were plated on coverslips coated with resorbable calcium phosphate (BD BioCoat™ Osteologic™), treated with vehicle, or CAL-120 (1 μM) for 10 min, fixed, permeabilized and incubated with fluorescently tagged phalloidin (Fig. 2.8A). Notably, sealing zones generally had a smaller diameter than F-actin belts. Vehicle treatment yielded 40% of cells with sealing zones, where as CAL-120 treatment reduced the incidence of intact sealing zones to 10% of cells. To examine the disruption of sealing zones, we used the same protocol as quantifying F-actin

belts. CAL-120 caused a significant reduction in the number of actin rings in comparison to vehicle (Fig. 2.8B). It was determined that the PI3Kδ inhibitor, CAL-120 decreases the incidence of osteoclasts with sealing zones, consistent with the involvement of a specific PI3K isoform in regulating cytoskeletal remodeling in osteoclasts.

2.4.5 Inhibition of PI3K Suppresses the Effects of RANKL on Osteoclast Survival

Bone resorption is proportional to the number of osteoclasts present during any given time, and therefore, survival is a key factor that regulates bone loss *in vivo* (Manolagas, 2000). Therefore, we examined the effect of isoform-selective PI3K inhibitors on osteoclast survival. Rat osteoclasts were placed on FBS-coated coverslips and incubated the absence (control) or presence of RANKL (100 ng/ml), along with vehicle, 1 μM CAL-120, 1 μM TGX221 or 100 nM PIK75. Survival was quantified by counting the number of osteoclasts before and after an 18 h incubation period and the proportion of surviving cells was calculated. Osteoclasts were identified by phase-contrast microscopy as multinucleated cells (≥ 3 nuclei). There were no significant effects of inhibitors on survival under control conditions (Fig. 2.9). However, RANKL significantly enhanced osteoclast survival, and CAL-120, TGX221 and PIK75 suppressed the stimulatory effect of RANKL on survival. These data illustrate a key functional role for PI3K in mediating the effects of RANKL on osteoclast survival.

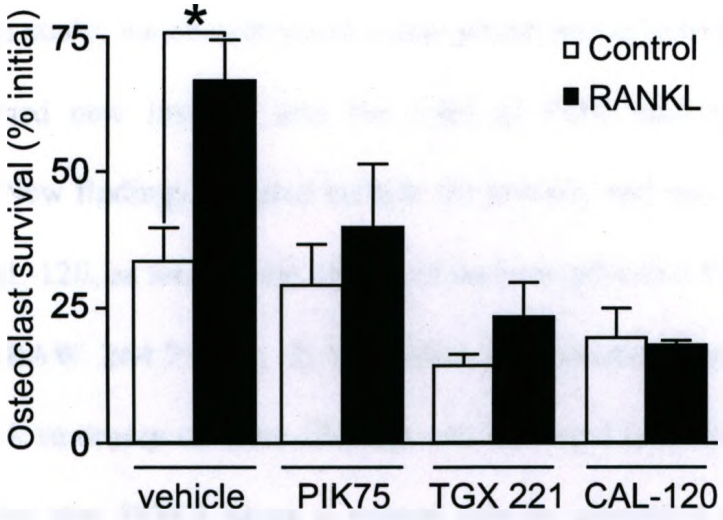

Fig. 2.9. The effects of isoform-selective PI3K inhibitors on osteoclast survival. Rat osteoclasts were plated on coverslips in HCO_3^--buffered M199 with 15% FBS and antibiotics. As indicated, samples were treated with RANKL (100 ng/ml) or its vehicle (Control). In addition, samples were treated with vehicle, PIK75 (100 nM), TGX221 (1 μM) or CAL-120 (1 μM). Survival was assessed by counting (using phase-contrast microscopy) the number of osteoclasts before and after 18 h of culture. As expected, RANKL increased osteoclast survival compared to vehicle. In contrast to their selective effects on cytoskeletal organization, all inhibitors tested (PIK75, TGX221 and CAL-120) suppressed RANKL-induced survival. Data are the number of surviving osteoclasts on each coverslip at 18 h expressed as a percentage of the initial number of osteoclasts on the same coverslip (means ± S.E.M., $n = 3$ independent experiments with a total of 2529 osteoclasts). Data were analyzed by a two-way ANOVA followed by a Bonferroni's multiple comparisons test. * indicates $p < 0.05$ for the effect of RANKL.

