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Antibody mediated “Universal” Osteoclast targeting platform

by

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ABSTRACT

Osteoclasts are the sole cells responsible for bone resorption and their activity is central to the process of bone remodeling. Excessive osteoclast activity leads to increased bone resorption, predisposing individuals to bone conditions such as osteoporosis, Paget's disease and the focal bone erosions seen in rheumatoid arthritis. Hence, the pharmacological arrest of osteoclasts is the mainstay of treating many bone diseases. However, none of the current antiresorptive therapies for bone loss target osteoclast cells directly. As osteoclasts express the Receptor Activator of Nuclear factor Kappa B (RANK) receptor, the essential signaling receptor for osteoclast differentiation, it was hypothesized that antibody-like molecules generated with specificity against RANK could selectively target and deliver conjugated drug cargo to the osteoclast.

The objectives of this thesis were to generate, characterize and evaluate monoclonal antibodies against the osteoclast RANK receptor, in order to develop a pharmaceutical platform capable of selective and targeted drug delivery to osteoclast cells. Using hybridoma technology, a specific monoclonal antibody against recombinant human RANK receptor was generated. Synthesis of osteoclast-targeting bioconjugates with that antibody was conducted using the antiresorptive peptide hormone calcitonin. Conjugate characterization was undertaken and its efficacy

tested on osteoclast cell cultures using various osteoclast specific assays. Both the conjugate as well as the antibody itself showed remarkable inhibition of osteoclast activity. The desirable result obtained with the mere binding of the antibody to the receptor led us towards new research work, focusing on valuable scale-up production and therapeutic use.

As a new research direction, single-chain Fraction variable (scFv) antibody-like molecules was expressed against the human RANK receptor using phage display technology, to circumvent complications associated with murine-derived antibodies. The Anti-RANK scFv showed specificity towards osteoclast RANK receptors and also showed an inhibitory effect on osteoclast activity.

With the increase in development trends for biologics as therapeutics and the growing knowledge on the importance of osteoclast targeted therapy, this novel biologic reagent may find utility as “universal osteoclast targeting platform”. This may provide a meaningful strategy in terms of osteoclast targeting and drug delivery with the aim of treating or controlling the progression of osteoclast related bone disorders.

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LIST OF ABBREVIATIONS

Ab	Antibody
ADC	Antibody Drug Conjugate
ADEPT	Antibody-directed enzyme prodrug therapy
ANOVA	Analysis of variance
AP-1	Activator protein-1
APS	Ammonium persulfate
ATCC	American type cell culture
ATPase	Adenosine triphosphatase
BCA	Bicinchoninic acid
BMD	Bone mineral density
BMU	Basic multicellular unit
BP	Bisphosphonates
BMPs	Bone morphogenic proteins
BRM	Biological response modifier
BRON	BP related osteonecrosis
BSA	Bovine serum albumin

cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CDR	Complementary determining Region
CFA	Complete Freund’s adjuvant
CT	Calcitonin
CTR	Calcitonin receptor
Cys	Cysteine
DAPI	4',6-diamidino-2-phenylindole
DC-STAMP	Dendritic cell-specific transmembrane protein
DEXA	Dual energy X-ray absorptiometry
Disodium-EDTA	Disodium ethylenediaminetetraacetic acid
DMARDs	Disease modifying anti-rheumatic drugs
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DTNB	5,5'-dithio-*bis*-(2-nitrobenzoic acid)
ECL	Enhanced chemiluminescence

E. coli	Escherichia coli
EGFR	Epidermal growth factor receptor
EIA	Enzyme immune assay
ELISA	Enzyme linked immuno sorbent assay
FBS	Fetal bovine serum
FcRγ	Fc receptor common γ subunit
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GAM-HRPO	Goat anti-mouse IgG conjugated with horseradish peroxidase
HA	Hydroxyapatite
HAMA	Human anti-mouse antibody
HAT	Hypoxanthine aminopterin thymidine
HBSS	Hank's balanced salt solution
HC	Hypercalcaemia of bone cancers
HER	Human Epidermal growth factor Receptor
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horse radish peroxidise

HRT	Estrogen hormonal replacement therapy
BMX	3-isobutyl-1-methyl-xanthine
Ig	Immunoglobulin
IL-1	Interleukin-1
IM	Intramuscular
IPTG	Isopropyl thiogalactopyranoside
ITAM	Immunoreceptor tyrosine based activation motif
IV	Intravenous
kDa	Kilodalton
Lys	Lysine
mAb	Monoclonal antibody
mAb-sCT	Monoclonal antibody-salmon calcitonin conjugate
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
µl	Microlitre
M-CSF	Macrophage-colony-stimulating factor
MITF	Microphthalmia-associated transcription factor
MMPs	Matrix metalloproteinases

mRNA	Messenger ribonucleic acid
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide
MWCO	Molecular weight cut off
NaCl	Sodium Chloride
NFATc1	Nuclear factor of activated T cells c1
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NHS-PEG-MAL	Maleimide-PEG-N-hydroxy succinimidyl carboxy methyl ester
nM	Nanomolar
nm	Nanometer
Ni-NTA	Nickel-nitrilotriacetic acid
NSAIDs	Non-steroidal anti-inflammatory agents
OA	Osteoarthritis
OC	Osteoclast
OD	Optical density
OP	Osteoporosis
OPG	Osteoprotegerin
OSCAR	Osteoclast specific immunoreceptor associate receptor

OVX	Ovariectomized
PBS	Phosphate buffer solution
PBST	Phosphate buffer containing tween 80
PD	Paget's disease
PEGs	Polyethylene glycols
PMOP	Postmenopausal osteoporosis
pNPP	Para-nitrophenyl phosphate
PPi	Inorganic pyrophosphate
PSG	Penicillin, Streptomycin, L-Glutamine
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor Kappa B
RANKL	Receptor activator of nuclear factor Kappa B-ligand
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI-1640	Roswell park memorial institute-1640
s. c.	Subcutaneous
scFv	Single chain variable fraction

sCT	Salmon calcitonin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERMs	Selective estrogen receptor modulators
TGF−β	Transforming growth factor- β
TK	Thymidine kinase
TMB substrate	3, 3', 5, 5'- Tetramethylbenzidine
TNF	Tumor Necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	Tumor necrosis factor receptor associate factor
TRAP	Tartrate resistant acid phosphatase

CHAPTER 1: BACKGROUND

1.1. Osteoclast and bone biology

Bone is a dynamic tissue that is constantly remodeled throughout life. From a biological point of view, bone is a complex living tissue in which this composite material of organic and mineral components is created and maintained by at least three major cell types, namely osteoclasts, osteoblasts and osteocytes (Bilezikian *et al.*, 2002). Multinucleate osteoclasts are responsible for bone resorption. Their chief functional characteristic is the ability to pump acid into specialized resorption pits to dissolve bone mineral as well as to provide an optimum environment for the enzymatic degradation of demineralized extracellular bone matrix. Osteoblasts are known for their osteogenic properties. They form a tight layer of cells on bone surface and are responsible for the production of the extracellular matrix and its mineralization (Lecanda *et al.*, 1998). Osteocytes are fully differentiated osteoblasts. They respond to stress by signalling to osteoblast to initiate bone resorption or bone formation (Donahue *et al.*, 1995). Once entrapped in the bone matrix (osteoid), osteoblasts undergo a morphological change to become osteocytes (Han *et al.*, 2004).

In mature healthy bone, the amount of bone formation by osteoblasts balances the amount of bone resorption by osteoclasts. During bone modeling, formation and resorption can occur independent of

one another. The volume of bone that is resorbed is replaced by an equal volume of new bone and hence, does not result in a net gain or loss of bone. Approximately 10% of the total bone content is replaced per year in adult humans (Alliston & Derynck, 2002).

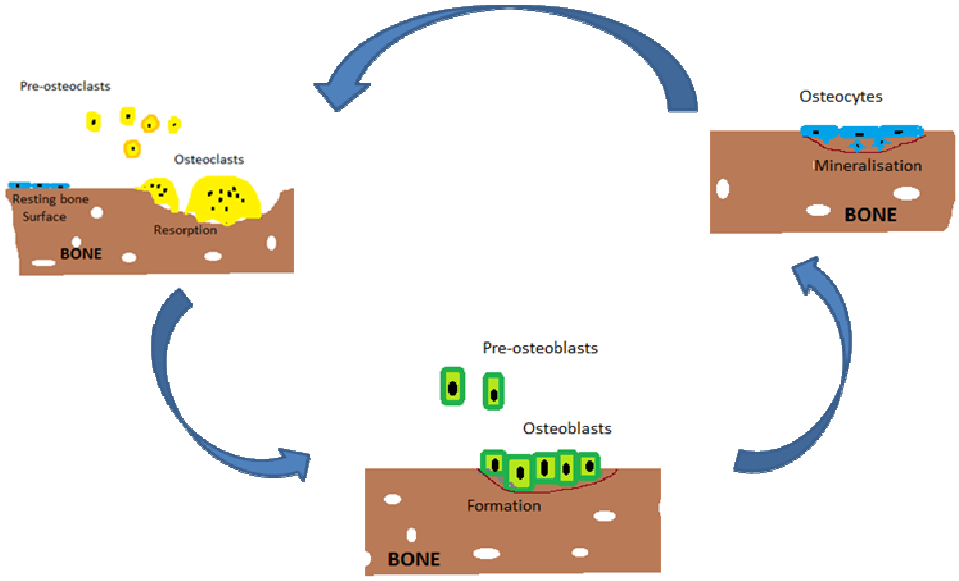

Figure 1: *Representation of the bone remodeling cycle.*

A general change in bone shape is termed bone “modeling” while an internal change and replacement of bone is termed “remodeling”. The replacement of older bone by newer bone occurs by bone remodeling and is accomplished by a discrete temporary anatomical structure in bone, the “basic multicellular unit” (BMU), sometimes referred to as the bone remodeling unit. The bone remodeling process consists of 4 phases: activation, resorption, reversal and formation. During activation,

osteoclast-precursor cells interact with lining cells (derived from osteoblasts) covering bone surfaces. The precursor cells settle on the exposed bone surface and respond to signals from various cytokines to fuse and form multinucleated osteoclasts. Once the osteoclasts are activated, the 2nd phase of bone remodeling i.e bone resorption occurs. After bone resorption has ended, reversal phase occurs where osteoclasts disappear from the bone surface and osteoblast-precursor cells come into action. Osteoblast-precursor cells attach to the resorbed bone surface, differentiate into mature osteoblasts and participate in bone formation, the last phase of bone remodeling (Dempster & Lindsay, 1993; Teitelbaum & Ross, 2003; Raisz, 2005).

The bone formation phase is significantly longer than the previous 3 phases and it involves osteoblasts producing new organic matrix at the resorbed surface. The average lifespan of an osteoclast is about 12 days. The resorbed bone begins to be replaced by a team of new osteoblasts, which takes approximately 3 months. During this process, the osteoblasts become progressively flatter and wider; some become entombed in the bone as osteocytes, some die by apoptosis; and those remaining when the process is completed become the lining cells that cover the new quiescent surface (Bilezikian *et al.*, 2002). Thus, the lifespan of an individual matrix-synthesizing osteoblast varies from a few days to about 100 days (Jilka, 2003). Resorption phases are shorter in duration and last

2-3 weeks, while the formation phase require a longer time lasting 2-3 months.When the very fine balance between bone resorption and bone formation is disturbed, it can lead to degenerative bone diseases like osteoporosis. Since bone resorption and bone formation are tightly coupled processes that are kept under homeostasis during normal conditions, a disruption to this equilibrium will result in an imbalance.In the case of osteoporosis, this imbalance leads to a significant increase in bone resorption with no change or even a slight decrease in bone formation. This imbalance can be caused by a combination of age-related factors such as secondary hyperparathyroidism, reduced mechanical loading and estrogen deficiency in the case of postmenopausal women (Sambrook & Cooper, 2006).

Biochemical markers of bone turnover are broadly divided into two categories: markers of bone resorption, which reflect osteoclast activity and markers of bone formation, which reflect osteoblast activity. Since bone resorption and bone formation are coupled processes, any of these markers will reflect a change in bone turnover in most situations. According to Eastell and Hannon 2007, the most commonly used biochemical markers of bone formation are by-products of collagen synthesis (procollagen type I C-terminal propeptide, procollagen type I N-terminal propeptide), matrix protein (osteocalcin), osteoblast enzyme (total alkaline phosphatase, bone alkaline phosphatase). The most commonly

used biochemical markers for bone resorption are collagen degradation products (hydroxyproline, pyridinoline, deoxypyridinoline), cross linked telopeptides of type I collagen (N-terminal cross-linked telopeptide, C-terminal cross-linked telopeptide, C-terminal cross-linked telopeptide generated by matrix metalloproteinase), osteoclast enzyme (tartrate-resistant acid phosphatase, cathepsin K).

1.2. Bone disorders and current therapies

Osteoporosis

Osteoporosis (OP) is a disease of low bone mass and the micro-architectural deterioration of bone structure, which in combination, predispose the patient to enhanced bone fragility and fracture risk (Raisz, 2005). Osteoporosis is a condition that causes bones to become thin and porous, decreasing bone strength and leading to increased risk of breaking a bone**.** It is often called the 'silent thief' because bone loss occurs without symptoms unless one has suffered a fracture. Osteoporosis can strike at any age and affects both men and women. Women and men begin to lose bone in their mid-30s; as they approach menopause, women lose bone at a greater rate, from 2-3 per cent per year. There are no symptoms in the early stages of the disease. Symptoms occurring late in the disease include bone pain or tenderness, fractures with little or no trauma, loss of height (as much as 6 inches) over

time, low back pain due to fractures of the spinal bones, neck pain due to fractures of the spinal bones, stooped posture or kyphosis, also called a "dowager's hump".

No single cause for osteoporosis has been identified. Risk factors include age, sex, vertebral compression fracture, fragility fracture after age 40, either parent has had a hip fracture, more than three months use of glucocorticoid drugs, medical conditions that inhibit absorption of nutrients and other medical conditions or medications that contribute to bone loss. Other risk factors related to this disease include low bone mass, being thin or having a small frame, a sedentary lifestyle, estrogen deficiency as a result of menopause (especially early or surgically induced), anorexia nervosa, cigarette smoking, excessive alcohol intake etc. Osteoporosis is categorised into three types: Type I (postmenopausal), Type II (senile) and Type III (secondary) osteoporosis (Burckhardt & Michel, 1989; Gallagher, 1992). Type I postmenopausal osteoporosis occurs in women between 51-75 years of age. The main cause for this type is due to estrogen deficiency leading to more of bone resorption than formation. Estrogen plays a vital role in maintaining bone strength. Estrogen level decreases in women at menopause causing an increase in the rate of bone loss. Type II senile osteoporosis occurs at later stage in life, around 75 years of age. It is caused by age related decline in renal production of vitamin D and the failure of the body to absorb calcium. This results in the loss of both the

cortical and trabecular bone. Type III or secondary osteoporosis can affect even young and middle aged people and is caused by medications (e.g. Prednisone), cancers, endocrine disorders, chronic liver or kidney diseases.

OP is most commonly diagnosed by measuring bone density using Dual Energy X-ray Absorptiometry (DEXA). The results of a bone density measurement (DEXA scan) are reported in two ways: as T-scores and as Z-scores. A T-score compares the bone density to the optimal peak bone density of a healthy young adult of the same gender. It is reported as the number of standard deviations below the average. A T-score of greater than -1 is considered normal. A T-score of -1 to -2.5 is considered osteopenic, and at risk for developing osteoporosis. A T-score of less than -2.5 is diagnostic of osteoporosis. A Z-score is used to compare the results with the same age, weight, ethnicity, and gender. This is useful to determine if there is something unusual contributing to the bone loss. Other techniques that are sometimes used to diagnose osteoporosis include quantitative computed tomography, magnetic resonance imaging and microcomputed tomography (Zaidi *et al.*, 2002).

According to Osteoporosis Canada, fractures from osteoporosis are more common than heart attacks, stroke and breast cancer combined. At least 1 in 3 women and 1 in 5 men suffer from an osteoporotic fracture

during their lifetime. Over 80% of all fractures in people over 50 years are caused by osteoporosis. Osteoporosis causes 70-90% of 30,000 hip fractures annually. Twenty-eight per cent of women and 37% of men who suffer a hip fracture die within the following year. Both vertebral and hip fractures are associated with an increased risk of death. A Canadian study showed that 14% of persons with a wrist fracture suffered a repeat fracture within three years. One in three hip fracture patients re-fracture at one year and over 1 in 2 suffer another fracture within 5 years. The risk of suffering a second spine fracture within the first 12 months following an initial vertebral fracture is 20%. A 50-year-old woman has a 40% chance of developing hip, vertebral or wrist fractures during her lifetime (Melton *et al.*, 1992). 1 in 4 women who have a new vertebral fracture will fracture again within one year (Lindsay *et al.*, 2005). Patients are at highest risk for subsequent fracture in the first few months following a vertebral fracture (Johnell *et al.*, 2001; Kanis *et al.*, 2001). The lifetime risk of hip fracture is greater (1 in 6) than the 1 in 9 lifetime risk of developing breast cancer (Cummings *et al.*, 1989). Osteoporotic hip fractures consume more hospital bed days than stroke, diabetes, or heart attacks. Fewer than 20% of fracture patients in Canada currently undergo diagnosis or adequate treatment for osteoporosis. Without bone mineral density (BMD) testing, 80% of patients with a history of fractures are not given osteoporosis therapies. Hundreds of thousands of Canadians unfortunately fracture each year because their osteoporosis goes undiagnosed and untreated. A

study recently reported that only 44% of people discharged from hospital for a hip fracture return home; of the rest, 10% go to another hospital, 27% go to rehabilitation care, and 17% go to long-term care facilities.

The overall yearly cost to the Canadian healthcare system of treating osteoporosis and the fractures it causes was over $2.3 billion as of 2010. This cost includes acute care costs, outpatient care, prescription drugs and indirect costs. This cost rises to $3.9 billion if one includes the proportion of Canadians that were assumed to be living in long-term care facilities because of osteoporosis (Hopkins *et al.*, 2012; Tarride *et al.*, 2012a; Tarride *et al.*, 2012b). Each hip fracture costs the health care system $21,285 in the 1st year after hospitalization and $44,156 if the patient is institutionalized (Tarride *et al.*, 2012a; Tarride *et al.*, 2012b). The consequences of this disease are far-reaching, costing billions of dollars in disease management. Additional costs include family stress, absence from work, job loss, poor quality of life, lost productivity, premature loss of life etc. Osteoporosis can result in disfigurement, lowered self-esteem, reduction or loss of mobility, and decreased independence.

Current therapies for osteoporosis and their therapeutic concern

Anabolic and antiresorptive therapies are the two strategies currently used in the treatment of osteoporosis.

Parathyroid hormone (PTH) is commonly used as an anabolic therapy. This hormone is the most important regulator of blood calcium ion concentrations in mammals and is synthesized in the parathyroid glands. When circulating levels of calcium is low, it acts by activating PTH-1 receptors expressed in bone and kidney tissues, thereby resulting in the release of calcium from bone as well as increasing the retention of calcium from the glomerular filtrate. Teriparatide, a parathyroid hormone analogue, is a recombinant amino terminal fragment of parathyroid hormone comprised of the first 34 amino acids of parathyroid hormone (PTH). It regulates calcium homeostatis. Depending on the degree of exposure, it can stimulate both bone formation and bone loss. Too much of PTH accelerates bone loss while intermittently given low doses of PTH can increase bone mass and strengthen bone. It is considered an anabolic agent and helps build new bone by increasing the activity and number of bone-building osteoblasts. This osteoporosis medication helps to build up bone in the hips and spine. It can be used for up to two years to prevent spinal fractures. Parathyroid hormone has the potential to induce osteosarcoma (Palmer *et al.*, 1988; Wermers *et al.*, 1998).