2.5 Discussion

In the present study, we characterized a new potent and selective PI3Kδ inhibitor as well as determined new insights into the roles of PI3K isoforms in regulating osteoclast function. New findings reported include the potency and specificity of a novel PI3Kδ inhibitor, CAL-120, as well as the effects of isoform-selective PI3K inhibitors on 1) the viability of RAW 264.7 cells, 2) osteoclast cytoskeletal organization, and 3) osteoclast survival. A summary of these findings can be found in Table 1. For the first time, we demonstrate that PI3Kδ plays a central role in osteoclast morphology and cytoskeletal reorganization, and that PI3Kα, PI3Kβ and PI3Kδ all contribute to osteoclast RANKL-induced survival. Finally, we reported the IC_{50} and EC_{50} values of CAL-120, a potent, reversible and selective cell permeable inhibitor of PI3Kδ. The other PI3K inhibitors used in the present study have been characterized previously (Marone *et al.*, 2008).

The expression of mRNA encoding PI3K isoforms has been documented in monocyte-macrophage-like RAW 264.7 cells with levels from highest to lowest being PI3Kα, PI3Kδ and then PI3Kβ (PI3Kγ not examined) (Grey *et al.*, 2010). Since osteoclasts are isolated with a number of other cell types, it is difficult to accurately determine the expression levels of PI3K in authentic osteoclasts. The finding that PI3Kδ expression is high in cells of hematopoietic lineage (Chantry *et al.*, 1997) suggests that the δ isoform may serve important function in osteoclasts.

Genetically modified mice, generated either by deletion of PI3K p110δ or by mutation of the kinase domain, exhibit severely altered immune and inflammatory responses compared to their wild-type controls (Vanhaesebroeck *et al.*, 2005). For

PI3K Inhibitor	Target	Promote Retraction	Disrupt F-actin Belts	Suppress Survival
PIK75	α	-	-	+
TGX221	β	-	-	+
AS252424	γ	-	n/t	n/t
CAL-120	δ	+	+	+
Wortmannin	Pan-PI3K	+	+	n/t

Table 2.2. Summary of findings. + symbol represents positive, – symbol represents a negative and n/t represents not tested. Wortmannin, LY294002, CAL-120, GDC0941 and IC87114 induced osteoclast retraction, whereas TGX221, PIK75 and AS242525 did not, consistent with involvement of the PI3Kδ isoform in regulating cytoskeletal remodeling in osteoclasts. CAL-120 and wortmannin, but not TGX221 and PIK75, disrupted F-actin belts which are important for osteoclast function. Further, CAL-120 decreased sealing zone number in osteoclasts on calcium phosphate surfaces. CAL-120, TGX221 and PIK75 all suppressed RANKL-induced osteoclast survival. In conclusion, these data are consistent with a key role for the PI3Kδ in regulating osteoclast morphology and cytoskeletal function. In contrast, a non-redundant role of Class IA PI3K isoforms was observed in osteoclast survival.

instance, studies of mice with a loss of function genetic alteration in the PI3Kδ subunit exhibit defects in B- and T-cell signaling, including improper maturation, defective antigen receptor signaling, and impaired humoral immune responses (Ramadani *et al.*, 2010; Uno *et al.*, 2010). Recent data in macrophages demonstrate that PI3Kδ is localized around the Golgi membrane and seems to be important in vesicle trafficking (Low *et al.*, 2010). Moreover, inhibition of PI3Kδ in neutrophils leads to defects in chemotaxis (Afonso & Parent, 2010). Our study is the first to establish roles for PI3Kδ in osteoclast survival and function.

PI3K has been shown to participate in lamellipodia spreading of osteoclasts (Palacio & Felix, 2001) and other cell types (Di Marzio *et al.*, 2005; Bagorda *et al.*, 2006; Weiger *et al.*, 2009). The results of our study show that blocking PI3Kδ pharmacologically causes retraction of osteoclast lamellipodia; at the same time, peripheral pseudopod ruffling is maintained. PI3K signaling might initiate osteoclast spreading through interactions with other signaling pathways, including Rho, Rac and Cdc42. It has been shown by others that activation of Rho and inhibition of Rac induces retraction of macrophage-derived multinucleated cells (Ory *et al.*, 2000). In keeping with this idea, RhoA and Rac have been shown to be regulated negatively and positively by PI3Kδ, respectively (Eickholt *et al.*, 2007; Papakonstanti *et al.*, 2008). Consistent with our findings, others have shown acute morphological contraction in macrophages upon PI3Kδ inactivation observed in parallel with decreased Rac activity and increased Rho activity (Papakonstanti *et al.*, 2007). Therefore, it would be of interest in future studies to examine the possible role of Rho and Rac in mediating the lamellipod retraction elicited by blocking PI3Kδ in osteoclasts.

Osteoclast retraction is also caused by the hormone calcitonin, and by the bioactive lipid mediators lysophosphatidic acid (LPA) and platelet-activating factor (PAF) which signal through GPCRs (Wood *et al.*, 1991; Gravel *et al.*, 1994; Lapierre *et al.*, 2010). Specifically, calcitonin-induced retraction of lamellipodia is sustained while osteoclasts remain quiescent (Komarova *et al.*, 2003), whereas LPA-induced retraction of lamellipodia is sustained and peripheral pseudopod ruffling is sustained (Lapierre *et al.*, 2010). Furthermore, PAF-induced retraction of osteoclast lamellipodia is not sustained over time resulting in re-spreading of lamellipodia (Wood *et al.*, 1991). Interestingly, the morphological responses observed during inactivation of PI3Kδ are similar to LPA-induced retraction and distinct from the responses elicited by calcitonin and PAF. Further studies are required to determine if there is possible cross-talk between LPA signaling and PI3Kδ signaling. In the present study, we found that TGX221 induced partial retraction of osteoclasts. TGX221 is a potent and selective cell permeable inhibitor of PI3K p110β relative to all other PI3K isoforms except PI3Kδ (IC_{50} values: p110α 1000 nM; p110β 9 nM; p110δ 210 nM) (Jackson *et al.*, 2005). Likely, the concentration used in our study (1 μM) fully inhibits PI3Kβ and partially blocks PI3Kδ accounting for its effect on retraction.

Activation of the αvβ3 integrin in osteoclasts initiates a signaling cascade which is known to involve a c-Src, Pyk2 and c-Cbl complex, and PI3K, resulting in actin polymerization and cytoskeletal reorganization (Chellaiah & Hruska, 1996; Faccio *et al.*, 2002; Novack & Faccio, 2009). Since osteoclasts lacking β3 have an abnormal cytoskeleton, fail to spread and do not have sealing zones *in vitro* (McHugh *et al.*, 2000), it is possible that αvβ3 integrins are required to activate PI3Kδ to mediate its effects on

the cytoskeleton. Further study is needed in order to elucidate the possible association of PI3K isoforms and αvβ3 integrins.