Strontium ranelate is similar to calcium and shows an anabolic as well as antiresorptive effect in osteoporosis. It is considered as a “dual action bone agent” since it increases bone formation by osteoblasts and

decreases bone resorption by osteoclasts, thus causing a rebalance of bone turnover in favour of bone formation. Diarrhea, seizures and memory loss are the potential side effects of strontium (O'Donnell *et al.*, 2006).

The current antiresorptive agents for the treatment of osteoporosis include bisphosphonates, estrogen, selective estrogen receptor modulator (SERM), calcium, vitamin D, calcitonin, and a monoclonal antibody.Bisphosphonates are synthetic analogues of natural inorganic pyrophosphate (PPi). They have a methylene carbon, a P-C-P backbone structure rather than an oxygen atom (P-O-P) as PPi. This structural feature makes bisphosphonate more stable and more resistant to hydrolysis under acidic conditions or by pyrophosphatases (Francis & Valent, 2007). Bisphosphonates(BPs) are divided into two groups, first generation or non-nitrogen-containing (etidronate, tiludronate) and second generation or amino- bisphosphonates/nitrogen-containing (alendronate, risedronate and zoledronate) which are more potent than their non-nitrogen containing counterparts (Rogers *et al.*, 1999; Russell *et al.*, 1999a; Russell & Rogers, 1999; Russell *et al.*, 1999b; Licata, 2005). Bisphosphonates carry a negative charge that is associated with the phosphate groups, and as a result they adhere to the positively charge surface of hydroxyapatite crystals of bone (Francis & Valent, 2007). First generation BPs inhibit osteoclastic activity by incorporating metabolically into non-hydrolyzable analogues of ATP which accumulates within

osteoclasts, preventing their normal function and causing apoptosis (Rogers *et al.*, 2000). Second generation BPs inhibit the enzyme farnesyl diphosphate synthetase which leads to induction of apoptosis in osteoclasts (Luckman *et al.*, 1998; Rogers, 2003). BP related osteonecrosis (BRON) in the alveolar bone of the jaw, development of osteomalacia (Malden & Pai, 2007) and patient compliance is a major concern for BP therapeutics.

The occurrence of osteoporosis is high after menopause because the ovaries stop producing estrogen, a hormone that plays a major role in the bone repair process. Hormone replacement therapy (HRT) using estrogen is capable of controlling bone loss but also stimulates uterine and breast tissue, potentially resulting in cancer, and is also associated with increased coronary heart disease (Espie *et al.*, 2007). SERMs have agonistic activity in estrogen receptors of bone and antagonistic activity in other estrogen receptors (Katzenellenbogen & Katzenellenbogen, 2002). However, they are less potent than estrogen and are associated with an increased incidence of vascular thromboembolism (Reid *et al.*, 2004).

Calcium is a mineral that is required for healthy bone. When the body does not get enough calcium from the diet, calcium gets removed from the bone, thus making the bone weak and fragile. Osteoporosis Canada recommends 1,000 mg of elemental calcium daily for men and

women between the ages of 19 and 50 years, and 1,200 mg for men and women over the age of 50 years. Vitamin D is required by the body for the absorption of calcium. If the body is not getting enough vitamin D from sunlight and food, vitamin D supplement should be taken. Osteoporosis Canada recommends vitamin D in daily doses of 400 IU to 1,000 IU for adults without osteoporosis under 50 years of age, and 800 IU to 2,000 IU for both adults over the age of 50 and people with osteoporosis.

Salmon calcitonin is the most potent form of calcitonin in terms of the inhibition of osteoclast activity (Zaidi *et al.*, 2002). It is a peptide that reduces blood calcium by increasing urinary calcium excretion and inhibiting bone resorption, thereby opposing the effects of parathyroid hormone (Boron, 2004; Bouyer *et al.*, 2004). Conventional calcitonin therapy is characterised by poor bioavailability and the undesired uptake of the drug by the calcitonin receptors present in tissues other than bone.

Osteoarthritis

The articular joint is made up of several tissues, the main ones involved during osteoarthritis (OA) being the cartilage, synovial membrane, and the subchondral bone, all of which are closely linked. The cartilage is the tough elastic material that covers and protects the ends of bone. In a healthy joint, it acts as a shock absorber when weight is exerted on the joint and its slippery surface allows the joints to move smoothly.

Joint degeneration does occur, however, and OA is among the most common joint disorders, affecting about 65% of individuals over 60 years of age. The disease causes pain and functional disability, resulting in a significant social and economic burden (Kong *et al.*, 1999b). Current treatments and drugs for OA include medications (acetaminophen, non-steroidal anti-inflammary drugs, narcotics), physical therapy, cortisone shots, lubrication injections and surgical procedures (realigning bones, joint replacement).

Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory disorder of joints, typically affecting the lining of the bone leading to painful bone erosion and swelling. It involves the smaller joints of hands, feet and later on progresses to larger joints like knees, ankle and elbows. Current treatment of rheumatoid arthritis includes non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease modifying anti-rheumatic drugs (DMARDs). DMARDs include methotrexate, sulfasalazine and antibodies like infliximab, adalimumab, certolizumab pegol, golimumab etc. These antibodies act as Tumor Necrosis Factor (TNF) inhibitor which is the dominant cytokine involved in the inflammation of rheumatoid arthritis.

Paget's disease

Paget's disease of bone is caused by the excessive breakdown and formation of bone tissue resulting in weak, painful, enlarged and misshapen bones, fractures, and arthritis in the joints near the affected bones. It is a chronic disorder and typically localized, affecting just one or a few bones, as opposed to osteoporosis, which usually affects all the bones in the body. US FDA approved drug therapies in Paget's disease to relieve pain and prevent progression of the disease include bisphosphonates and calcitonin. Osteosarcoma, multiple myeloma, hypercalcemia are some of the other prevalent bone disorders.

1.3. Osteoclast: the key player in bone disease

Osteoclasts are the cells uniquely responsible for dissolving both the organic and inorganic components of bone during development, and as part of the bone remodeling cycle throughout life. These cells originate from hematopoietic precursors of the monocyte/macrophage lineage that are present both in the bone marrow and peripheral circulation, and their numbers and/or activity are frequently increased in a wide range of clinical disorders that are associated with excessive bone loss and which affect millions of people (Roodman, 1996). The increased bone resorption in osteoporosis is due both to increased osteoclastogenesis and to decreased osteoclast apoptosis (Manolagas, 2000; Riggs *et al.*, 2002).

Some clinical studies in OA patients have suggested that the indices of bone resorption are increased early in the disease process. This concurs with the in vivo findings of a study performed on an induced OA dog model allowing the chronological analysis of the disease progression. At an early stage of the disease process, increased bone loss and resorption with subchondral bone exhibiting an increased number of osteoclasts, as well as production of catabolic factors including cathepsin K and MMP-13 were observed (Pelletier *et al.*, 2004). Increased bone resorption results in increased articular damage (Han *et al.*, 2004). Osteoclasts are considered as the essential link between synovial inflammation and bone destruction. Increased osteoclast functional activity is directly responsible for the generalized bone loss that occurs in rheumatoid arthritis (Hirayama *et al.*, 2002). Activated $CD4^+$ T-cells are capable of inducing osteoclastogenesis (Kong *et al.*, 1999a; Kong *et al.*, 1999b; Kong *et al.*, 1999c). As the T cells are activated in autoimmune diseases like rheumatoid arthritis (Waalen *et al.*, 1987; Maurer *et al.*, 1992; Kotake *et al.*, 2001; Brennan *et al.*, 2002; Ogawa *et al.*, 2003; Harigai *et al.*, 2004; Aerts *et al.*, 2008; Nakou *et al.*, 2009; Kuca-Warnawin *et al.*, 2011) and osteoarthritis, an antiresorptive strategy that selectively carries an active drug cargo to bone would be a highly desirable therapeutic approach to treat bone diseases involving upregulated osteoclast activity. Interestingly, the antiresorptive peptide, calcitonin has been reported to be beneficial in treating rheumatoid arthritis (Aida *et al.*, 1994; Pappalardo *et*

al., 1994; Kotaniemi *et al.*, 1996; Ide & Suzuki, 2001). Also, the studies have shown that osteoclasts are more active in Paget's disease and multiple myelomas, characterized by increased numbers of osteoclasts and markedly increased bone resorption at the sites of the disease (Roodman, 1996; Roodman & Windle, 2005).

Osteoclast exhibits unique cytoskeleton with its ruffled membrane and actin rings. The ruffled membrane is the product of intracellular acidified vesicles. The actin ring is a circumferential structure that surrounds the ruffled membrane and isolates the acidified resorptive microenvironment from the general extracellular space (Faccio *et al.*, 2003b). The mature osteoclast is a functionally polarized cell that attaches via its apical pole to the mineralized bone matrix by forming a tight ring-like zone of adhesion, the sealing zone. This attachment involves the specific interaction between adhesion molecules in the cell membrane (integrins) and some bone matrix proteins. The integrins are a family of transmembrane proteins whose cytoplasmic domains interact with the cytoskeleton while their extracellular domains bind to bone matrix proteins, enabling them to mediate cell–substratum and cell–cell interactions (Hynes, 1987; Yang *et al.*, 1995).The space contained inside this ring of attachment and between the osteoclast and the bone matrix constitutes the bone resorbing compartment. Acidification of the extracellular bone-resorbing compartment is one of the most important features of osteoclast

action. The osteoclast is highly enriched in carbonic anhydrase (Gay & Mueller, 1974). Carbonic anhydrase generates protons and bicarbonate from carbon dioxide and water, providing the cells with protons to be extruded across the cell membrane into the bone-resorbing compartment by proton pumps (H^+ ATPases) located in the ruffle border apical membrane. Regulation of H^+ transport at the apical surface of the osteoclast, which is tightly linked to the regulation of intracellular pH and membrane potential, is mostly accomplished by ion exchangers, pumps and channels present in the basolateral membrane of the cell **(Figure 2).**

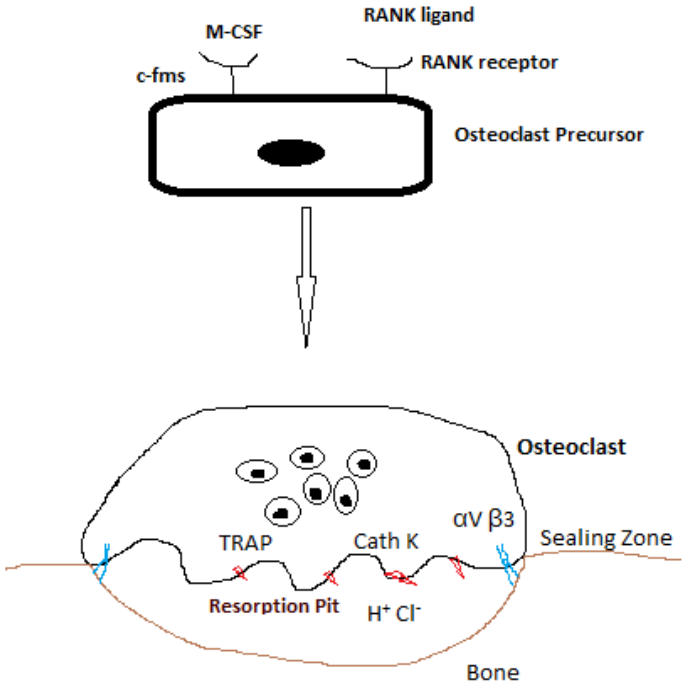

Figure 2. *Osteoclast differentiation and function.*

Osteoblast lineage cells produce the osteoclastogenic cytokines RANKL and M-CSF, which recognize their respective receptors RANK and c-fms on macrophages, principally of marrow origin. Osteoprotegerin (OPG), also synthesized by osteoblast lineage cells, is a soluble decoy receptor that binds RANKL, thus preventing its interaction with RANK. In osteoblastic precursor cells, it was found that RANKL is expressed at high levels while OPG is secreted at low levels. This high RANKL-to-OPG ratio favors osteoclastic generation and activity. On the other hand, as osteoblasts mature, this ratio reverses and osteoclast activity is in turn reduced or lost (Hofbauer & Heufelder, 2001).

RANKL and M-CSF are sufficient to promote the osteoclast phenotype. A co-culture system of bone marrow cells and osteoblasts, which serves as an in vitro osteoclast formation system, was established in the late 1980s (Takahashi *et al.*, 1988a; Takahashi *et al.*, 1988b; Suda *et al.*, 1992). The difficulty in isolating or culturing bone cells left the molecular analysis of osteoclastogenesis unexplored for a long period, but later on, researchers had a great breakthroughs in this field (Karsenty & Wagner, 2002; Takayanagi, 2005). Several groups independently cloned the long-sought osteoclast differentiation factor in 1997–98 (Anderson *et al.*, 1997; Wong *et al.*, 1997; Lacey *et al.*, 1998; Yasuda *et al.*, 1998), which is now widely called RANKL. Recombinant RANKL and M-CSF efficiently induce osteoclastogenesis, which has allowed this process to be

extensively investigated in a culture of a relatively pure population of osteoclast precursor cells. In addition to the identification of M-CSF as an essential survival and proliferation factor for osteoclast precursor cells, the cloning of osteoclast differentiation factor, RANKL, enabled the reconstitution of dynamic differentiation processes, including cell fusion, in a culture system (Theill *et al.*, 2002; Takayanagi, 2005). Now, under direct observation, osteoclasts can be generated from RANKL stimulated monocyte/macrophage lineage cells in the presence of M-CSF (Wiktor-Jedrzejczak *et al.*, 1990; Yoshida *et al.*, 1990). This osteoclast differentiation system has developed into one of the most sophisticated culture systems available, allowing an extremely useful system for osteoclast related studies.

The osteoclast is a multinucleated cell created by the differentiation of monocyte/macrophage precursor cells. The two hematopoietic factors, Receptor activator of nuclear factor κβ ligand (RANKL) and macrophage colony stimulating factor (M-CSF) are the two cytokines that are essential and sufficient to induce osteoclastogenesis and to regulate osteoclast activity (Lacey *et al.*, 1998; Yasuda *et al.*, 1998). RANKL mediates the differentiation of bone-resorbing osteoclasts from monocyte-macrophage precursors and modulates the survival and function of mature osteoclasts (Li *et al.*, 2000; Teitelbaum & Ross, 2003). Mice deficient in RANKL, or its receptor RANK, exhibit a complete lack of osteoclasts, resulting in severe

osteopetrosis and hypocalcemia. Clinically, RANKL has been implicated in the pathogenesis of postmenopausal osteoporosis. In this disorder, estrogen deficiency leads to enhanced expression of TNF and RANKL, as well as decreased production of osteoprotegerin (OPG, a soluble TNF receptor (TNFR) family decoy receptor for RANKL) (Hofbauer *et al.*, 1999; Cenci *et al.*, 2000; Hofbauer & Heufelder, 2001). RANKL are both secreted by or are present on bone marrow stromal cells and osteoblasts. M-CSF induces the expression of RANKL's receptor (RANK), in osteoclast precursors thus priming these cells to differentiate into osteoclasts in the presence of RANKL. The deletion of M-CSF, RANK, or RANKL results in the inhibition of osteoclastogenesis, thus leading to osteopetrosis in mice (Suda *et al.*, 1992; Teitelbaum, 2000).

The major criteria generally used to identify osteoclasts are multinuclearity, positive staining for tartrate resistant acid phosphatase (TRAP), expression of calcitonin receptors and the ability to resorb calcified matrices (Takahashi *et al.*, 1988a; Takahashi *et al.*, 1988b; Shinar *et al.*, 1990). Osteoclasts express high amounts of TRAP, also known as type 5 acid phosphatase. TRAP has long been used as a histochemical marker of the osteoclast (Burstone, 1959; Minkin, 1982). Expression of TRAP is associated with the activation and differentiation of osteoclasts. Osteoclasts secrete TRAP into the circulation, suggesting that serum TRAP may be a useful resorption marker. The activity of the

enzyme is pathologically increased in conditions where bone resorption is enhanced, including Paget's disease and hyperparathyroidism (Lau *et al.*, 1987; Chamberlain *et al.*, 1995). TRAP is localised in the transcytotic vesicles of resorbing osteoclasts, and that it can generate highly destructive reactive oxygen species (ROS) able to destroy collagen (Halleen *et al.*, 1999). This activity, together with the co-localisation of TRAP and collagen fragments in transcytotic vesicles, suggests that TRAP functions in further destruction of matrix degradation products in the transcytotic vesicles. The observed mild osteopetrosis in TRAP-knockout mice supports this hypothesis.

The osteoclast is a giant cell polykaryon. The multinucleation characterizing the osteoclast is the most striking morphological feature distinguishing the osteoclast from its precursor. The mammalian osteoclast normally contains up to eight nuclei, but around 100 nuclei are found in osteoclasts of Paget's disease patients (Roodman & Windle, 2005). In vitro diameter can reach around 300 μm as compared to 10–20 μm for a macrophage and this big size enables the osteoclast to cover a relatively large matrix area. It is hypothesized that multinucleation increases resorption efficiency. Avian osteoclasts contain many more nuclei than mammalian osteoclasts, and have a higher capacity for resorbing bone (Vaananen, 2005). This high resorption capacity of the bird

cells is probably associated with the need for rapid release of calcium to calcify eggshells in laying hens.

Dendritic cell-specific transmembrane protein (DC-STAMP), the membrane protein, was found to be critical for fusion of the mononuclear precursors to form the multinucleated osteoclast. DC-STAMP expression was rapidly upregulated when mouse cells were cultured in the presence of OC-promoting cytokines such as receptor activator of nuclear factor-kβ (NF-kβ) ligand, RANKL (Kukita *et al.*, 2004). DC-STAMP -deficient cells do not fuse but exhibit characteristics like that of osteoclasts, including formation of an actin ring and ruffled border. However, the resorption efficiency in vitro was reduced in DC-STAMP-deficient mononuclear osteoclasts and DC-STAMP-deficient mice exhibited increased bone mass (Yagi *et al.*, 2005).

For resorption activity, osteoclast needs to attach to the bone surface and to assume a polarized morphology. On contact with bone, the osteoclast polarizes via matrix derived signals transmitted by the αvβ3 integrin, enabling the cell to form an isolated microenvironment between itself and the bone surface. The microenvironment is acidified by H^+ATPase-mediated extracellular transport of protons. Intracellular pH is maintained by an electroneutral HCO_3^-/Cl^-exchanger. The Cl^-entering the cell is released into the resorptive microenvironment by an ion channel

charge coupled to the H^+ATPase. The acidified microenvironment mobilizes the bone mineral, thereby exposing the organic phase of bone that is degraded by cathepsin K (Teitelbaum & Ross, 2003). After solubilization of the mineral phase, several proteolytic enzymes degrade the organic bone matrix. The high levels both of expression of matrix metalloproteinase-9 (MMP-9) and cathepsin K and of their secretion into the resorption lacuna suggest that these enzymes play a central role in the resorption process (Tezuka *et al.*, 1994; Drake *et al.*, 1996).

Osteoclast receptors are categorized into (i) nuclear receptors (sex hormone, glucocorticoid and retinoid receptors) (ii) cell surface receptor (RANK, M-CSF, Calcitonin receptor), (iii) integrin receptors and (iv) cell death receptors (TNF-related apoptosis inducing ligand: TRAIL receptor). These receptors function together to control osteoclast activity and prevent both reduced and increased bone resorption (Del Fattore *et al.*, 2008). Here, more focus will be given on cell surface receptors: RANK, M-CSF and Calcitonin receptor.