Mature osteoclasts exhibit two types of specialized actin structures, F-actin belts and sealing zones, when adherent to non-mineralized or mineralized substrata, respectively. It is suggested that osteoclasts only degrade substrate within the area defined by specialized actin structures (Badowski *et al.*, 2008) and, therefore, bone resorption by osteoclasts is dependent on the integrity of the actin cytoskeleton (Jurdic *et al.*, 2006; Ory *et al.*, 2008). The results from our study show that the incidence of F-actin belts and sealing zones in osteoclasts is markedly lower when PI3Kδ is inhibited. Live cell imaging on osteoclasts shows gradual disruption of F-actin belts into puncta along with break down of actin structures. This finding is in accordance with the observation that inhibition of PI3K disrupts ring-like F-actin structures in murine osteoclast-like cells (Nakamura *et al.*, 1995; Lakkakorpi *et al.*, 1997). Our results indicate that PI3K p110δ inhibition causes both retraction and disruption of F-actin belts and sealing zones, suggesting that the two events are correlated. However, as discussed previously, the exact mechanisms by which PI3K affects the actin cytoskeleton are currently unknown. Rho and Rac have an antagonistic relationship affecting cell spreading, and both have been implicated in regulating the formation of F-actin belts and sealing zones (Razzouk *et al.*, 1999; Chellaiah *et al.*, 2000; Ory *et al.*, 2008). Specifically, a constitutively activated form of Rho stimulates gelsolin-associated PI3K activity resulting in podosome assembly in osteoclasts (Chellaiah *et al.*, 2000). F-actin belts and sealing zones have been shown to regulate contractility of osteoclasts adherent to non-mineralized or mineralized substratum in part through myosin II (Chabadel *et al.*, 2007). Furthermore, compounds

that affect osteoclast retraction also disrupt sealing zones and the ability of osteoclasts to form pits on dentine slices, such as the hormone calcitonin (Suzuki *et al.*, 1996; Yamamoto *et al.*, 2006).

The lifespan of osteoclast precursors and mature osteoclasts, in conjunction with their differentiation and activation, contribute to the control of bone resorption (Manolagas, 2000). Several ligands may activate PI3K and regulate osteoclast survival. For example, in previous studies, RANKL has been shown to activate PI3K/Akt/mTOR signaling pathway, thereby enhancing osteoclast survival (Glantschnig *et al.*, 2003; Aeschlimann & Evans, 2004). RANKL also activates NF-κB signaling and activation of calcineurin and NFATc1, promoting osteoclastogenesis and enhancing cell survival (Aeschlimann & Evans, 2004; Novack & Teitelbaum, 2008). According to our results, all Class IA PI3K isoforms play a role in regulating RANKL-induced cell survival, but do not affect basal levels of survival. In support of our findings, others have shown that the activity of any Class IA PI3K isoform can sustain survival of other cell types (Foukas *et al.*, 2010). Specifically, leukocytes and fibroblasts continue to proliferate and survive during PI3K0α or PI3Kδ inactivation by increasing p110β signaling and enhancing input from the ERK pathway (Foukas *et al.*, 2010). Similar results in embryonic fibroblasts show that PI3Kα or PI3Kβ alone can sustain cell proliferation and survival (Foukas *et al.*, 2010; Matheny & Adamo, 2010). These data demonstrate functional redundancy of PI3K isoforms and that only a small subset of Class IA PI3K activity is necessary for osteoclast survival. In contrast, it has been suggested that PI3Kα is most important for the survival of other cell types (Niedermeier *et al.*, 2009).

Overall, our findings provide evidence for redundant and non-redundant functions of different PI3K isoforms and show for the first time an important role for PI3Kδ in regulating osteoclast morphology and cytoskeletal function. Knowledge of the important roles of each Class I PI3K family member in various physiological and pathophysiological processes may allow isoform-selective PI3K inhibitors to be used therapeutically. In particular, the PI3Kδ isoform, which has more limited tissue distribution than PI3Kα and PI3Kβ is an attractive target for novel anti-resorptive therapeutics.

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CHAPTER THREE

General Discussion

3.1 Summary and Conclusions

Objective #1: To characterize the effects of isoform-selective PI3K inhibitors on the viability of monocyte-macrophage-like cells.

Effects of isoform-selective PI3K inhibitors on the viability of RAW 264.7 cells were investigated using an MTT assay. High concentrations of PIK75 and LY294002 decreased viability, whereas GDC0941, TGX221, CAL-120, IC87114, AS252424 and wortmannin had no significant effects.

Objective #2: To investigate the effects of isoform-selective PI3K inhibitors on osteoclast morphology and motility.

Live cell microscopy revealed that inhibition of the PI3Kδ isoform induces retraction of lamellipodia in primary rat osteoclasts under basal conditions. Although lamellipodia retracted, peripheral pseudopods still remained motile.