1.3.1. RANKL and RANK receptor

RANKL, a member of the TNF superfamily, is a membrane bound protein on osteoblasts and their precursors that recognize its receptor RANK, on marrow macrophages, prompting them to assume the osteoclast phenotype. Like TNF, RANKL is a homotrimer but contains four

unique surface loops that distinguish it from other TNF family cytokines (Lam *et al.*, 2001). RANKL activity is negatively regulated in the circulation by osteoprotegerin (OPG), which competes with RANK as a soluble decoy receptor (Simonet *et al.*, 1997). RANKL is the only known ligand for RANK. RANK and RANKL are crucial in bone metabolism, lymph node formation in the immune system, and mammary gland development in pregnancy. Parathyroid hormone, prostaglandin E2, dexamethasone, inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNFα), or 1, 25- dihydroxyvitamin D_3 can stimulate RANKL expression. By contrast, estrogen or transforming growth factor b (TGFb) attenuate RANKL expression. RANKL exists both as a transmembrane protein in osteoblasts/stromal cells and as a soluble protein (Bilezikian *et al.*, 2002; Boyle *et al.*, 2003; Teitelbaum & Ross, 2003; Lerner, 2004). Soluble forms of RANKL (sRANKL) have more potent activity (Nakashima *et al.*, 2000) and have important roles in osteolysis induced by tumors (Lynch *et al.*, 2005). sRANKL derives from the membrane form as a result of proteolytic cleavage (Ikeda *et al.*, 2001). The proteolytic cleavage of RANKL requires a disintegrin and metalloprotease domain family members and matrix metalloproteases (MMP).

RANK is a type I transmembrane single pass protein molecule anchored to the lipid membrane with N-terminus exposed to the extracellular or luminal space. It consists of around 620 amino acids with

~85% homology between mouse and human homologs. The ectodomain of RANK is formed by 4 cysteine rich pseudorepeats (Anderson *et al.*, 1997). All TNFRSF members possess ectodomains comprised of from one to five cysteine-rich pseudorepeats (Locksley *et al.*, 2001). Each pseudorepeat is characteristically 40 amino acids in length and contains 6 conserved cysteine residues that form 3 intrachain disulfide bonds. Based on structural analysis of other TNFR, the four pseudorepeats of RANK are projected to form an elongated structure capable of binding to one of the three grooves formed by the trimerization of RANK-L (Lam *et al.*, 2001) **(Figure 3).** Thus, each RANK-L trimer engages three molecules of RANK (Chung *et al.*, 2002). RANK-trimer and RANKL-trimer is necessary in order to successfully generate signaling cascade and form fully functional osteoclast.

Researchers have different views on the order of RANK/RANKL trimerization, Some believe that RANKL first trimerizes, binds to RANK, which in turn brings 2 units of RANK within close proximity to initiate trimerization (Zhang *et al.*, 2009) while some suggest that RANK is capable of trimerizing without the aid of RANKL (Kanazawa & Kudo, 2005). According to Iwamoto et al, dimerization of RANK and RANKL is also capable of inducing a signaling cascade, but the resulting effect is much less significant in that the resorption activity and expression of

osteoclast markers normally induced by RANKL are greatly reduced (Iwamoto *et al.*, 2004).

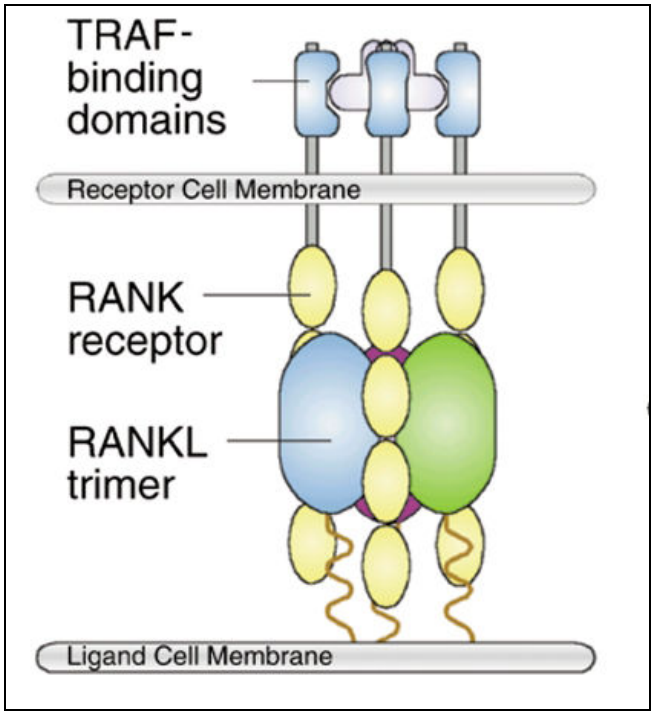

Figure 3 : *Receptor contact regions of RANKL. Schematic depiction of a RANKL trimer (green, cyan, and magenta monomers) docked with its receptor RANK, drawn here as four Cysteine Rich Domains, yellow. Image taken from (Lam et al., 2001).*

Binding of RANKL to RANK induces various intracellular signaling cascades including TNF receptor associated factor 6 (TRAF6) and nuclear factor of activated T cells c1 (NFATc1) activation. TRAF family proteins

are adapter molecules. They mediate intracellular signaling of various cytokine receptors including TNF receptor superfamily and Toll/interleukin (IL)-1 receptor family members (Naito *et al.*, 1999; Inoue *et al.*, 2000). Although RANK recruits several members of the TRAF family, only TRAF6 is indispensable for osteoclastogenesis signaling capability. TRAF6 is pivotal not only in the formation of the osteoclast but also in the bone resorbing function of mature osteoclasts (Kobayashi *et al.*, 2001; Gohda *et al.*, 2005; Kadono *et al.*, 2005). TRAF6-induced intracellular signaling includes NF- κβ, c-Jun NH_2-terminal kinase, and p38 mitogen-activated protein kinase, which all are critically involved in osteoclast development (Tanaka, 2007). RANKL-inducible genes specifically required for the terminal differentiation of osteoclasts, NFATc1 was shown to be strongly induced by RANKL (Ishida *et al.*, 2002; Takayanagi, 2005). The process of osteoclast differentiation can be divided into three stages in the context of transcriptional control by NFATc1, (i) initial induction of NFATc1 (ii) autoamplification of NFATc1 (iii) induction of osteoclast specific genes. The binding of RANKL to RANK results in the activation of downstream molecules such as NF-κβ. As RANK was named after its ability to activate NF-κβ, it is well recognized that NF-κβ activation is among the very early molecular events induced by RANK (Anderson *et al.*, 1997). NF-κβ proteins reside in the cytoplasm of non-stimulated cells but rapidly enter the nucleus upon cell stimulation with a variety of agonists, including RANKL (Anderson *et al.*, 1997; Boyle *et al.*, 2003).

NFATc1 is the critical target gene of NF-κβ in the early phase of osteoclastogenesis. The NFAT transcription factor family was originally identified in T cells and is now comprised of five members including NFATc1, NFATc2, NFATc3, NFATc4 and NFAT5. The induction of NFATc1 is a hallmark event in the cell fate determination of osteoclasts (Takayanagi *et al.*, 2002; Asagiri *et al.*, 2005). NFATc1 induction was shown to be impaired in TRAF6−/− cells (Takayanagi *et al.*, 2002). Also, NF-κβ inhibitor suppressed RANKL-stimulated induction of NFATc1 (Takatsuna *et al.*, 2005). Hence, it has been suggested that NFATc1 is one of the key target genes of NF-κβ in the early phase of osteoclastogenesis. NFATc2 is present prior to RANKL stimulation and is involved in the initial induction of NFATc1 in cooperation with NF-κβ. NFATc2 and NF-κβ cooperatively activate the NFATc1 promoter within minutes of RANKL stimulation, which is an important step toward the robust induction of NFATc1 at the next stage of differentiation. Loss of NFATc2 might be compensated for by other NFATs as NFATc2−/− mice display no defect in osteoclastogenesis (Asagiri & Takayanagi, 2007).

Osteoclast-specific immunoreceptor osteoclast associated receptor (OSCAR) is an immunoglobulin-like receptor expressed by osteoclasts and is involved in the cell–cell interaction between osteoblasts and osteoclasts (Kim *et al.*, 2005). OSCAR associates with an adaptor

molecule, Fc receptor common γ subunit FcRγ (Koga *et al.*, 2004; Merck *et al.*, 2004) and FcRγ harbors an immunoreceptor tyrosine-based activation motif (ITAM), which is critical for the activation of calcium signaling in immune cells (Reth, 1989). The RANKL–RANK signaling and the stimulation of the immunoglobulin-like receptors cooperatively phosphorylate ITAM leading to calcium signaling, which is critical for NFATc1 induction and activation. Stimulated by calcium signaling, NFATc1 is activated and binds its own promoter. This leads to the robust induction of NFATc1 (i.e., autoamplification). An activator protein-1 (AP-1) is critical for this autoamplification. RANK activates the transcription factor complex AP-1 partly through an induction of its critical component, c-Fos (Takayanagi, 2005; Wagner & Eferl, 2005).

A number of osteoclast-specific genes are directly regulated by NFATc1 such as TRAP (Matsuo *et al.*, 2004; Kim *et al.*, 2005; Takayanagi, 2005), calcitonin receptor (Anusaksathien *et al.*, 2001; Matsuo *et al.*, 2004; Kim *et al.*, 2005; Takayanagi, 2005), cathepsin K (Matsuo *et al.*, 2004; Kim *et al.*, 2005), β3 integrin genes (Crotti *et al.*, 2006) and osteoclast-specific immunoreceptor osteoclast associated receptor (OSCAR) (Kim *et al.*, 2005). They are activated by a transcriptional complex containing NFATc1 and cooperators such as AP-1, PU.1 and microphthalmia-associated transcription factor (MITF). The NFAT: AP-1 complex is important for the induction of the TRAP and

calcitonin receptor genes as well as the robust autoamplification of NFATc1 (Takayanagi, 2005). It has also been shown that NFATc1 cooperates with PU.1 and MITF on the cathepsin K and the OSCAR promoters (Matsuo *et al.*, 2004; Kim *et al.*, 2005). Both PU.1 and MITF, which are thought to be important for the survival of osteoclast precursor cells, also participate in osteoclast-specific gene induction at the terminal stage of differentiation. Thus, NFATc1 forms an osteoclast specific transcriptional complex containing AP-1 (Fos/Jun), PU.1 and MITF for the efficient induction of osteoclast-specific genes (Asagiri *et al.*, 2005).

RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass. $RANK^{-/-}$ mice lacked osteoclasts and showed a profound defect in bone resorption and remodeling (Li *et al.*, 2000). RANK was chosen as the knock down target to suppress osteoclast-mediated bone resorption (Wang & Grainger, 2010). Sordillo and Pearse reported that administration of RANK-Fc, a recombinant RANK-L antagonist, limits multiple myeloma (MM)-induced osteoclastogenesis, development of bone disease, and MM tumor progression (Sordillo & Pearse, 2003).

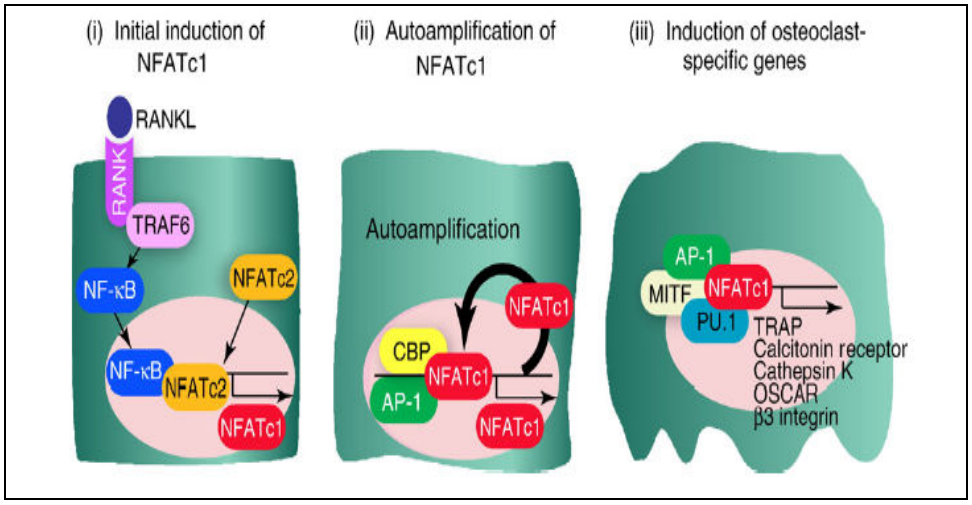

Figure 4: *A schematic diagram of three stages of osteoclast differentiation governed by NFATc1. Image taken from (Asagiri & Takayanagi, 2007)*

Suppression of RANK expression by RANK small hairpin RNA, a sequence of RNA that can be used to silence target gene expression via RNA interference, brought about a marked inhibition of osteoclast formation and bone resorption as demonstrated by tartrate resistant acid phosphatase (TRAP) staining and osteoclast resorption assay (Ma *et al.*, 2012).The critical role of RANK/RANKL interaction in osteoclastogenesis makes RANK an attractive target for potential therapies.

1.3.2. M-CSF and c-fms receptor

Activation of c-fms by M-CSF promotes the proliferation and survival of osteoclasts and osteoclast precursors. According to Faccio et al., M-CSF binding the c-fms receptor modulates α vβ3 integrin function to

attach cells to extracellular matrices and aid the cell in bone resorption (Faccio *et al.*, 2003a; Faccio *et al.*, 2003b). M-CSF also plays a role in regulating osteoclast survival by preventing apoptosis. M-CSF signaling triggers the anti-apoptotic Akt kinase in osteoclasts. This signaling pathway promotes osteoclast precursor proliferation and survival of the differentiating and differentiated osteoclast. Akt activity is necessary for cell survival (Datta *et al.*, 1999; Kelley *et al.*, 1999). Akt has been shown to target the apoptotic machinery by phosphorylating downstream pro-apoptotic molecules such as caspase-9, thus preventing apoptosis of osteoclasts.

In addition, M-CSF is essential for the expression of the receptor activator of NF κβ (RANK), which upon binding to its ligand RANKL, results in osteoclastogenesis (Arai *et al.*, 1999). M-CSF signaling also induces cytoskeletal reorganization, cell spreading, and increased motility of mature osteoclasts (Fuller *et al.*, 1993; Insogna *et al.*, 1997a; Insogna *et al.*, 1997b).

1.3.3. Calcitonin and Calcitonin receptors

Calcitonin (CT) is a 32-amino acid peptide secreted by thyroid C-cells (Foster *et al.*, 1964; Zaidi *et al.*, 2002) **(Figure 5).** It reduces blood calcium, thereby opposing the effects of parathyroid hormone. The

hypocalcaemic response is mainly due to inhibition of bone resorption (Friedman & Raisz, 1965), caused by activation of CT receptors (CTRs) in mature osteoclasts leading to contraction, reduced motility and decreased bone-resorbing activity (Chambers *et al.*, 1984). Calcitonin inhibits osteoclasts from synthesizing and secreting degradative enzymes such as TRAP (Yumita *et al.*, 1991). Calcitonin has also been shown to reduce the amount of acid secreted by osteoclasts by altering Na^{+}-K^{+}-ATPase activity and carbonic anhydrase localization (Akisaka & Gay, 1986; Chambers *et al.*, 1987; Moonga *et al.*, 1990).

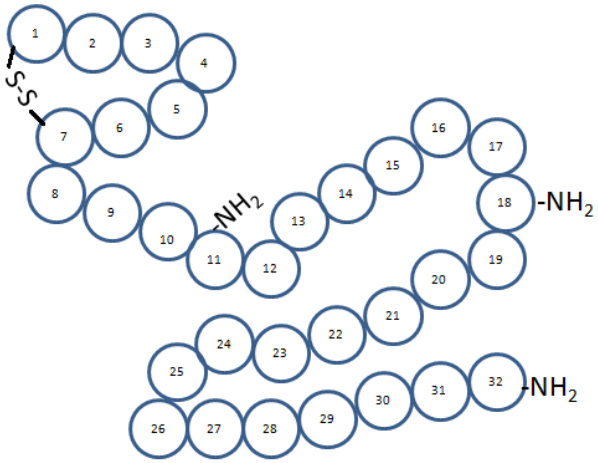

Figure 5: *Primary structure of salmon calcitonin, a 32 amino acid peptide with Lys 11, 18 or the N-terminal amine*

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Mature osteoclasts express the calcitonin receptor on their surface. Calcitonin receptors are of the G protein-coupled receptor family (comprising seven transmembrane-spanning receptor domains) whose signals inhibit osteoclast activity both in vitro and in vivo (Breimer *et al.*, 1988). Upon binding of calcitonin, calcitonin receptors are activated initiating a cascade of events. G proteins can activate adenylyl cyclase to generate cyclic adenosine monophosphate (cAMP) **(Figure 6).**

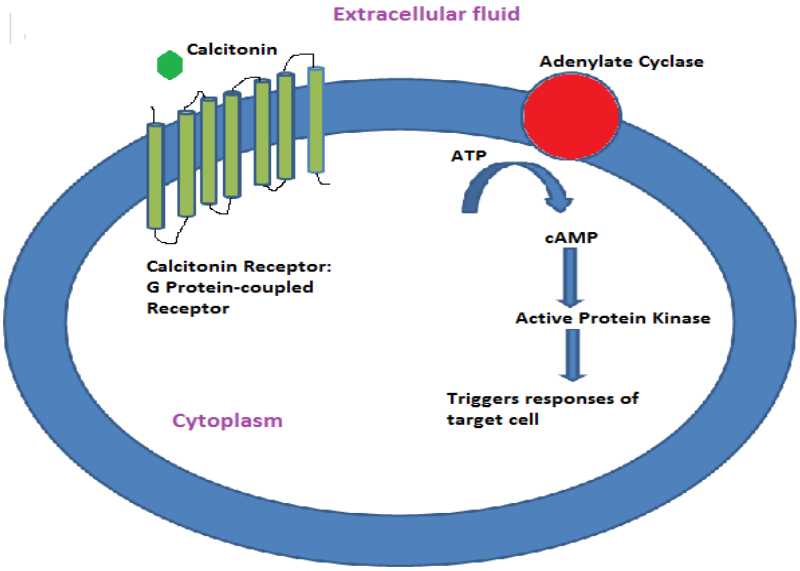


Figure 6: *Calcitonin receptor belongs to a subfamily of seven transmembrane-spanning G protein-coupled receptors. Binding of calcitonin to the receptor stimulates cyclic AMP production.*

Their interaction with calcitonin rapidly induces loss of ruffled border and immobility followed by cell retraction and arrest of bone resorption. Calcitonin receptor signals also alter ion transporter distribution, impair enzyme activity (Moonga *et al.*, 1990) and inhibit the osteoclastogenic effects of RANKL (Mancini *et al.*, 2000). M-CSF and NF κβ regulate the expression and function of the calcitonin receptor in human osteoclast-like cells (Samura *et al.*, 2000). However, one interesting study showed that treatment of osteoclasts with CT induced a prolonged but temporary down-regulation of CTR expression (Wada *et al.*, 1997). CT inhibited transcription of CTR mRNA by inhibiting its transcription or by reducing its stability (Wada *et al.*, 1997; Inoue *et al.*, 1999).