Objective #3: To determine the effect of isoform-selective PI3K inhibitors on cytoskeletal organization in osteoclasts on varying substrata.

Fluorescence microscopy revealed that the PI3Kδ isoform is important in F-actin organization in rat primary osteoclasts plated on glass and on calcium phosphate surfaces.

Objective #4: To evaluate the effect of isoform-selective PI3K inhibitors on osteoclast survival.

All isoform-selective Class IA PI3K inhibitors suppressed RANKL-induced survival.

3.2 Contributions of the Research to the Current State of Knowledge

General significance—Prior to the work reported in this thesis, knowledge of the effects of isoform-selective PI3K inhibitors on osteoclasts was limited. The overall objective of this research was to investigate the effect of isoform-selective PI3K inhibitors in osteoclasts. The key implication of this study is that PI3Kδ plays an important role in regulating osteoclast morphology and cytoskeletal organization; whereas, multiple PI3K isoforms contribute to the control of osteoclast survival. Thus, the PI3Kδ isoform, which has more limited tissue distribution than PI3Kα and PI3Kβ, is an attractive target for reducing bone loss in diseases, such as metastatic bone cancer and inflammatory bone diseases.

Cancer metastasis and osteolytic lesions—Common human cancers, including lung, prostate and breast often metastasize to bone due to its blood vessel density and abundance of growth factors, which support the invasion and proliferation of cancer cells (Mundy, 2002). Tumours in bone frequently promote the formation and activation of osteoclasts, leading to bone resorption and subsequent tumour growth. Cancer cell proliferation in bone mainly occurs because osteoclastic resorption releases growth factors from the matrix, such as IGF-1, TGFβ, fibroblast growth factor and platelet-derived growth factor (Mohan & Baylink, 1991; Mundy, 2002, Kingsley, 2007 #97). Interestingly, all of the above growth factors signal through receptor tyrosine kinases, which directly or indirectly activate the PI3K/Akt pathway (Brader & Eccles, 2004; Stitt *et al.*, 2004; Assinder *et al.*, 2009). Furthermore, tumor progression is often associated with excessive activation of the PI3K pathway, whether it is due to activating mutations

of the PI3K catalytic p110 subunit, regulatory p85 subunit, or deletion of the PTEN inhibitory protein (Liu *et al.*, 2009; Vanhaesebroeck *et al.*, 2010).

There is extensive evidence that the PI3K pathway affects osteoclast survival, in particular through RANKL signaling (Yang *et al.*, 2008; Yi *et al.*, 2008). Tumour cells release factors which act on neighbouring cells (such as T cells or osteoblasts) to release RANKL. In turn, RANKL binds RANK receptors expressed on tumour cells which promote migration and proliferation (Huang *et al.*, 2002; Jones *et al.*, 2006). As mentioned above, RANKL also binds to RANK on osteoclasts to promote bone resorption and the release of bone-derived growth factors.

Recently developed isoform-selective PI3K inhibitors show considerable promise for the treatment of cancer and are making their way into the early phases of clinical trials (Lannutti *et al.*, 2011). The findings in Chapter Two suggest a novel therapy for skeletal metastases – it may be possible to identify isoform-selective PI3K inhibitors that suppress metastatic tumour cell growth, as well as inhibit bone resorption. Thus, pharmacological inhibition of the PI3K signaling axis may reduce tumor proliferation in bone, and limit the progression of osteolytic lesions.

PI3K and inflammatory bone diseases— Patients suffering from arthritis have a marked decrease in quality of life. For example, rheumatoid arthritis (RA) is a chronic, progressive, debilitating inflammatory disease that affects approximately 1% of the world's population (Rommel *et al.*, 2007). Matrix-degrading enzymes such as metalloproteinases were initially thought to be the sole cause of bone and cartilage destruction in arthritis. However, cell-mediated mechanisms, involving osteoclast activation, are now thought to be important contributors (Takayanagi, 2009).