1.4. PEGylation of protein molecules

Over the last few years, the modification of proteins with polyethylene glycol (PEG) has become a well established technique. PEG is a chemical compound composed of repeating ethylene glycol units. PEGylation is the process of covalent attachment of polyethylene glycol polymer chains to drug molecule or therapeutic protein.

According to Chapman (2002), a number of benefits of PEGylation have been found. PEGylation reduces antigenicity and immunogenicity of the molecule to which PEG is attached and markedly improves circulating half-lives in vivo due to either evasion of renal clearance as a result of the

polymer increasing the apparent size of the molecule to above the glomerular filtration limit, and/or through evasion of cellular clearance mechanisms. PEGylation improves bioavailability via reduced losses at subcutaneous injection sites and also shows improved thermal and mechanical stability of PEGylated molecule. It confers enhanced proteolytic resistance of the conjugated protein (Cunningham-Rundles *et al.*, 1992). PEGylation enhances enzymatic stability of peptide drugs including calcitonin from proteolysis by forming an effective shield against degrading enzymes (Diwan & Park, 2001; Veronese & Pasut, 2005a; Anabousi *et al.*, 2006; Youn *et al.*, 2006a; Kang *et al.*, 2009; Jevsevar *et al.*, 2010; Park *et al.*, 2010; Palm *et al.*, 2011; Yang *et al.*, 2011). The addition of the PEG moiety can have beneficial effects on the immunological profile of a molecule by reducing the ability of the compound to raise antibodies in humans (Mehvar, 2000). The conjugation of PEG to the target protein has been reviewed in the areas of protein conjugates (Zalipsky, 1995) and drugs and prodrugs (Greenwald *et al.*, 2000).

PEG is widely used as an excipient in medicines that are administered by the intravenous, oral, rectal, and topical routes. PEG is also found in non-pharmaceutical products and so, humans are routinely exposed to PEG. Toothpaste, shampoo, moisturizers, colorants, foods, drinks, and deodorants are some examples of PEG-containing non-

pharmaceutical products. Exposure to PEG via these products will be by the oral and topical routes. The acute or chronic administration of PEG with a range of molecular weights by a range of routes has not led to any major toxicities, and signs of toxicity that do occur are only apparent at high dose. PEG can be considered to have a toxicological profile of very low concern in animals. A review of the toxicology information has suggested an acceptable daily intake of PEG for humans. This estimate is up to 10 mg/kg or 0.7 g/70-kg human/day (Fruijtier-Polloth, 2005). For low molecular weight PEGs, this acceptable dose could, in theory, give rise to a systemic (absorbed) dose of approximately 400 mg/day. The amount of PEG a human is exposed to from a PEGylated biological product is significantly less than the acceptable daily intake. The PEG exposure resulting from PEGylated biologicals is comparable to or lower than the intravenous exposure of PEG as an excipient. PEG associated with a protein or other biological molecule does not represent an additional unquantified risk to humans because of 1) the low exposures involved, 2) the low toxicity profile of PEG, and 3) the similarity of the metabolites that are formed in all species (Webster *et al.*, 2007). The metabolism of PEG itself is simple. Urinary excretion of unchanged material will be the major route of clearance of any PEG released by degradation of the conjugate. Biliary excretion of unchanged material would be expected to be a minor route.

There are now numerous functionalised PEG molecules commercially available. Thermo Scientific SM $(PEG)_n$ reagents are heterobifunctional crosslinkers with *N*-hydroxysuccinimide (NHS) ester and maleimide groups that allow covalent conjugation of amine and sulfhydryl containing molecules. Crosslinkers having polyethylene glycol (PEG) spacers are convenient alternatives to reagents with purely hydrocarbon spacer arms. PEG spacers improve water solubility of the reagent and conjugate, reduce the potential for aggregation of the conjugate, and increases flexibility of the crosslink, resulting in reduced immunogenic response to the spacer itself. N-hydroxysuccinimde (NHS) esters react with primary amines to form amide bonds, while maleimides react with sulfhydryl groups to form stable thioether bonds. NHS/maleimide crosslinkers can be used to prepare protein conjugates in a two-step reaction scheme. First, the amine-containing protein is reacted with a several-fold molar excess of the crosslinker, followed by removal of excess (nonreacted) reagent by desalting or dialysis; finally, the sulfhydryl-containing molecule is added to react with the maleimide groups already attached to the first protein. Generally, a 10- to 50-fold molar excess of crosslinker over the amount of amine-containing protein results in sufficient maleimide activation to enable several sulfhydryl-containing proteins to be conjugated to each amine-containing protein **(Figure 7).**

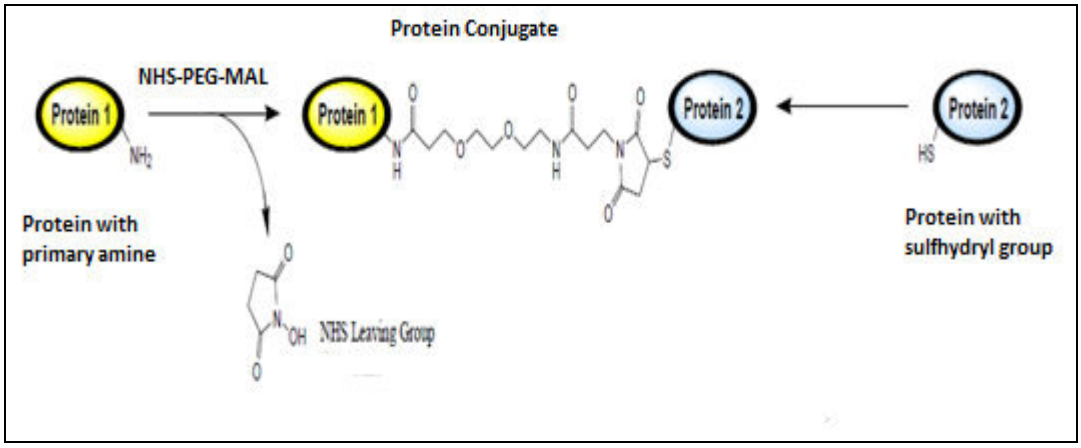


***Figure 7**: Synthesis of protein conjugate using NHS-PEG-MAL*

1.5. Antibody in drug delivery and therapeutics

An antibody (Ab) is a large Y-shaped protein produced by B-cells that is used by the immune system to identify and neutralize foreign objects like bacteria and viruses. The antibody recognizes a unique part of the foreign target, called an antigen. Each tip of the "Y" of an antibody contains a paratope that is specific for one particular epitope on an antigen, allowing these two structures to bind together with precision.

An antibody, also known as an immunoglobulin (Ig), binds to antigen via the antigen binding domain formed between the heavy and light chain variable domains. Each IgG molecule contains two identical heavy chains and two identical light chains. The antibody structure has evolved to accommodate the diverse antigen binding specificities through

the ‘variable region'. The antigen binding site is formed by the intertwining of the light chain variable domain (V_L) and the heavy chain variable domain (V_H). Each V domain contains three short polypeptide sequences known as the complementarity determining regions (CDRs). The CDRs are the prominent determinants of antigen binding affinity and specificity and the pocket formed by these CDRs enables the antibody molecule to bind its antigen. The light chain contains one constant domain: C_L. The heavy chain contains three constant domains: C_H1, C_H2, and C_H3. This is schematically shown in the **Figure 8**. Antibodies, because of their inherent specificity, seem to be ideal for targeting and drug delivery.

The immunoglobulins can be divided into five different classes, based on differences in the amino acid sequences in the constant region of the heavy chains: IgG - Gamma heavy chains, IgM - Mu heavy chains, IgA - Alpha heavy chains, IgD - Delta heavy chains, IgE - Epsilon heavy chains**.** IgM is the first antibody to be produced in an immune response and forms a pentameric complex comprised of Ig monomers. IgA is the main class of antibody in external secretions, where it is found as a dimer that protects the body’s mucosal surfaces from infection; it is also found as a monomer in serum. IgD is the main antibody on the surface of B cells. IgE is found bound to cells that secrete histamines after antigen binding. IgG is the main antibody in serum. The IgG is often referred to as “therapeutics antibody” and this class of antibody is the most stable and

has a serum half-life of 20 days, whereas IgM and IgA persist for only 5–8 days (Stockwin & Holmes, 2003). Immunoglobulin G (IgG) antibodies are large heterodimeric molecules, approximately 150 kDa and are composed of two different kinds of polypeptide chain, called the heavy (~50kDa) and the light chain (~25kDa). There are two types of light chains, kappa (κ) and lambda (λ). The two heavy chains are linked to each other and to a light chain each by disulfide bonds.

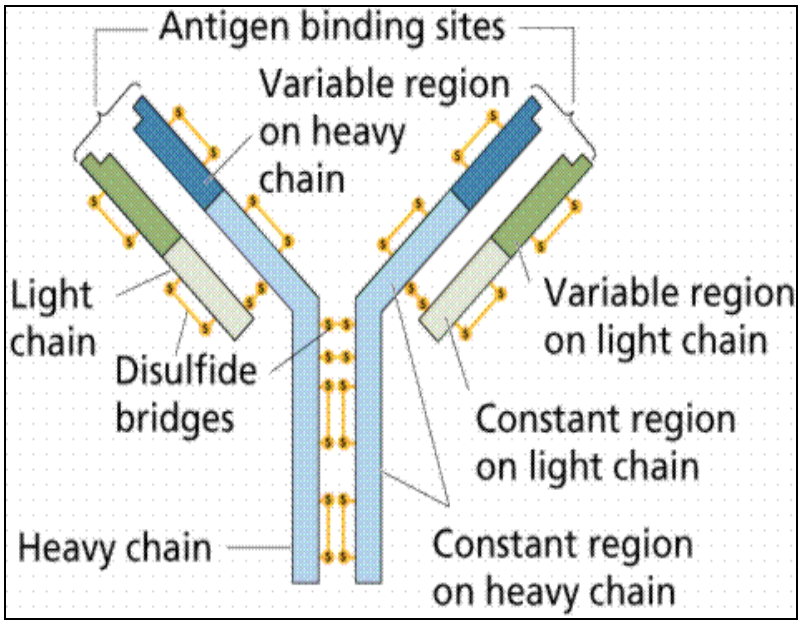


Figure 8: *Structural regions of an antibody molecule. Image taken from Purves et al., Life: The Science of Biology, 4th Edition (Purves, 2004)*

Monoclonal antibodies (mAbs) are an increasingly important class of therapeutic agents. Twenty two mAbs have been approved for therapeutic use in the United States, and over 200 mAb candidates are in the clinical pipeline (Reichert, 2008; 2011). Monoclonal antibody therapy is a rapidly-expanding immunotherapeutic treatment platform for cancer,

and at least 12 therapeutic mAbs for oncologic indications have already been approved by the FDA (Scott *et al.*, 2012) while several more are in clinical development (Reichert, 2011). The majority of marketed antibodies are approved for IV, SC and IM administration. Oral administration of antibodies is not preferred as its absorption is limited by presystemic degradation in the gastrointestinal tract and by inefficient diffusion or convection through the gastrointestinal epithelium. Systemic absorption includes convective transport of antibodies through lymphatic vessels and into the blood, and diffusion of antibodies across blood vessels distributed near the site of injection. Its bioavailability is determined by the relative rates of presystemic catabolism and systemic absorption. Presystemic catabolism may be dependent on rates of extracellular degradation (e.g., via proteolysis), rates of antibody endocytosis (e.g., receptor-mediated, fluid phase), and rates of recycling through interaction with the Brambell receptor (FcRn). Renal elimination, which is a primary pathway of clearance of small-molecule drugs, is relatively unimportant for IgG, as its large size prevents efficient filtration through the glomerulus. The majority of IgG elimination occurs via intracellular catabolism, following fluid-phase or receptor mediated endocytosis (Wang *et al.*, 2008).

1.5.1. Monoclonal Antibody and receptor mediated endocytosis and endosomal trafficking dynamics

IgG differs from most proteins in that a significant fraction of endocytosed IgG is not sorted to the lysosome but is redirected to the cell surface and released into the plasma or interstitial fluids. The recycling of IgG is mediated by the Brambell receptor, FcRn. FcRn is a receptor present within the fluid phase of endosomes which binds to IgG with pH dependent affinity (Brambell *et al.*, 1964; Junghans, 1997). Within the acidified environment of the early endosome, IgG binds tightly to FcRn. The IgG–FcRn complexes are not delivered to the lysosome for catabolism but rather are sorted to the cell surface for fusion with the cell membrane. The receptor shows virtually no affinity for IgG at physiological pH and, upon fusion of the sorting vesicle with the cell membrane, IgG dissociates from the receptor and is rapidly released into extracellular fluid. FcRn-mediated recycling is capacity limited as FcRn expression is limited. High concentrations of IgG are able to saturate the recycling system, decreasing recycling efficiency and leading to an increase in the fractional catabolic rate of IgG. IgG affinity for FcRn is species specific. Human FcRn shows high affinity for human IgG and low affinity for mouse IgG. This causes the very rapid elimination of murine mAbs in humans. Approved murine monoclonal IgGs such as Muromononab has a half-life of around 1 day in patients.

1.5.2. Antibody Drug Conjugates

Over the past decade, the effectiveness of antibodies in treating patients with cancer has been increasingly recognized. Many of these antibodies are specific for antigens expressed by the tumour itself. The use of antibody-drug conjugates (ADCs) as a therapeutic platform to treat cancer has recently gained substantial momentum. Antibodies conjugated to radioactive isotopes or chemotherapeutic drugs have shown therapeutic efficacy.

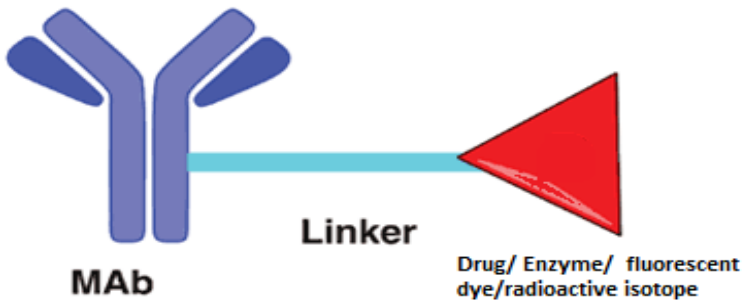


Figure 9: *Antibody Drug Conjugates*

Many of the tumour-expressed targets for therapeutic antibodies are growth factor receptors that show increased expression during tumorigenesis. Cetuximab (trade name Erbitux) is an epidermal growth factor receptor (EGFR)-specific monoclonal antibody which functions by preventing binding of activating ligand (Sunada *et al.*, 1986) and by

preventing receptor dimerization, a crucial step for initiating EGFR-mediated signal transduction (Li *et al.*, 2005).

Therapeutic antibodies currently provide clinical benefit to patients with cancer and have been established as 'standard of care' agents for several highly prevalent human cancers. Anti-cancer monoclonal antibodies can be targeted against malignant cells by several mechanisms. In cancer therapy, an antibody with specificity for a tumor-associated antigen is used to deliver a lethal dose of radiation to the tumor cells. Tositumomab, anti-CD20 monoclonal antibody is a drug for the treatment of follicular lymphoma. Iodine (^{131}I) tositumomab is the same antibody covalently bound to the radionuclide iodine-131.

Antibody-directed enzyme prodrug therapy (ADEPT) is a strategy to overcome the problems of lack of tumor selectivity. An antibody designed/developed against a tumor antigen is linked to an enzyme and injected to the blood, resulting in selective binding of the enzyme in the tumor. When the discrimination between tumor and normal tissue enzyme levels is sufficient, a prodrug is administered into the blood circulation, which is converted to an active cytotoxic drug by the enzyme, only within the tumor (Bagshawe, 2006). Another aspect of antibody based therapeutics is the development of antibody-conjugated liposomes called

immunoliposomes. Liposomes can carry drugs when conjugated with monoclonal antibodies, may be directed against malignant cells.

Cell surface receptors show a broad range of basal and antibody-induced internalization rates and this can affect ADC efficacy. On comparing the efficacy of ADCs targeting CD19 and CD21, which are cell surface receptors present on normal and tumorigenic B-cells, Ingle (2008) found that CD21 does not appreciably internalize upon antibody binding, even when expressed at very high levels. Moreover, CD19 did undergo endocytosis and efficiently internalized anti-CD19 ADCs, only in the absence of CD21. CD21 and CD19 form a complex on the surface of B cells where CD21 prevents the internalization of CD19 (Ingle *et al.*, 2008). The results of this study therefore emphasise the need to comprehensively understand the molecular mechanisms governing receptor endocytosis and intracellular trafficking properties of the respective target when designing an ADC.

Another factor that affects the efficacy of an ADC is the choice of linker in the efficient release of the drug from the antibody into the cell cytosol. This is achieved through the disruption of the chemical linkage between the antibody and the toxic payload. Non-cleavable linkers, such as thioether or amide bonds, have been utilized more recently and are intended to retain stability throughout the plasma and most of the

intracellular space. Trastuzumab emtansine (T-DM1), composed of trastuzumab linked to the cytotoxin maytansinoid DM1 (DM1) via a thioether linker, is undergoing evaluation in multiple Phase 3 trials for HER2-positive metastatic breast cancer and a marketing application for T-DM1 was submitted to the Food and Drug Administration in August 2012 (LoRusso *et al.*, 2011).

1.5.3. Biological response modifiers

Biological response modifiers, also known as BRMs, are substances that the human body produces naturally, as well as the substances that are created artificially to arouse the body's response to an infection. Infliximab (trade name Remicade), Rituximab (trade name Rituxan), Adalimumab (trade name Humira), Etanercept (trade name Enbrel) belong to the class of medications called biological response modifiers ("biologics") or Tumor Necrosis factor (TNF) blockers. They are indicated for rheumatoid arthritis where a patient body overproduces (TNF), which causes pain, inflammation, and damage. The antibodies/ protein therapeutics bind specifically to TNF-alpha and block its interaction with cell surface TNF receptors.Rituximab (trade names Rituxan and MabThera) is a monoclonal antibody against the protein CD20, primarily found on the surface of B cells. Rituximab destroys B cells, and is therefore used to treat diseases which are characterized by excessive numbers of B cells, overactive B cells, or dysfunctional B cells. This

includes many lymphomas, leukemias, transplant rejection, and some autoimmune disorders. Denosumab (trade names Prolia and Xgeva) is a monoclonal antibody for the treatment of osteoporosis and is designed to target RANKL (RANK ligand), a protein that acts as the primary signal for bone removal. With the advent of this novel RANKL-scavenging antibody-based antiresorptive strategy, it may further prove efficacious to target the RANK receptor directly with an antibody.