Identification of osteoclast-like giant cells at the interface between the synovium and bone in rheumatoid joints dates back to the early 1980's (Takayanagi, 2009). It has been determined that osteoclasts are responsible for the resorption of mineralized extracellular tissue, which contributes to the damage, pain and deformity associated with RA (Schett, 2008). In the pathogenesis of bone destruction associated with RA, the synovium is a site of active interplay between immune and bone cells. Furthermore, RA synovial fluid contains osteoclast precursors as well as cells that support osteoclastogenesis (Takayanagi, 2009). It is important that osteoclast activity be tightly regulated for skeletal and joint homeostasis. Osteoclast activation and apoptosis represents a critical point at which bone resorption can be minimized during RA. In osteoclasts, PI3K is activated by RANKL and MCSF, two cytokines that are critical in osteoclast development (Novack & Teitelbaum, 2008; Mandal *et al.*, 2009).

Interestingly, selective PI3Kδ and PI3Kγ inhibitors suppress joint inflammation and bone erosion in mouse and rat RA models (Rommel *et al.*, 2007). Evidence so far suggests that PI3Kδ and PI3Kγ operate as partners in distinct, yet co-dependent signaling pathways in many immune cells, such as B cells, T cells, macrophages and mast cells (Rommel *et al.*, 2007). This strongly indicates that, in addition to RA, these enzymes are potential therapeutic targets for inflammatory disease. Therefore, it is possible that isoform-selective PI3K inhibitors may be useful for the treatment of inflammatory bone diseases such as RA by suppressing the inflammatory response and inhibiting osteoclast activity.

3.3 Limitations of the Research and Suggestions for Future Studies

In vitro experiments – *In vitro* studies are important for understanding the mechanisms of cell physiology. The main advantages of *in vitro* experiments are that they allow for the precise control of concentrations of studied compounds and that results are usually swift. *In vitro* studies do have their limitations, such as culture artifacts. However, it is difficult to monitor the activity of PI3Ks in cells *in situ* (Vanhaesebroeck *et al.*, 2005).

Pharmacological inhibitors were used in determining the roles of PI3K isoforms in osteoclasts and it is possible that inhibitors had non-specific effects. It would have been ideal to perform full concentration-response studies for the effects of inhibitors on the morphology of primary osteoclasts. However, it should be noted that these studies involve lengthy time-lapse recordings of single osteoclasts, precluding us from carrying out full concentration-dependence studies. It would be desirable to validate the findings in Chapter Two using knockdown techniques such as shRNA targeting PI3Kδ, or by obtaining osteoclasts from PI3Kδ 'knockout' or 'kinase-deficient knock in' mice. Moreover, studies of such genetically modified mice would allow for determination of a bone phenotype, which has not yet been documented. Further study is needed to elucidate the possible effects of genetic modification of PI3K isoforms on the skeleton *in vivo*.

Another limitation of the present studies is the possibility that contaminating cell types may release signaling factors that act on osteoclasts and, thus, produce indirect effects in response to PI3K inhibitors.

A number of questions remain. These include: whether certain PI3K isoforms produce specific pools of PIP_3 located on the plasma membrane, endosomes (Sato *et al.*,

2003) or nucleus (Lindsay *et al.*, 2006); and whether PI3K isoforms interact with other proteins. The roles of many phosphatases that regulate and diversify PI3K signaling are undetermined and further complicate the situation. Future investigations should include lipid analysis, for instance by mass spectrometry, to study the phospholipid profile in primary cells (Vanhaesebroeck *et al.*, 2005).

RAW 264.7 cells (rather than primary osteoclasts) were selected for Objective #1 because they are an osteoclast precursor cell model and because primary cultures of osteoclasts always include other cell types.

PI3K signaling in osteoclasts – The data presented in Chapter Two do not fully explain the PI3K signaling pathway and the ligands which activate it. Further studies are required to determine the mechanisms leading to retraction of osteoclast lamellipodia and the influence of PI3Kδ, as well as the signaling pathways in which multiple PI3K isoforms decrease osteoclast survival.