Some examples of FDA approved monoclonal antibodies/protein therapeutics targeting receptor are Muromonab-CD3, Anakinra and Trastuzumab. Muromonab-CD3 (trade name Orthoclone OKT3), a monoclonal antibody generated by hybridoma technology, is an immunosuppressant drug given to reduce acute rejection in patients with organ transplants. It was the first monoclonal antibody to be approved for clinical use in humans. It targets at the CD3 receptor, a membrane protein on the surface of T cells. T cells recognise antigens primarily via the T cell receptor. This receptor needs various co-receptors to function, one of which is CD3. The T cell receptor-CD3 complex transduces the signal for the T cell to proliferate and attack the antigen. Muromonab-CD3 binds to the T cell receptor-CD3-complex on the surface of circulating T cells, inducing blockage of the T cells and thus protecting the transplant against the T cells. Antibodies can be engineered to carry effector moieties, such as enzymes, toxins, radionuclides, cytokines or even DNA molecules, to

the target cells, where the attached moiety can then exert its effect. Radioimmunotherapy uses an antibody labeled with a radionuclide to deliver cytotoxic radiation to a target cell (Milenic *et al.*, 2004). Newer monoclonal antibodies with the same mechanism of action include otelixizumab, teplizumab and visilizumab.Trastuzumab (trade name Herceptin) is a monoclonal antibody approved by the Food and Drug Administration (FDA) for patients with invasive breast cancers that overexpress Human Epidermal growth factor Receptor 2 (HER2). The antibody binds to the extracellular juxtamembrane domain of HER2 and inhibits the proliferation and survival of HER2-dependent tumors. In contrast to EGFR, HER2 has no known ligand, and antibodies targeting this receptor function mainly to inhibit receptor homo- and hetero-dimerization and internalization, rather than by blocking ligand-binding (Chen *et al.*, 2003). Anakinra (trade name Kineret) is recombinant protein consisting of 153 amino acids with a molecular weight of 17.3 kilodaltons and is produced by using an Escherichia coli (E. coli) bacterial expression system. Anakinra is an interleukin-1 (IL-1) receptor antagonist. Anakinra blocks the biologic activity of naturally occurring IL-1, including inflammation and cartilage degradation associated with rheumatoid arthritis, by competitively inhibiting the binding of IL-1 to the Interleukin-1 type receptor, which is expressed in many tissues and organs.

Therapeutic antibodies can thus function by three principal modes of action: by blocking the action of specific molecules, by targeting specific cells or by functioning as signalling molecules. The blocking activity of therapeutic antibodies is achieved either by the antibody binding to the factor itself or to its receptor, thereby preventing growth factors, cytokines or other soluble mediators reaching their target receptors. Targeting involves directing antibodies towards specific populations of cells. Antibodies, because of their remarkable specificity, have long had appeal as the “magic bullet”. The past century has witnessed the evolution of the 'magic bullet' from concept to clinical reality. The attributes of high target specificity, versatility, low toxicity and favorable pharmacokinetics suggest the continuing promise of therapeutic antibodies.

1.6. Thesis proposal

1.6.1. Objective

Osteoclasts (OC) are the sole bone cells responsible for bone resorption. None of the current antiresorptive therapies directly targets the osteoclast. Receptor Activator of Nuclear factor Kappa B (RANK) receptors are highly expressed on osteoclasts and the receptors potentially serve as useful molecular targets for the specific delivery of therapeutic drugs predominantly to osteoclast cells.Monoclonal antibodies have been recognized as a promising tool for the site-specific delivery of

drugs. Hence, the objective of this research was to develop a monoclonal antibody as an osteoclast targeting agent for the therapeutic delivery of salmon calcitonin, an antiresorptive hormone. Salmon calcitonin was chosen as a model drug as it acts on the Calcitonin receptors expressed on bone-resorbing osteoclasts.

1.6.2. Hypothesis

Osteoclasts are the sole cells responsible for bone resorption and the pharmacological arrest of the increased osteoclast activity is the mainstay of treating various bone disorders. Since antibodies are renowned for their exquisite specificity of target recognition and generate highly selective outcomes following their systemic administration, we hypothesised that antibodies against the osteoclast receptor RANK can be a “universal” osteoclast-targeting platform as a drug-delivery strategy.

1.6.3. Specific aims:

Aim #1:

To generate & characterize monoclonal antibody against RANK to target osteoclasts.

Aim #2:

To synthesise & characterize conjugate of Anti-RANK antibody and salmon calcitonin as a model therapeutic molecule to deliver calcitonin on to osteoclasts.

Aim #3:

To evaluate in vitro efficacy of the conjugate

Commercially available human RANK receptor will be used as the desired target antigen. Monoclonal antibodies against the target receptor will be generated by using hybridoma technology. The RANK receptor targeting antibody will then be conjugated with the antiresorptive hormone salmon calcitonin. The synthesised bioconjugate will then be characterised followed by examining its efficacy using osteoclast specific assays.

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1. Generation & characterization of antibody against RANK to target osteoclasts

Hybridoma Technology: In 1975, Georges J.F. Köhler and César Milstein described a hybridoma technique for the generation of monoclonal antibodies (Kohler & Milstein, 1975). Fusion with tumor cells allowed the immortalization of antibody producing cells, thereby letting an unlimited production of a specific antibody molecule. Monoclonal antibodies were produced for diagnostic and therapeutic use and the discovery was a major breakthrough, underscored by the 1984 Nobel Prize in Physiology or Medicine which was awarded to Köhler and Milstein.

2.1.1. Preparation of anti-RANK monoclonal hybridoma cell lines

A monoclonal antibody (mAb) to the RANK receptor was generated using Hybridoma technology, as previously performed in our laboratory (Shahhosseini *et al.*, 2007; Kammila *et al.*, 2008) **(Figure 10).** Briefly, 6–8 week old female BALB/c mice were immunized intraperitoneally 3 times with 25µg of recombinant human sRANK receptor (Peprotech, USA) on day 0, and 14 using complete and incomplete Freund's adjuvant respectively, and once with 10µg of antigen on day 28 using phosphate buffered saline (PBS, pH 7.3). Freund's adjuvant is a solution of antigen

emulsified in mineral oil and used as an immunopotentiator. Complete Freund's adjuvant (CFA) is composed of inactivated and dried mycobacteria (usually M. tuberculosis) and incomplete Freund's adjuvant lacks the mycobacterial component. Complete Freund's adjuvant is used for initial injections as the mycobacteria in CFA attract macrophages and other cells to the injection site thereby enhancing immune response. Incomplete Freund's adjuvant is used for the boosts to minimise side effects.

The immune response to the antigen was assessed by measuring the titer of polyclonal antibody in mouse serum using an indirect enzyme linked immunosorbent assay (ELISA). Mice with the highest antibody titer were euthanized and splenectomized 3 days after the final injection of antigen. Spleen cells were fused with SP2/0 myeloma cells at a ratio of 4:1 using 50% (w/v) polyethylene glycol (PEG) (Sigma, USA) according to the technique described previously by Köhler & Milstein (Kohler & Milstein, 1975). The hybridoma cells were suspended in culture medium (RPMI 1640), supplemented with Penicillin, Streptomycin, l-Glutamine (PSG), Hypoxanthine Aminopterin Thymidine (HAT), and 20% fetal bovine serum (FBS) (Sigma, USA). During DNA synthesis, normal cells synthesize purine nucleotides and thymidylate in a pathway requiring tetrahydrofolate. Aminopterin blocks the activation of tetrahydrofolate and inhibits the synthesis of purines in this pathway. Aminopterin treated cells can use an alternative pathway to synthesize purine nucleotides if hypoxanthine is

supplied externally. The enzyme present in cells that catalyses the alternative pathway is hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Thymidylate can also be synthesized if thymidine is supplied externally. The enzyme that catalyses this reaction is thymidine kinase (TK). Normal cells therefore grow normally in the presence of aminopterin if the culture medium is also supplemented with hypoxanthine and thymidine. Myeloma cells are made defective in HGPRT or TK by mutation of their DNA and therefore cannot use the alternative pathway and will die in HAT medium. However, if normal B cells are fused to HGPRT negative or TK negative myeloma cells, the B cells provide the necessary enzymes so that the hybridoma cells can grow in HAT medium. Unfused B cells cannot survive for more than 1 to 2 weeks because they are not immortalised. HAT medium therefore provides the ideal environment for the selection of fused myeloma and B cells i.e. hybridomas.

Cells were seeded in 96-well tissue culture plates and incubated in a humidified 37°C, 5% carbon dioxide incubator for 2 weeks. Clones were maintained in HAT medium for a further 2 weeks. Hybridoma cell lines were screened by an indirect ELISA. The cell lines producing specific antibodies were recloned successively 3–7 times by limiting dilution to ensure monoclonality and stability of the cell line. Hybridoma cell lines were propagated in large 175 cm^2 tissue culture flasks and the conditioned supernatant was then collected.

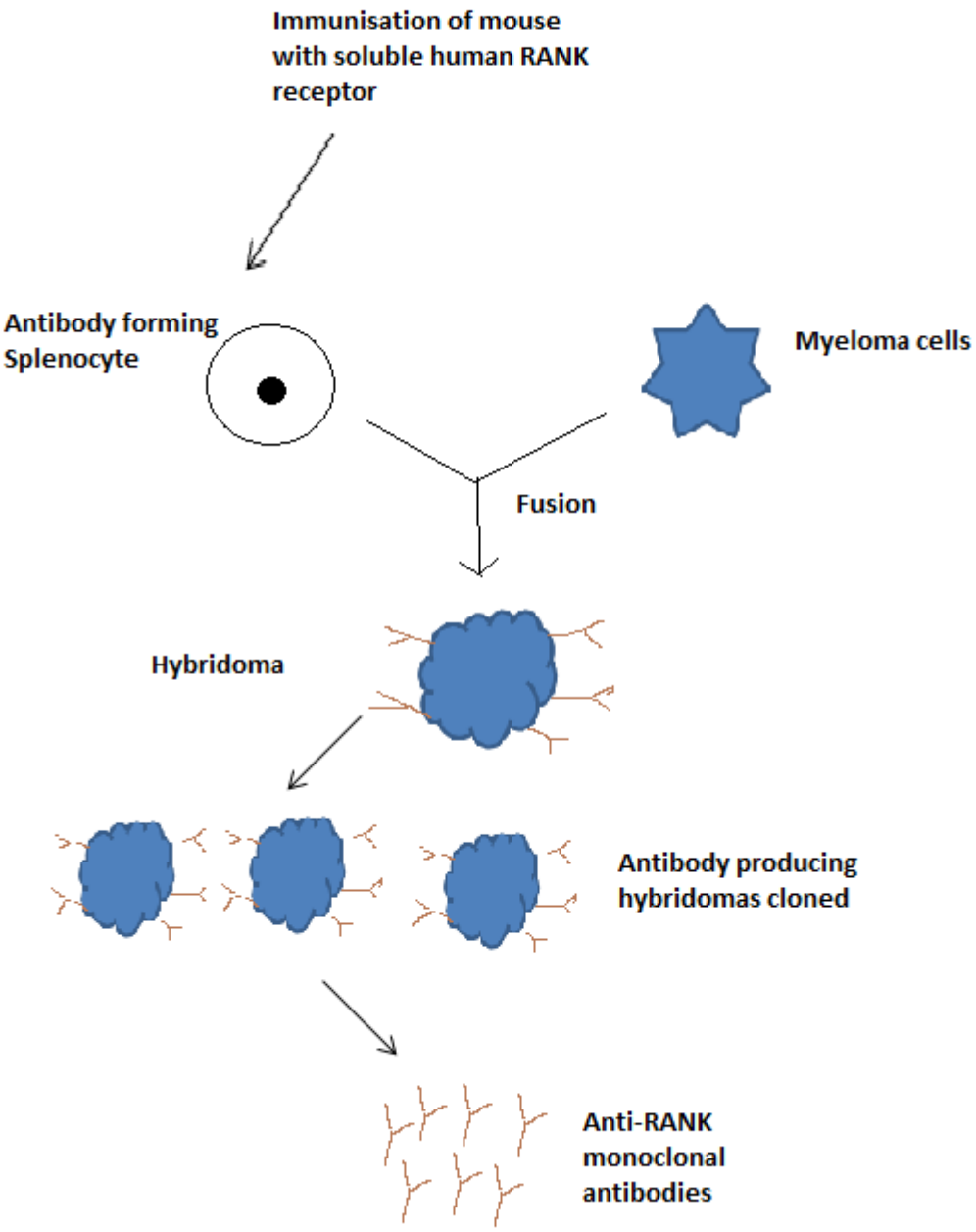


Figure 10: *Production of anti-RANK monoclonal antibodies by the hybridoma method*

Purification of IgG mAbs was achieved by affinity chromatography, using Protein G agarose (Sigma, USA). Protein G is an immunoglobulin-binding protein expressed in Streptococcal bacteria. Protein G binds preferentially to the Fc portion of IgG, but can also bind to the Fab region. After binding, the column is washed with PBS to remove the unbound components. Acidic elution buffer 0.1M Glycine-HCl (pH 2.8) was added to the column to elute the antibodies. The low-pH condition dissociates the antibody from the immobilized Protein G, and the IgG is recovered in its purified state. The collected fraction is immediately neutralized with 1M Tris-HCl (pH 9). All eluted antibodies are dialyzed against PBS at 4˚C. Antibody concentration was determined by Micro BCA protein assay. Briefly, an aliquot of 100 µl suitably diluted sample was mixed with 100 µl of working reagent (micro BCA reagent A, B and C in a volume ratio of 50: 48: 2). The mixture was incubated at 37°C for 2 hours and absorbance at 562 nm was measured using 96-well microplate reader. The antibody protein concentration was calculated by converting the absorbance into mass using the standard curve.

2.1.2. Indirect enzyme linked immunosorbent assay (ELISA)

All ELISAs were performed in flat bottomed 96 well plates (Nunc-Immuno MaxisorbTM plates, Nunc). Antibody secretion by hybridoma cells was detected by indirect ELISA. Briefly, 100 µl of antigen (human sRANK

receptor) was used for coating at a concentration of 1µg/100µl, overnight at 4^{o}C. The wells were washed three times with PBS (pH 7.3), and to avoid nonspecific binding, incubated with 3% BSA for 1 hr at room temperature. After washing, the wells were incubated with 100µl supernatant from each hybridoma clone for 1 hr at room temperature. After washing, bound antibodies were detected using secondary goat anti-mouse IgG conjugated with horseradish peroxidase (GAM-HRPO) at a 1:5000 dilution for 1 hr at room temperature. After final washing, 100 µl of 3, 3', 5, 5'- tetramethylbenzidine (TMB substrate) was added to each well and incubated for 15 min at room temperature. The optical density (OD) was measured at 650 nm using an ELISA Vmax kinetic microplate reader (Molecular Devices Corp., California, USA). The clones showing ELISA values five times higher than the negative control were considered positive. Sera of unimmunized mice and irrelevant antibody were used as negative controls.

2.1.3. Characterization: SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on purified antibody under both reducing and non-reducing conditions. The antibody was mixed with loading buffer and run in 10% polyacrylamide gel, with the thiol reagent 2-mercaptoethanol added to the loading buffer for reducing conditions (to cleave the disulfide bonds between the polypeptides). Gels were stained with Coomassie blue

for protein band detection of individual heavy and light chains of the antibody.

2.1.4. Characterization: ELISA

ELISA was performed as mentioned above, using 100µl RANK receptor in bicarbonate/carbonate coating buffer (100mM) for coating at a concentration of 1µg/100µl, overnight at 4˚C. To avoid nonspecific binding, the wells were incubated with 3% BSA in PBS for 1 hr at room temperature. After washing, the wells were incubated with different concentration of antibody (0.25 µg, 0.5 µg, 1 µg, 2 µg, 4 µg) in PBS for 1 hr at room temperature. The wells were washed with PBS and the bound antibodies were detected using secondary antibody, goat anti-mouse IgG conjugated with horseradish peroxidise (GAM-HRPO) at a 1:5000 dilution in PBS for 1 hr at room temperature. After final washing, 100 µl of TMB substrate was added to each well and incubated for 15 min. The optical density (OD) was measured at 650 nm.

2.1.5. Characterization: Western blot

For Western blot analysis, 2 µg of commercially available soluble RANK receptor (19.3 kDa) was electrophoresed on SDS-PAGE using 12% polyacrylamide gel and transferred onto nitrocellulose membrane using mini trans-blot apparatus. The membrane was blocked with 5% skim milk in PBS-T overnight at 4°C. After washing with PBST, it was cut into strips,

and incubated with 40 μg of purified mAb solution in PBS for 1 hr at room temperature. After washing, strips were reacted with GAM-HRPO at a 1:5000 dilution for 1 hr at room temperature, and the binding of anti-RANK monoclonal antibody to the RANK receptor was detected using enhanced chemiluminescence. For the negative control, the primary antibody was omitted and the antigen strip incubated with PBS.

2.1.6. Characterization: Immunocytochemistry

To confirm the RANK receptor targeting potential of the generated mAb *in vitro*, osteoclast-like cells were generated in a Lab Tek II chamber slide system (Nunc) from RAW 264.7 cells, as described below for the generation of osteoclasts. The osteoblast-like cell MG-63 cell line was used the negative control. Likewise, osteoclast cell culture, omitting the primary mAb, was also used as a negative control. Cell cultures were washed with PBS, fixed in 4% paraformaldehyde in PBS (pH 7.4) for 5 minutes and rinsed thoroughly. After blocking with 3% BSA in PBS for 1hr, the cells were incubated with our anti-RANK mAb (4 μg/ml) overnight at 4°C. Antibody detection was carried out using rabbit anti mouse IgG-FITC (1:100; Sigma, USA) for 60 min at 4°C. To visualize cell nuclei, slides were counterstained with 1.5 μg/ml 4-6-diamidino-2 phenylindole (DAPI, Sigma) diluted in PBS. Culture slides were separated from their chambers, mounted and photographed using confocal microscopy (Zeiss LSM 710 with ZEN software and the microscope the Observer.Z1).

2.2. Synthesis of Anti-RANK mAb-Calcitonin Conjugate

2.2.1. Thiolation of antibodies using Traut's reagent

No free sulfhydryl moieties were found to be present in the generated IgG mAb, as determined by Ellman's assay (Jue *et al.*, 1978). Ellman's reagent 5,5'-dithio-*bis*-(2-nitrobenzoic acid), also known as DTNB, is a versatile water-soluble compound for quantitating free sulfhydryl groups in solution. A solution of this compound produces a measurable yellow-colored product when it reacts with sulfhydryls. No such color reaction was detected when 50 µl of 4 mg/mL Ellman's Reagent Solution was added to 250 µl of 1 mg/ml antibody solution.

In order to generate an anti-RANK mAb-salmon calcitonin conjugate (mAb-sCT), free thiol groups were generated on anti-RANK mAb by reacting it in a 10 molar excess of Traut's reagent at room temperature for 1.5 hr **(Figure 11).** Traut's reagent (2-Iminothiolane or 2-IT, Sigma USA), a cyclic thioimidate compound, reacts spontaneously and efficiently with primary amines at pH 7-9 resulting in sulfhydryl addition (Traut *et al.*, 1973).

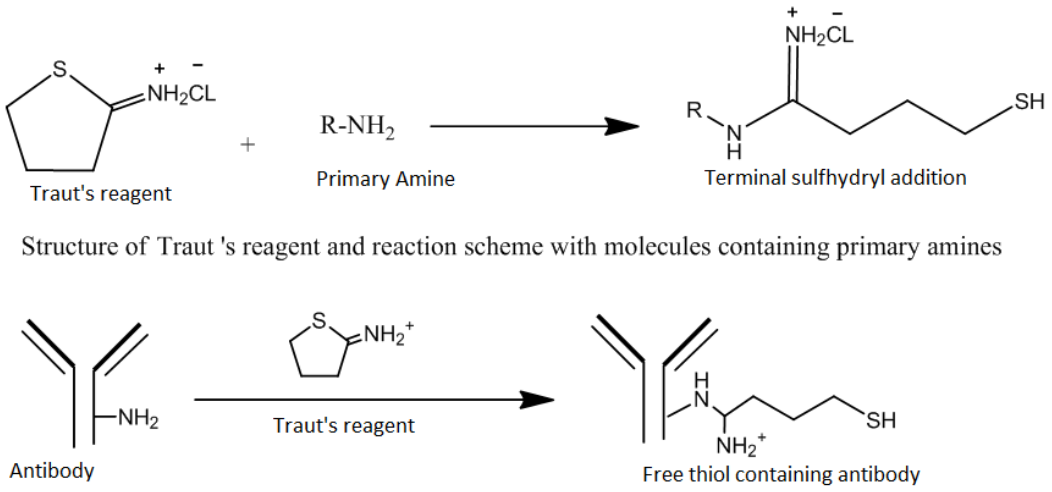


Figure 11: *Thiolation of antibody using Traut's reagent*

2.2.2. Synthesis of a thiol-reactive calcitonin analogue

Salmon calcitonin has three primary amines at residues Lys 11, Lys 18 and the N-terminus, which can react with the NHS functional group of NHS-PEG-MAL to generate three intermediate conjugates: mono-, di- and tri-substituted thiol reactive sCT analogues. N-hydroxysuccinimide(NHS) esters of NHS-PEG-MAL react with primary amine to form amide bonds. Molecules with primary amines were reacted with three fold molar excess of the crosslinker and the unreacted excess reagent was removed by desalting.

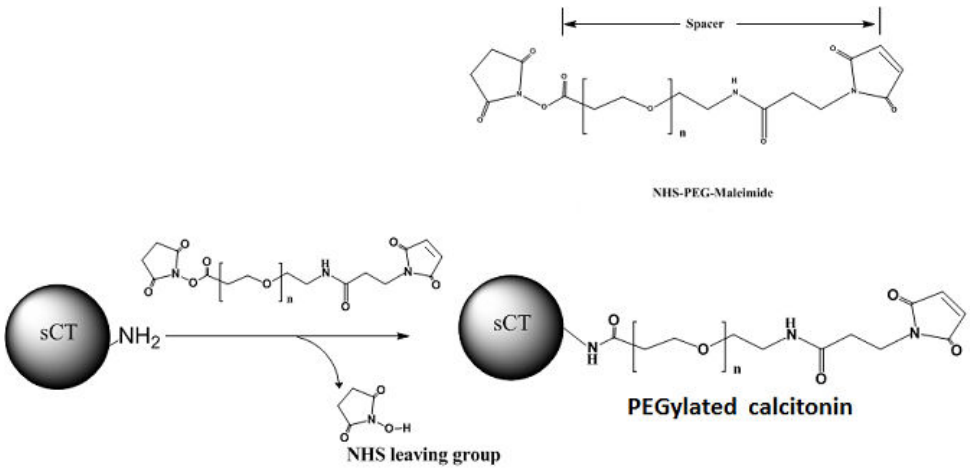
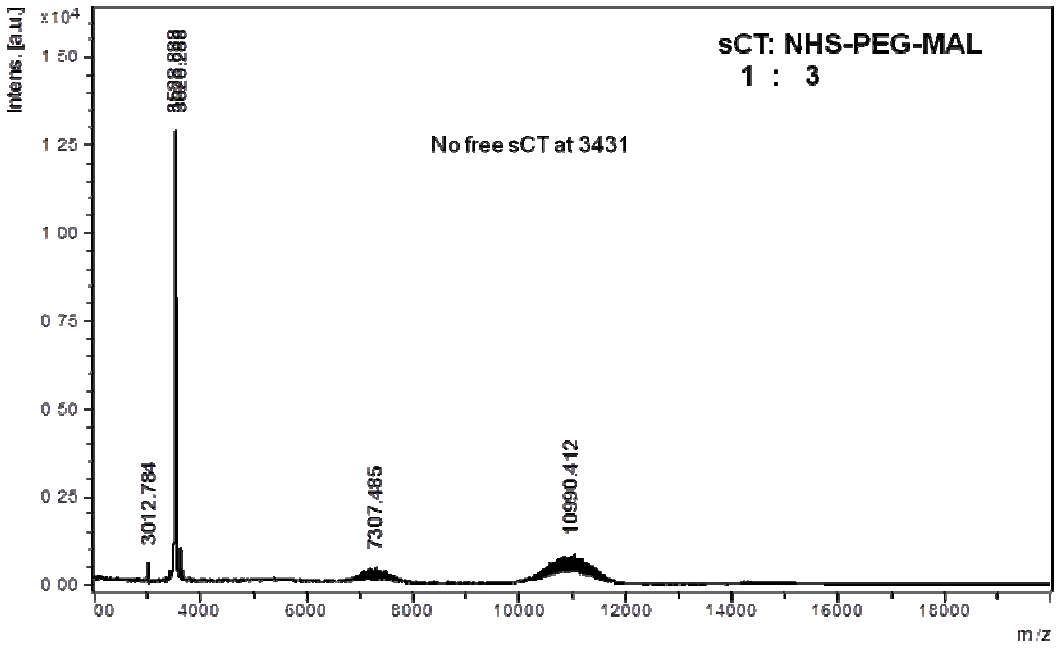


Figure 12: *Synthesis of thiol reactive sCT analogue by reacting sCT with NHS-PEG-MAL.*

To generate a thiol-reactive calcitonin analogue, synthetic salmon calcitonin (sCT, Calbiochem, USA) in DMSO (13.72 mg/ml) was mixed with NHS-PEG-MAL (Creative Biochem, USA) in DMSO (51 mg/ml) (Sigma,USA) in 1:3 molar ratio **(Figure 12).** DMSO was chosen as the reaction medium because of the instability of NHS and sCT in aqueous solutions. sCT is highly soluble and highly stable in DMSO (Stevenson & Tan, 2000). The reaction between the primary amines in sCT and NHS group of NHS-PEG-MAL was allowed to proceed at room temperature with constant stirring for 45 minutes. The reaction was optimised in Doschak's lab.

The effect of various concentration of NHS-PEG-MAL was studied to optimise the reaction using sCT: NHS-PEG-MAL at 1:1, 1:2, 1:3, 1:5 and 1:7 mol/mol ratios. MALDI-TOF spectra showed the reaction to be incomplete for sCT: NHS-PEG-MAL at 1:1, 1:2 molar concentration with the peak at 3430 representing sCT and the reaction was complete for sCT: NHS-PEG-MAL at 1: 3 mol/mol as shown by the loss of the sCT peak **(Figure 13)**. With increase in NHS-PEG-MAL concentration, formation of di and tri-substituted products also increased. Hence, sCT: NHS-PEG-MAL at 1:3 mol/mol ratio was chosen based on those results (Bhandari *et al.*, 2012)



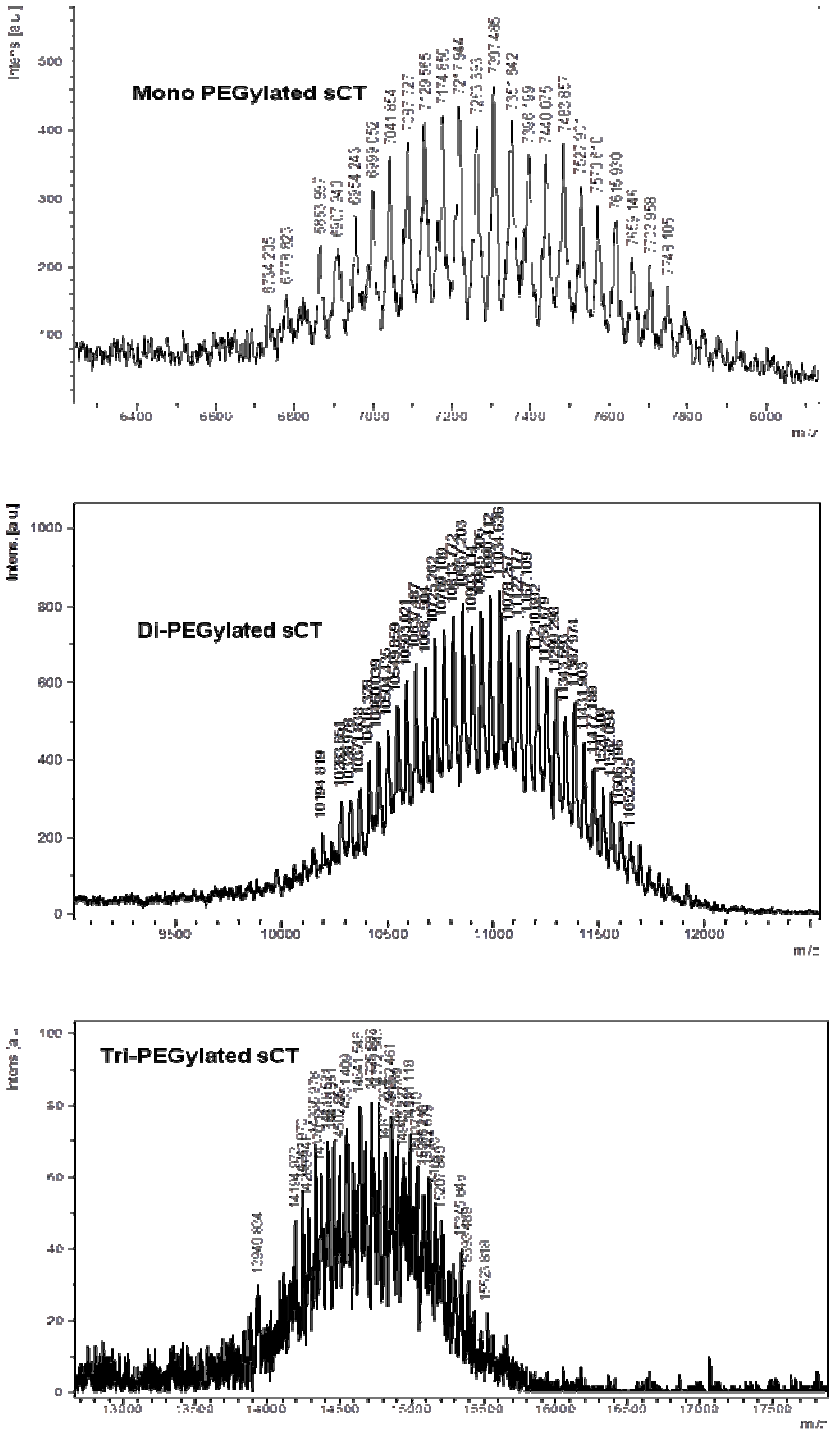
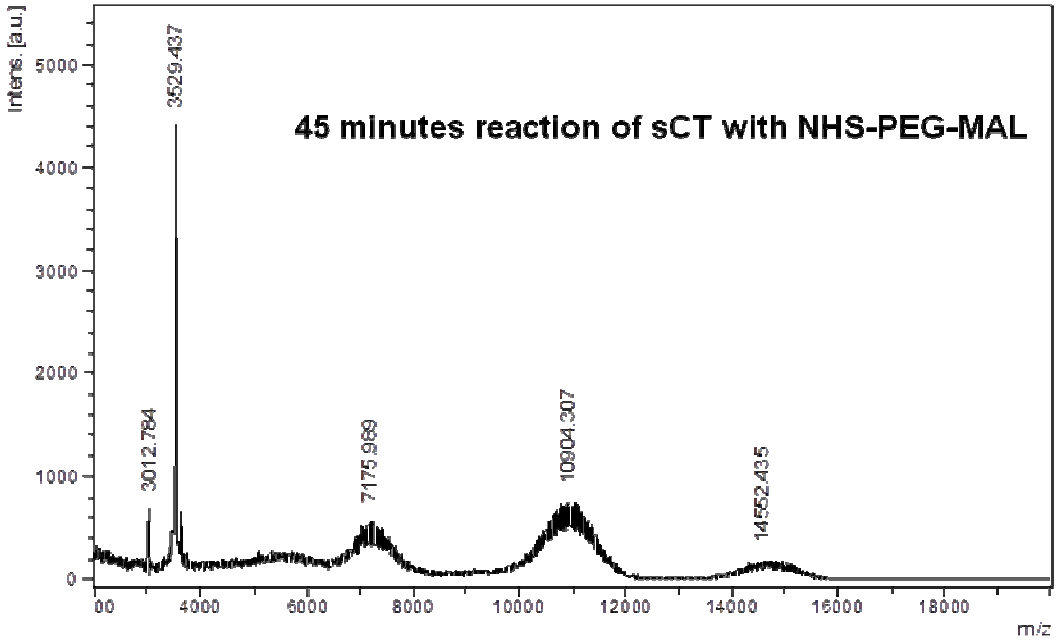


Figure 13: *MALDI- TOF spectra for the effect of NHS-PEG-MAL concentration in its reaction with sCT: 1:3 mol/ mol ratio of sCT to NHS-PEG-MAL. No free sCT at 3431. Image taken from (Bhandari et al., 2012).*

The effect of reaction time was studied at various time points; 15, 30, 45 and 60 minutes. Reaction was found to be incomplete before 30 minutes as sCT peak was seen on MALDI -TOF. No sCT peak was observed at the reaction time of 45 minutes thus indicating the reaction to be complete **(Figure 14)**. With the increase in reaction time, formation of di and tri-substituted products also increased. Hence, reaction time of 45 minutes was chosen based on those results (Bhandari *et al.*, 2012).



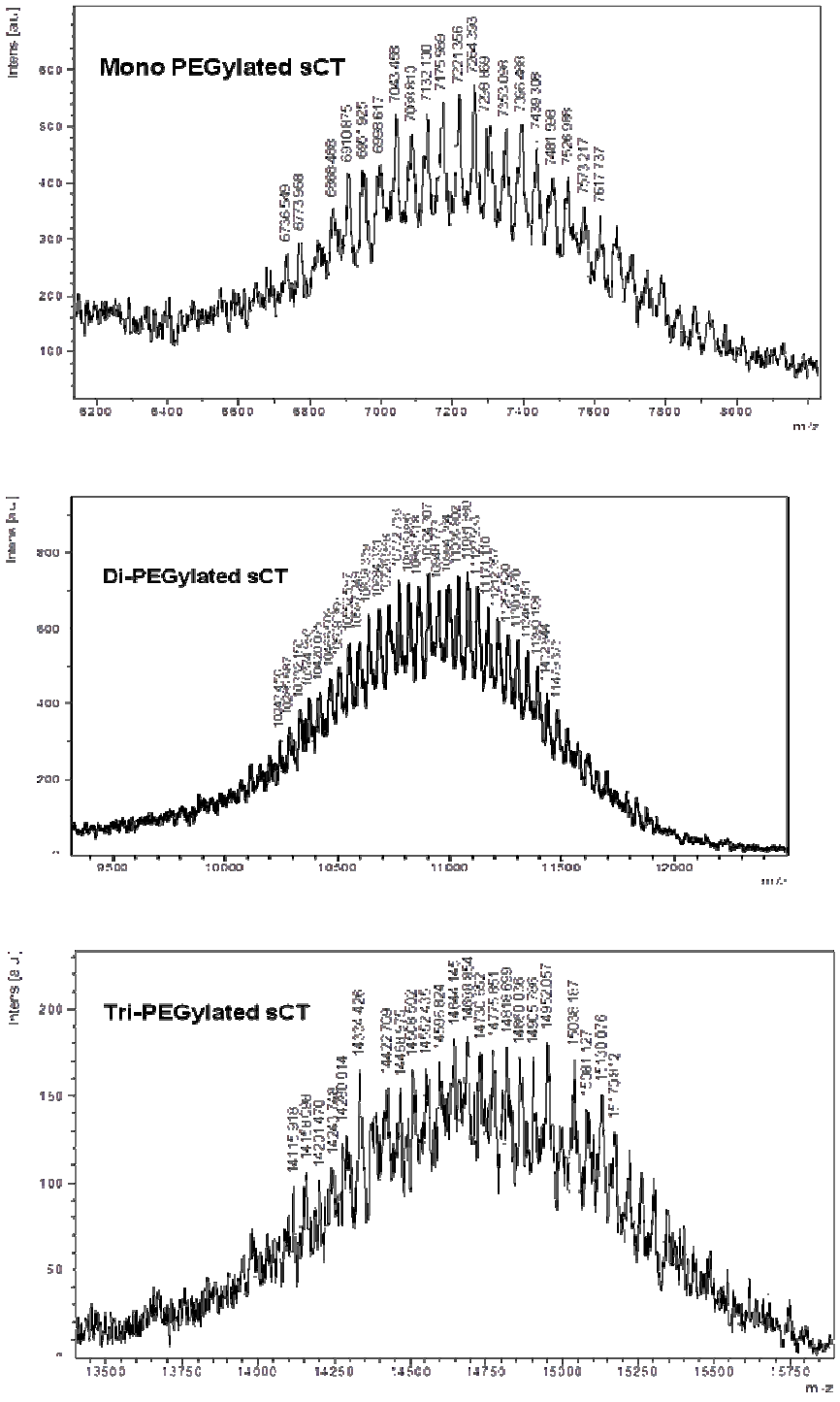


Figure 14: *MALDI- TOF spectra for the reaction of sCT with NHS-PEG-MAL at 45 minutes. No free sCT at 3431. Image taken from (Bhandari et al., 2012).*

2.2.3. Coupling free Thiol-containing mAb with functionalized Thiol-reactive sCT analogue

The functionalized thiol-reactive sCT analogue was added intermittently with constant stirring to the thiol-mAb solution at a 10:1 molar ratio and the reaction between the thiol-reactive MAL groups in functionalized sCT and the SH group of the thiol-mAb was allowed to proceed at room temperature, in the dark, with constant stirring for 2 hours. Unreacted sCT-PEG was removed by dialysis at 4˚C **(Figure 15).**

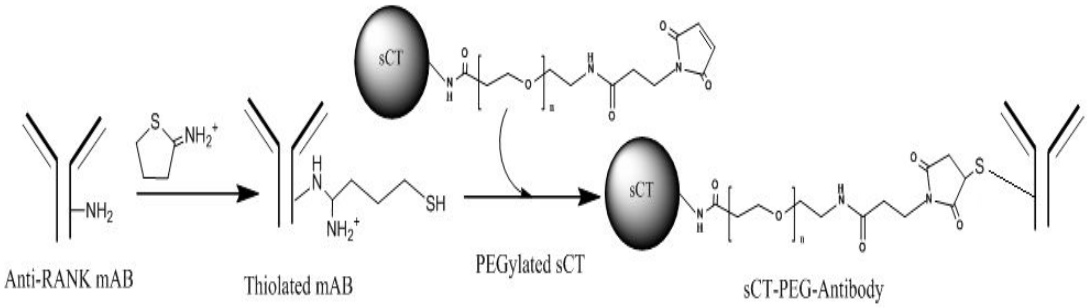


Figure15: *Coupling free thiol containing mAb with functionalized thiol reactive sCT analogue.*

2.3. Characterization of Anti-RANK mAb-sCT conjugate

2.3.1. Characterization: SDS-PAGE

To confirm the conjugation of calcitonin on to the antibody molecule, SDS-PAGE analysis was performed on this synthesised anti-RANK mAb-sCT conjugate under both reducing and non-reducing conditions. The conjugate was mixed with loading buffer and run in 10% polyacrylamide gel, with the thiol reagent 2-mercaptoethanol added to the loading buffer for reducing conditions and gels were stained with Coomassie blue for protein band detection.

2.3.2. Characterization: ELISA

ELISAs were performed, using 100µl RANK receptor for coating at a concentration of 1µg/100µl, overnight at 4˚C. To avoid nonspecific binding, the wells were incubated with 3% BSA for 1 hr at room temperature. The wells were incubated in triplicate with either mAb alone, functionalized sCT-PEG, or mAb-sCT conjugate for 1 hr. The wells were washed with PBST followed by incubation with rabbit anti-salmon calcitonin antibody (US Biologicals) at a 1:5000 dilution for 1 hr at room temperature. The wells were washed with PBST three times and incubated with HRPO conjugated goat anti-rabbit IgG (R&D Systems) at a 1:5000 dilution for 1 hr at room temperature. After final washing with

PBST, TMB substrate was added to each well and the OD measured at 650 nm.