Suggestions for future work include determining the relative expression levels of the different PI3K isoforms in osteoclasts but, as mentioned above, it is difficult to obtain pure preparations of osteoclasts. The purest system presently available is probably osteoclasts differentiated from bone marrow cell cultures. Using pharmacological approaches, we have identified important roles for specific PI3K isoforms in osteoclasts. However, we have not yet determined whether this is due to differences in i) the expression of these isoforms, ii) their intrinsic biochemical activity, or iii) their localization within the osteoclast. Further studies are clearly needed.

PI3K signaling in osteoclastogenesis – RANKL-RANK-mediated interaction activates tumour necrosis factor receptor-associated factor 6 (TRAF6), c-src and PI3K

and later c-fos, NF-κB and NFATc1 to drive osteoclastogenesis (Aeschlimann & Evans, 2004). The importance of c-fos, NF-κB and NFATc1 in osteoclastogenesis is demonstrated in knockout mice of each signaling molecule – all of which exhibit osteopetrosis (Grigoriadis *et al.*, 1994; Asagiri & Takayanagi, 2007). A recent study by Kang and colleagues report that genetic inactivation of the *p110γ* gene leads to an increase in bone mass, likely due to impairment in osteoclastogenesis (Kang *et al.*, 2010). PI3K p110γ and p110δ have been shown to activate NF-κB in other cell types so it is plausible that they could be having an effect on osteoclast precursors (Wang *et al.*, 2011). It would be interesting to know the bone phenotype produced by a *p110δ* knock out and the effects p110δ have on c-fos, NF-κB and NFATc1 expression. The mice have been generated but the effect on the skeleton has not yet been documented. It is possible that PI3K p110 plays a role in osteoclastogenesis, and further studies are required to investigate this process.

PI3K signaling in osteoclast survival and apoptosis – It will be of interest to extend our studies of osteoclast survival by looking at the effects of isoform-selective PI3K inhibitors on apoptosis. Apoptosis normally controls and regulates excessive proliferation and damage to cells by eliminating them (Chang *et al.*, 2003). When apoptosis is compromised, cell survival is prolonged and problems such as cancer can arise. The Bcl-2 family of proteins plays a critical role in the regulation of apoptosis. These proteins include both pro-apoptotic proteins such as, BAD and BAX as well as anti-apoptotic proteins such as Bcl-2 and Bcl-x_l (Chang *et al.*, 2003). The balance of anti-apoptotic and pro-apoptotic proteins is thought to dictate whether or not a cell will survive or undergo apoptosis. Stimulation of PI3K leads to activation of Akt, which has

been shown to increase cell survival in response to IGF-1 (Vivanco & Sawyers, 2002). Also, constitutively active Akt rescues cells during PTEN-mediated apoptosis (Li *et al.*, 1998). There are several possible mechanisms by which Akt may protect cells from apoptosis: i) phosphorylation of the pro-apoptotic protein BAD, which causes its dissociation from anti-apoptotic protein BCL-x_l (normally, BAD forms a non-functional heterodimer with the BCL-x_l (Vivanco & Sawyers, 2002)); ii) phosphorylation of the pro-apoptotic protease, caspase-9, inhibiting catalytic activity (Cardone *et al.*, 1998); and iii) phosphorylation of the Forkhead family of transcription factors, which inhibits translocation into the nucleus (Forkhead transcription factors normally cause up-regulation of several pro-apoptotic proteins such as FAS ligand (Vivanco & Sawyers, 2002)). Future studies should include apoptosis assays to determine the effect of PI3K inhibitors on osteoclast apoptosis. To elucidate the mechanism, it would be interesting to look at expression levels of anti-apoptotic proteins such as Bcl-2 or Bcl-x_l, or pro-apoptotic proteins such as caspase-9 during blockade or inactivation of specific PI3K isoforms.