2.4. In vitro testing of mAb-sCT conjugate efficacy

2.4.1. Generation of Osteoclasts

Osteoclast-like cells were generated in culture from RAW 264.7 cells (a transformed murine monocytic cell line), purchased from the American type culture collection (ATCC, VA, USA) (Rahman *et al.*, 2006). RAW 264.7 cells were cultured to confluence in a 75cm^2 flask in GIBCO High Glucose 1X Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, CA, USA) containing 4.5 g/L D-Glucose, L-Glutamine, and 110 mg/L Sodium Pyruvate, with the addition of 10% heat inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin added (10000 U/ mL; Invitrogen, USA). Confluent cells were harvested by scraping, centrifuged at 1500 RPM, and resuspended in 25 mL of culture medium. The number of cells in the suspension was measured using a Bright-Line Hemocytometer (Hausser Scientific, PA, USA), and seeded at a concentration of 2 X 10^3cells/well in 96-well culture plates (Corning) and placed in the CO_2 incubator overnight to allow the cells to attach to the surface. After 24 hr, the culture medium was replaced with media containing 25 ng/ml macrophage colony stimulating factor (M-CSF) and 50 ng/mL RANKL (PeproTech, NJ, USA). Osteoclasts were successfully

generated by dosing with M-CSF and RANKL every 48 hr over the course of 7 days.

To confirm the generation of multinucleated osteoclast-like cells, the cultured cells were stained for the enzyme tartrate-resistant acid phosphatase (TRAP) using the Leukocyte Acid Phosphatase (TRAP) Kit from Sigma-Aldrich (St.Louis, MO, USA), according to the manufacturer's instructions. TRAP-positive multinucleated osteoclasts were visualized by light microscopy and photographed.

2.4.2. Spectrophotometric assay of *In vitro* TRAP activity

To quantify the total TRAP activity from *in vitro* osteoclast cultures, RAW264.7 cells were seeded at a concentration of 2 X 10^3cells/well in 96-well culture plates and incubated for 24 hrs. Peptide factors RANKL (50 ng/ml) and M-CSF (25ng/ml) were added to the culture, with or without the addition of 100 nM of mAb, 100 nM of mAb-sCT, or 100 nM of sCT. The medium and factors were replaced every 48 hr. Osteoclastogenesis was assessed by the spectrophotometric measurement of TRAP activity on day 7.

Briefly, the medium was aspirated and the cell monolayer washed twice with PBS. The cells were then lysed with 100 μl of 0.2% Triton X-100 in water (v/v) for 10 min. TRAP activity in the cell lysate was determined

using an Acid Phosphatase Assay Kit (Cayman Chemical, USA). The assay utilizes para-nitrophenyl phosphate (pNPP) as a chromogenic substrate for the TRAP enzyme. In the first step, acid phosphatase dephosphorylates pNPP. L-tartrate, an inhibitor of non-tartrate resistant acid phosphatase, provided in the kit was used to measure TRAP enzyme activity. In the second step, the phenolic OH- group was deprotonated under alkaline conditions resulting in p-nitrophenolate that yields an intense yellow color which was measured at 405 nm using a microplate reader.

2.4.3. In *vitro* mineral resorption assay

To quantify the mineral resorptive activity of osteoclast-like cells in culture, RAW 264.7 cells were suspended in the cell culture medium and seeded in a 16-well BD Biosciences Osteologic® Slide (BD Biosciences, MA, USA) at a density of 2 X 10^3 cells/well and incubated for 24 hrs. Osteoclast-like cells were generated by dosing with 50 ng/ml RANKL and 25ng/ml M-CSF every 48 hr over the course of 7 days. The wells were incubated with 100 nM of either mAb alone, sCT alone, or mAb-sCT conjugate. Half the volume of the medium and factors were replaced every 48 hrs. After 7 days of culture, the medium was aspirated, the slide placed in a petri-dish and washed with double-distilled water. To remove the adherent cells, the slide was then soaked in bleach for 10 min and washed

with double-distilled water. Slides were air-dried, viewed under an inverted light microscope and low power images of the calcium phosphate mineral remaining on the slide acquired. To quantify the resorptive effect of osteoclasts, the images were analyzed using the Java-based image-processing program ImageJ (NIH, USA).

2.4.4. Cell viability test for mAb-sCT conjugate

The MTT assay was employed to test the effect of conjugate on cells other than osteoclasts. In this assay, mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of MTT yielding purple formazan crystals. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.

RAW 264.7 cells were seeded on 96 well plates at an initial density of 2 X 10^3 cells/well and incubated for 72 hr at 37^{o}C, 5% CO_2 until the cells were 80% confluent. The culture medium was replaced by 200 µl basic DMEM media without FBS and incubated for 30 minutes. Then the culture medium was replaced by 100 µl basic DMEM media containing 50, 100 and 200 nM of mAb, mAb-sCT and sCT respectively. The cells were incubated for 4 hr at 37°C. The medium was then replaced with 100 µl basic medium containing (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) at a concentration of 100 µg/well and

incubated for a further 4 hrs at 37°C. After removing the supernatant and washing twice with phosphate buffered saline, the newly formed purple formazan crystals were dissolved in 200 µl of solubilization solution using an *in vitro* toxicology assay kit (TOX-1, Sigma Aldrich, St. Louis, USA) and the absorbance was measured at 570 nm using a microplate reader.

2.4.5. In vitro bioactivity of mAb-sCT and calcitonin receptor binding affinity

To confirm that calcitonin bioactivity was not lost after conjugation to the mAb, and to confirm the ability for the mAb-sCT conjugate to trigger the calcitonin receptor, T47D human breast cancer cells (known to contain calcitonin receptors) were used, as previously described (Bhandari *et al.*, 2010).

Briefly, T47D cells (ATCC, VA, USA) were cultured in triplicate in RPMI-1640 culture medium containing 1% penicillin–streptomycin, 10% fetal bovine serum, and insulin (0.2 IU/mL). Cells were seeded on 48 well plates at an initial density of 5×10^4 cells/well and incubated in 95% air and 5% CO_2 at 37°C for 2 days. Cells were then washed with HBSS and pre-incubated in RPMI-1640 culture medium devoid of FBS, insulin and antibiotics. Cells were treated with the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, 1 mM) and incubated at 37 °C for 30 min. 100 nM of mAb, mAb- sCT and sCT were then added to the wells

except the control where T47D cells were treated with IBMX only. The plate was incubated for 20 min at 37 °C. After removing the supernatant, cells were washed three times in cold phosphate buffered saline and resuspended in 500 µl of Cell Lysis Buffer. Cells were frozen at -20°C and thawed with gentle mixing. The freeze/thaw cycle was repeated three times and the mixture was centrifuged at 600 g for 10 minutes at 2-8° C to remove cellular debris. The supernatant was collected and stored at -20°C. cAMP concentrations were then measured using the cyclic adenosine monophosphate (cAMP) Enzyme Immuno-Assay (EIA) kit (KGE002B, R & D systems, USA).

2.5. Statistics

Statistical Analysis were conducted using Graph Pad Prism 5 program (GraphPad Software, Inc..CA, USA). Results are presented as the mean ± standard deviation of three samples. Unpaired t-tests were used to assign significance between groups, with a P-value of less than 0.05 as the threshold for significance. Two way-ANOVA was used for MTT assay.

A version of this chapter has been published. Madhuri Newa, Krishna Hari Bhandari, Lili Tang, Rohit Kalvapalle, Mavanur Suresh,Michael R.Doschak: Antibody-Mediated "Universal" Osteoclast Targeting Platform using Calcitonin as a Model Drug;Pharmaceutical Research: Volume 28, Issue 5 (2011), Page 1131.

CHAPTER 3: RESULTS

Anti-RANK monoclonal antibody was generated by Hybridoma technique by immunising the mice with human sRANK receptor. Antibody was purified by Protein G affinity chromatography and antibody concentration was determined by Micro BCA protein assay. Antibody aliquots were made to avoid repeated freeze thaw cycle and stored at -20°C for further study.

3.1. SDS-PAGE analysis

SDS-PAGE analysis under non reducing condition showed the band at 150 kDa for unconjugated anti-RANK IgG and for the mAb-sCT conjugate, a band above 150 kDa was observed thus confirming the conjugation to have taken place. Under reducing condition, separate bands for heavy chain at 50 kDa and light chain at 25 kDa observed for the unconjugated antibody due to the cleavage of disulfide bond between heavy chain and light chain of the antibody. For the SDS PAGE analysis of the conjugate under reducing condition, some bands above 50 kDa and 25 kDa were observed, suggestive of conjugation of PEGylated calcitonin on heavy chain and light chain of antibody **(Figure 16).**

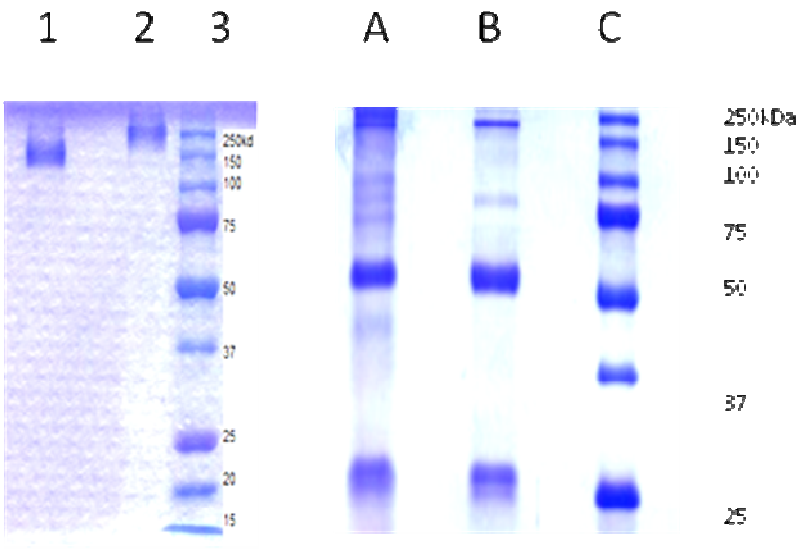


Figure 16: *SDS-PAGE analysis of mAb-sCT conjugate and unconjugated antibody. Lane 1: Unconjugated antibody (Non-reducing condition), Lane 2: mAb-sCT Conjugate (Non-reducing), Lane 3 Standard marker, Lane A: mAb-sCT Conjugate (Reducing), Lane B: Unconjugated antibody (Reducing), Lane C: Standard marker.*

3.2. ELISA for anti-RANK monoclonal antibody and its conjugate with calcitonin

ELISA was conducted with the different concentration of generated antibody on the RANK receptor coated well plates. The optical density was seen to linearly increase with the concentration of mAb **(Figure 17).** Spectrophotometric absorbance value for 0.25, 0.5, 1.0, 2.0 and 4.0 µg of the anti-RANK antibody was 0.025, 0.070, 0.131, 0.27 and 0.518 respectively.

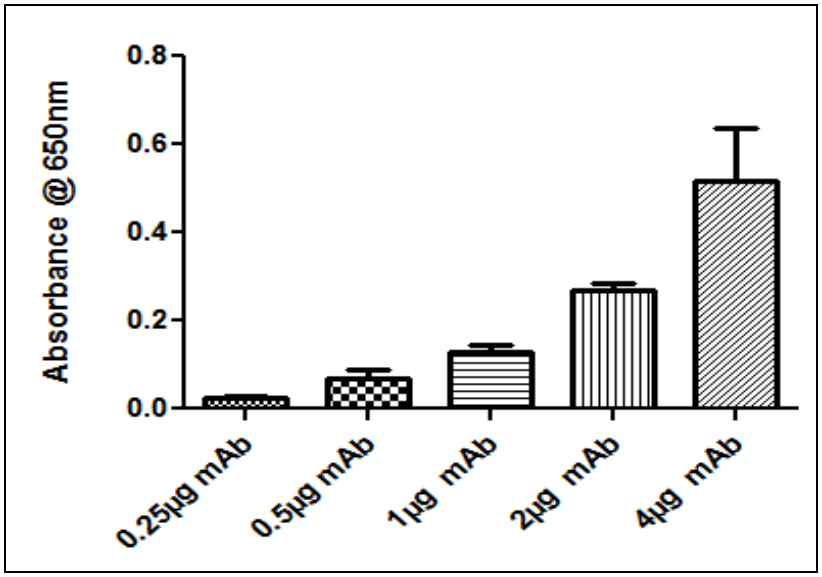


Figure 17: *ELISA of anti-RANK antibody conducted on RANK receptor coated plate. The optical density was seen to be proportional with the concentration of anti-RANK Antibody.*

ELISA carried out for the conjugate showed the binding of the conjugated antibody to the coated RANK receptor **(Figure 18).** The sCT attached to the conjugate was detected using an anti-calcitonin antibody, thus confirming the intactness of mAb-sCT conjugates. Optical density for mAb alone was negligible as it did not have calcitonin to be detected by the anti-calcitonin antibody. Similarly, the OD for sCT-PEG was also significantly low as it did not have the anti-RANK mAb targeting moiety necessary to bind to the RANK coated plate. ELISA derived spectrophotometric absorbance values for mAb, sCT-PEG and mAb-sCT conjugate were 0.002, 0.025 and 0.153 respectively.

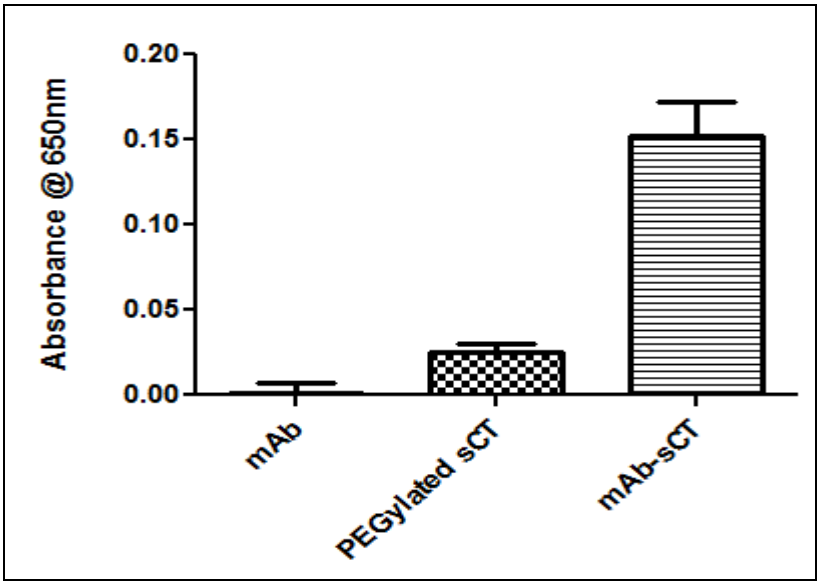


Figure 18: *ELISA of the mAb-sCT conjugate where anti-calcitonin secondary antibody reagent was used to detect immobilized calcitonin residues that were part of the primary anti-RANK conjugates that were binding to the RANK-coated wells.*

3.3. Western blot analysis

Recombinant human sRANK receptor (a 19.3 kDa polypeptide) was electrophoresed on a 12% polyacrylamide gel and transferred to nitrocellulose membrane. On incubation with generated anti-RANK monoclonal antibody, a band was detected between 15 to 20 kDa, after the chemiluminescent detection of anti-RANK IgG **(Figure 19).**

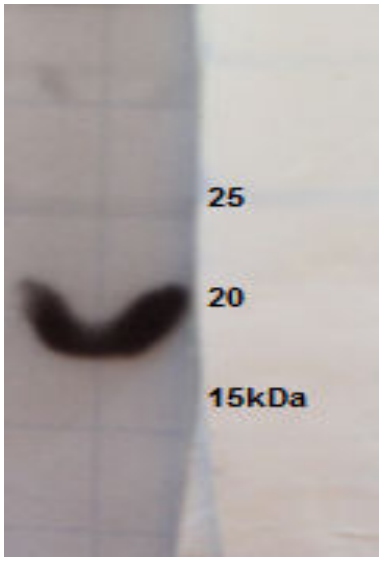


Figure 19: *Western blot analysis: Lane 1: A band seen between 20 kDa and 15 kDa as a result of chemiluminescent detection of Anti-RANK IgG binding to RANK receptor (19.3 kDa). Lane 2: Standard marker.*

3.4. Osteoclast generation and confirmation by TRAP staining

Osteoclasts were generated by dosing the RAW 264.7 cells with RANKL and M-CSF. The staining for TRAP is a technique commonly used to visualize osteoclasts. The principle behind staining of TRAP involves the use of napthol AS phosphates in conjunction with fast garnet GBC salts for the detection of acid phosphatase. This diazonium fast garnet GBC salt was selected because it couples rapidly at acid pH, forming insoluble dye deposits. Napthol AS-BI, released by enzymatic hydrolysis, couples immediately with the fast garnet GBC salt resulting in the

formation of insoluble maroon deposits at sites of activity. Tartaric acid was used in order to demonstrate the presence of tartrate-resistant acid phosphatase. Hence, cells containing tartrate-sensitive acid phosphatase are devoid of activity and only the cells containing tartrate acid resistant phosphatase show maroon dye deposits at the sites of activity. Staining confirmed that the presence of TRAP positive multinucleated osteoclast-like cells was limited to those wells dosed with exogenous M-CSF and RANKL, under the cell culture conditions described **(Figure 20).**

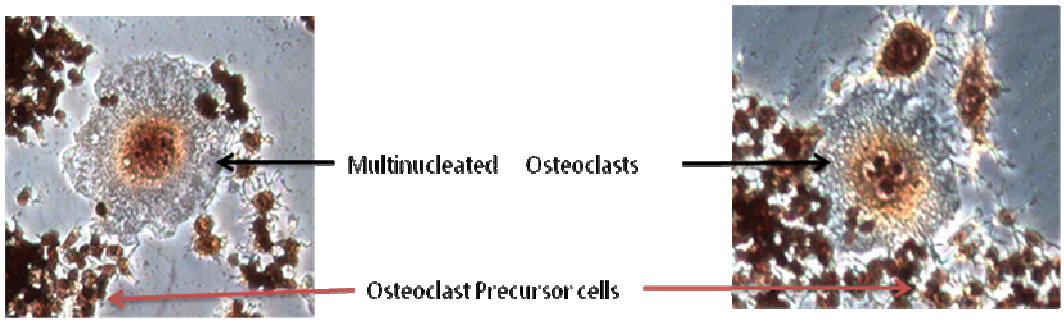
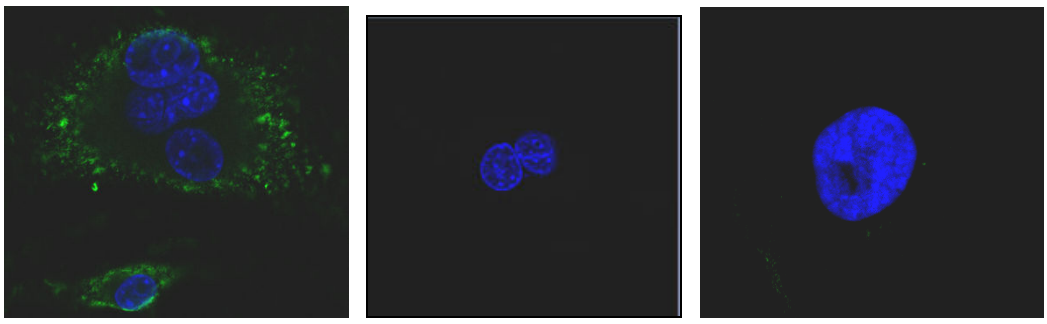


Figure 20: *Multinucleated TRAP positive osteoclast was observed by TRAP staining. RAW 264.7cells, a murine macrophage-like osteoclast precursor line, were stimulated with 50ng/ml RANKL and 25ng/ml M-CSF for osteoclast generation.*

3.5. Immunocytochemistry

Confocal microscopy confirmed that the anti-RANK receptor mAb staining (green color) was limited to surface receptors present on osteoclastic precursors as well as mature osteoclast-like cells **(Figure 21a).** Counterstaining with DAPI (blue color) confirmed the multinucleated phenotype of osteoclast-like giant cells. There was no demonstrable fluorescent fluorescein isothiocyanate (FITC) staining in negative control slides **(Figure 21b and 21c).** A commercially available anti-RANK mAb was used as a positive method control, to confirm that RAW 264.7 osteoclastic precursors as well as mature osteoclast-like cells indeed stained for surface RANK receptors using immunocytochemistry.