In summary, for the first time, we have described the potency and specificity of a novel PI3Kδ inhibitor, CAL-120. We also demonstrate the effects of isoform-selective PI3K inhibitors on the viability of RAW 264.7 cells and on the cytoskeletal organization and survival of primary osteoclasts. For the first time, we demonstrate that PI3Kδ plays a central role in regulating osteoclast morphology and cytoskeletal organization, and that PI3Kα, β and δ all contribute to RANKL-induced survival. PI3Ks are promising targets for therapeutic intervention in metabolic, immunological and oncological diseases (Vanhaesebroeck *et al.*, 2010). Interference with PI3K signaling should be targeted at a

specific PI3K isoform to reduce side-effects. The potential exists for identifying isoform-selective PI3K inhibitors that target both osteoclasts and tumor cells for the treatment of metastatic bone disease, or osteoclasts and immune cells for the treatment of inflammatory diseases such as RA. In particular, PI3Kδ, which has more limited tissue distribution than PI3Kα and PI3Kβ is an attractive target for the future development of anti-resorptive therapeutics.

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APPENDIX A

Ethics Approval of Animal Use

Western

2008-043-06::3:

AUP Number: 2008-043-06
AUP Title: Role of cytosolic calcium in the regulation of osteoclasts and bone resorption.
Ion Transport and Signalling in Skeletal Cells: P2 Nucleotide Receptor Function in Bone

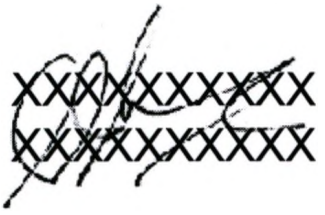
The YEARLY RENEWAL to Animal Use Protocol (AUP) 2008-043-06 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Thompson, Sharla H
on behalf of the Animal Use Subcommittee



The University *of* Western Ontario
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APPENDIX B

Supplementary Video Captions

Suppl. Video 1. No Effect of Vehicle on Osteoclast Morphology or Motility. Rat osteoclasts were bathed in HEPES-buffered M199 medium with 15% FBS medium at 35°C and imaged using time-lapse phase-contrast microscopy. Video begins at 0 min, vehicle was added at 24 min and video ends at 60 min. Image intervals are 1 min and frames are shown at 10 frames/s. Vehicle was added ~ 2 s into the video. Width of field is 320 μm. Selected frames from this video are shown in Fig. 2.2A.

Suppl. Video 2. PI3Kδ Isoform-Selective Inhibitor, CAL-120, Induces Osteoclast Retraction Without Inhibiting Pseudopod Motility. Rat osteoclasts were bathed in HEPES-buffered M199 medium with 15% FBS medium at 35°C and imaged using time-lapse phase-contrast microscopy. Video begins at 0 min, CAL-120 (1 μM) was added at 24 min and video ends at 60 min. Image intervals are 1 min and frames are shown at 10 frames/s. CAL-120 was added 2 s into the video. Width of field is 320 μm. Selected frames from this video are shown in Fig. 2.2E.

Suppl. Video 3. No Effect of Vehicle on Actin Organization in Osteoclasts. Rabbit osteoclasts were plated on FBS-coated MatTek glass-bottom culture dishes and transduced with adenoviruses expressing actin-EGFP fusion. Cells were then bathed in HEPES-buffered M199 medium with 15% FBS at ~26°C and imaged using confocal microscopy. Video begins at 0 min, vehicle was added at 7.5 min and video ends at 90 min. Image intervals are 1.5 min and frames are shown at 12 frames/s. CAL-120 was added at immediately into the video. Width of the field is 225 μm. Data obtained from this video are included in Fig. 2.7Ai.

Suppl. Video 4. PI3Kδ Isoform-Selective Inhibitor, CAL-120 Disrupts Actin Organization in Osteoclasts. Rabbit osteoclasts were plated on FBS-coated MatTek glass-bottom culture dishes and transduced with adenoviruses expressing actin-EGFP fusion. Cells were then bathed in HEPES-buffered M199 medium with 15% FBS at ~26°C and imaged using confocal microscopy. Video begins at 0 min, CAL120 (1 μM) was added at 7.5 min and video ends at 90 min. Image intervals are 1.5 min and frames are shown at 12 frames/s. CAL120 was added at immediately into the video. Width of the field is 225 μm. Data obtained from this video are included in Fig. 2.7Aii.