(a) (b) (c)

Figure 21 (a) *Confocal images of immunocytochemistry performed with anti-RANK mAb which detected RANK receptors, the surface receptors present on the osteoclasts as well as osteoclast precursors.*

*Counterstaining with DAPI showed the multinucleation in osteoclasts. (**b)** Confocal image of immunocytochemistry for osteoclast cell culture omitting primary antibody, as a negative control. **(c)** Confocal image of immunocytochemistry for MG-63 cell line as a negative control.*

3.6. TRAP activity assay

RAW 264.7 cell cultures treated with exogenous RANKL and M-CSF showed a 9.9 fold increase of TRAP activity compared to controls without cytokine treatment. The release of this osteoclast associated enzyme was potently inhibited by the synthesised mAb-sCT conjugate (and by the unconjugated mAb alone) as compared to sCT alone. Decrease in TRAP activity with 5.47 fold was observed after treatment with mAb-sCT conjugate. Also, the conjugate showed a 2.82 fold greater inhibition of TRAP compared to sCT treatment alone **(Figure 22).**

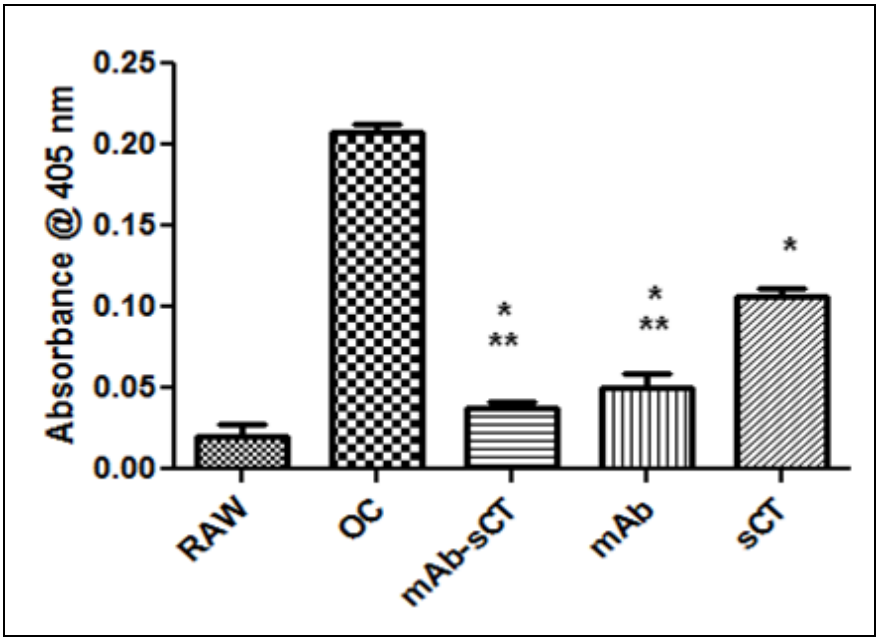


Figure 22. *Tartrate-resistant acid phosphatase (TRAP) activity Assay. mAb-sCT conjugate showed an inhibitory effect on TRAP activity, an important cytochemical marker of osteoclasts. RAW : cells cultured in media with 0ng/ml RANKL + 0ng/ml M-CSF; OC: cells cultured in media with 50ng/ml RANKL + 25ng/ml M-CSF; mAb-sCT: cells cultured in media with 50ng/ml RANKL + 25ng/ml M-CSF+100nM conjugate; mAb: cells cultured in media with 50ng/ml RANKL + 25ng/ml M-CSF+100nM mAb; sCT: cells cultured in media with 50ng/ml RANKL + 25ng/ml M-CSF+100nM sCT . * P < 0.05 versus OC; ** P < 0.05 versus sCT. No statistically significant difference was observed between naked antibody and the conjugate.*

3.7. In vitro mineral resorption assay

The osteoclast-like cells generated on Osteologic® calcium phosphate-coated culture wells were shown to be capable of resorbing the immediate calcium phosphate layer surrounding them **(Figure 23).** That result was in direct contrast to precursor RAW 264.7 cells (cultured in the absence of M-CSF and RANKL) that were shown not to affect the calcium phosphate coating in any manner. Osteoclast-like cell cultures treated with the mAb-sCT conjugate, or mAb alone, demonstrated a significant inhibitory effect on the resorptive ability of the osteoclast-like cells, as measured by the conservation of calcium phosphate layer on the Osteologic® slide. The integrated density value (IDV) of the remaining calcium phosphate layer for the culture treated with the mAb-sCT conjugate was significantly greater than that treated with sCT alone.

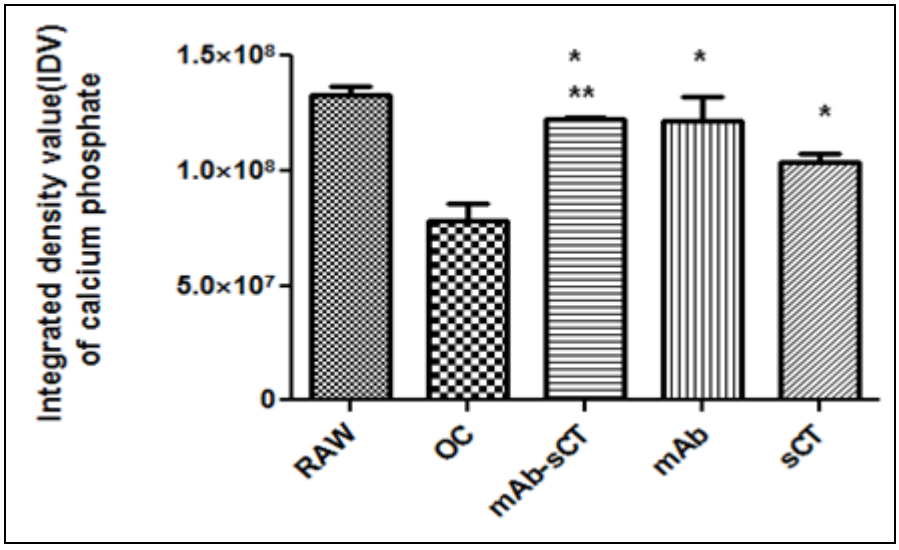


Figure 23: *Demonstration of resorption on an osteologic substrate plate. After 7 days of culture, adherent cells were removed and the integrated density value of the remaining osteologic layer was calculated using an alphaimager densitometer. RAW: cells cultured in media with 0ng/ml RANKL + 0ng/ml M-CSF; OC: cells cultured in media with 50ng/ml RANKL + 25ng/ml M-CSF; mAb-sCT: cells cultured in media with 50ng/ml RANKL + 25ng/ml M-CSF+100nM conjugate; mAb: cells cultured in media with 50ng/ml RANKL + 25ng/ml M-CSF+100nM mAb; sCT: cells cultured in media with 50ng/ml RANKL + 25ng/ml M-CSF+100nM sCT. *P< 0.05 versus OC; ** P<0.05 versus sCT. No statistically significant difference was observed for antibody versus conjugate and antibody versus calcitonin.*

3.8. Cytotoxicity of mAb-sCT conjugate

To test for potential cytotoxic effects of the generated mAb and/or the synthesised mAb-sCT conjugate, MTT assay was used to test for RAW 264.7 cell viability. Anti-RANK monoclonal antibody, mAb-sCT conjugate and sCT showed no demonstrable cytotoxicity on RAW 264.7 cells when treated for 4 hours at the concentrations tested (up to 200 nM), as measured by the absorbance of formazan solution. **(Figure 24).** Thus, the viability of these cells was not perturbed by the 4 hour incubation with the generated mAb and/or conjugate treatments.

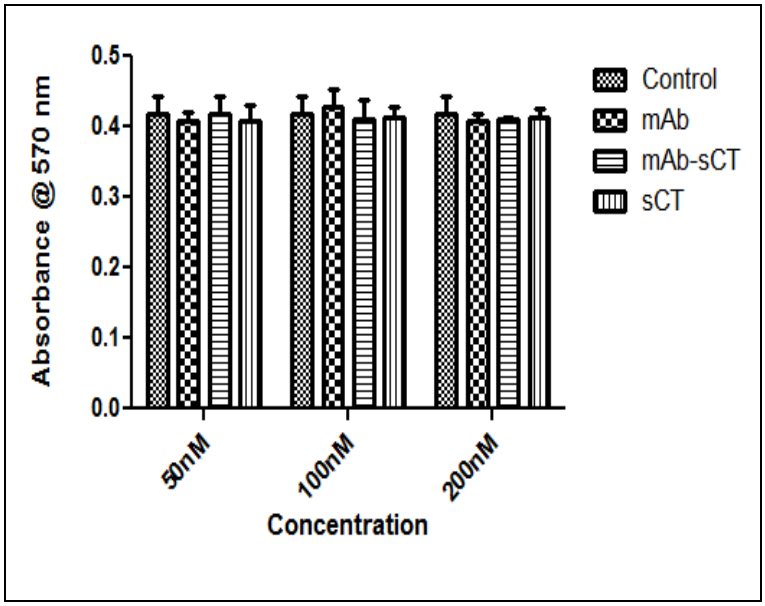


Figure 24*. In vitro cytotoxicity of mAb, mAb-sCT & sCT on RAW 264.7 cells was determined by MTT assay. No demonstrable cytotoxicity was seen as measured by the absorbance of formazan solution formed after 4 hours incubation with compounds, compared to that seen with untreated media. All samples were not statistically significant compared to the control at all concentrations.*

3.9. In vitro Bioactivity of mAb-sCT and Calcitonin Receptor Binding Affinity

Using an *in vitro* competitive binding assay, cAMP activity was determined in TD47 breast cancer cells known to contain the calcitonin receptor. The assay confirmed that sCT, as well as the mAb-sCT conjugate, were capable of stimulating cAMP production after binding the calcitonin receptor **(Figure 25).** The significantly lower absorbance values measured (due to the competitive nature of the assay) indicated the ability

for sCT and/or mAb-sCT to generate intracellular cAMP in the presence of a phosphodiesterase inhibitor. Conversely, the mAb treated and control wells exhibited negligible intracellular cAMP generation, and accordingly, high absorbance values in the competitive binding assay were observed.

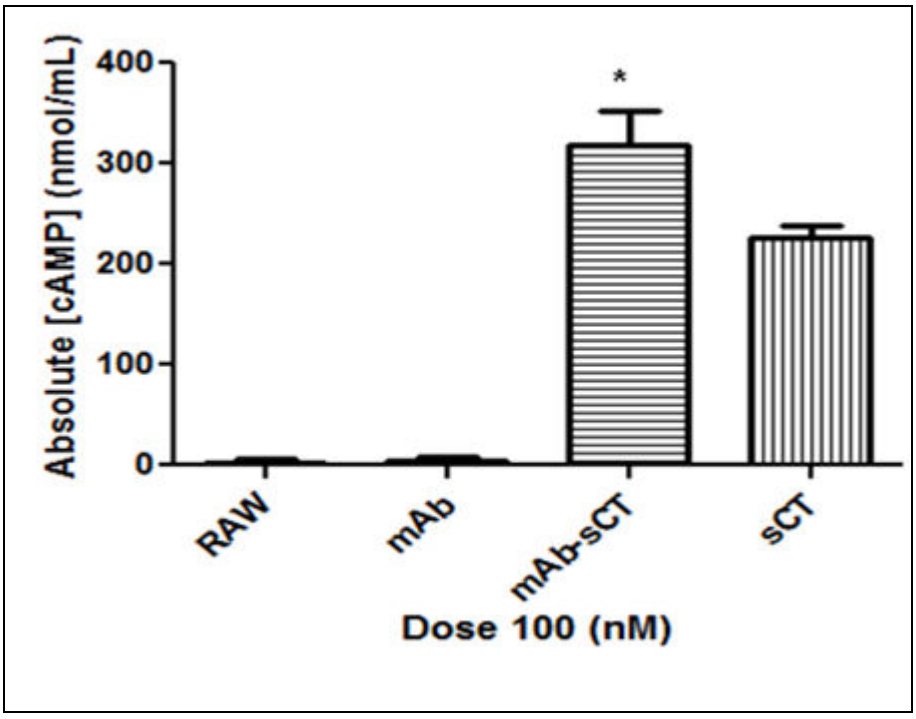


Figure 25. *Calcitonin receptor binding affinity and in vitro bioactivity of mAb-sCT determined by using intracellular cAMP stimulation in human T47D cells. 50,000 cells/well were cultured for 2 days in 48 well plates. To perform the cyclic AMP assay, the phosphodiasterase activity was blocked using 3-IBMX. Cells were then was treated with 100 nM of mAb, mAb-sCT conjugate and sCT and the generated cAMP was assayed by cAMP ELISA. * P < 0.05 versus all other cultures.*

3.10. Discussion

Using hybridoma technology, a specific monoclonal antibody was generated against the recombinant soluble human RANK receptor. The generated antibody was characterised by various techniques. SDS PAGE showed the protein band at 150 KDa under non reducing condition and the protein bands of light chain and heavy chain of the antibody at 25 and 50 KDa respectively under reducing condition. The binding ability of the antibody to the RANK receptor was confirmed by ELISA and Western Blot analysis. Functional osteoclasts were generated using RAW 264.7 cells by treating the cells with the necessary cytokines RANKL and M-CSF. TRAP staining and immunocytochemistry image confirmed the generation of multinucleated osteoclasts. The osteoclast targeting potential of the antibody was confirmed by immunocytochemistry experiment.

Salmon calcitonin, a single chain polypeptide hormone consisting of 32 amino acids, was chosen as a “model” drug for this study. A conjugation strategy was designed whereby calcitonin would be coupled to an antibody directed predominantly against osteoclast-specific receptors, in order to impart osteoclast specificity to the attached calcitonin “cargo”. The synthesized bio-conjugate was initially characterized using SDS-PAGE analysis and ELISA. A protein band above 150 kDa was observed for the conjugate under non reducing condition during SDS-PAGE analysis and specific bands were seen above 50 kDa and 25 kDa under reducing

conditions, thus suggesting that conjugation of PEGylated sCT with mAb had occurred. An ELISA further verified the successful conjugation of sCT to our mAb, as the anti-calcitonin secondary antibody reagent would only detect immobilized Calcitonin residues that were part of the primary anti-RANK conjugates that were binding to the RANK-coated wells of the ELISA plate. A slight signal was observed for PEGylated sCT and this could be due to the reaction of thiol reactive MAL functional group of PEGylated sCT with the free thiol group in BSA used as a blocking agent. In the case of the conjugate, those MAL groups were utilized in the reaction with free thiol groups generated in the antibody.

Binding of sCT with its receptor activates adenyl cyclase, an enzyme responsible for the generation of cAMP. The cyclic AMP assay confirmed the bioactivity of calcitonin in the conjugate. The conjugate showed higher activity than calcitonin alone and the exact reason for this effect is still not clear. The possible reason could be that this conjugation strategy allowed one mole of the antibody to become conjugated with more than one mole of calcitonin. It is reported that when IgG is treated with 10 molar excess of Traut's reagent, 3-7 thiol groups are generated, which could have led to the attachment of 3-7 PEGylated calcitonin molecules per molecule of IgG. That could be one possible reason why the conjugate showed a higher activity than calcitonin alone. In *vitro* cAMP assay results thus confirmed that the modification of sCT by conjugation did not interfere with its ability

to bind the calcitonin receptor and trigger its biological activity. Retention of bioactivity after conjugation is the central concern in the development of bioconjugates.

Salmon calcitonin is currently marketed as injectable and nasal spray dosage forms to treat bone diseases such as osteoporosis, Paget's disease and hypercalcemia. However, conventional CT therapy is associated with certain drawbacks like poor bioavailability and patient side-effects. Systemic bioavailability of a nasally administered doses of CT ranges from 0.3%-30.6%, which is very poor as compared to the same dose administered by intramuscular injection (71% bioavailability) or by subcutaneous injection (66% bioavailability) (Overgaard *et al.*, 1991; Chen *et al.*, 2000; Buclin *et al.*, 2002; Miyazaki *et al.*, 2003b; a). Furthermore, local nasal irritation or ulceration is associated with the nasal route of administration (Carstens & Feinblatt, 1991) and deleterious gastric and vascular side effects are also of concern in CT administration (Gruber *et al.*, 1984). Effective CT drug concentrations are also hampered by the rapid half-life of elimination. The terminal half-life is approximately 58 minutes for intramuscular administration and 59 to 64 minutes for s.c.administration (www.pharma.us.novartis.com/miacalcin_injection.pdf).

Numerous studies have been conducted on improving the solubility, stability and pharmacokinetic profiles of CT as a result of the PEGylation process (Yoo *et al.*, 2000a; Yoo *et al.*, 2000b; Na *et al.*, 2004; Youn *et al.*,

2006b). However, such improvements may not be beneficial if PEG-CT is not targeted to bone. Furthermore, PEGylation may cause an increase in unwanted side-effects as there would be a proportional increase in competition for CT uptake between all available CT receptors on other organs and tissues.

The skeleton of humans comprises only 4 to 5% of the total body mass (Heymsfield *et al.*, 2005). With unfavourable pharmacokinetic profiles of CT and the presence of calcitonin receptors on other parts of the body outside of bone, only a small proportion of the systemically bioavailable CT drug will be distributed to bone to elicit its therapeutic effect. Clearly, there is a strong clinical need in the targeting of this antiresorptive hormone to the site where it is mostly needed. To address this problem, research for bone targeting has focused mainly on the mineral composition of bone during the last decades. Bisphosphonates, due to their strong affinity to calcium phosphate, are used as bone targeting moieties to deliver non-specific bone therapeutics to the skeleton. BPs have been used as targeting structures for delivering anti-resorptive proteins such as salmon calcitonin (sCT) and RANK decoy receptor osteoprotegerin (OPG) in order to enhance bone-specificity (Doschak *et al.*, 2009; Bhandari *et al.*, 2010; Bhandari *et al.*, 2012).

Clinical indications of sCT is to inhibit bone resorption by regulating both the number and activity of osteoclasts (Suzuki & Takahashi, 2001;

Yamamoto *et al.*, 2005; Yamamoto *et al.*, 2006; Granholm *et al.*, 2007; 2008; Karsdal *et al.*, 2008). Antiresorptive effects are mediated by the sCT interaction with calcitonin receptors (CTRs) found primarily on bone-resorbing OC (Sexton *et al.*, 1999) via activation of adenyl cyclase enzyme activity (Jansen-Olesen *et al.*, 1996) leading to greater calcium retention in bone and an increase in bone density (Casez *et al.*, 2003; Matuszkiewicz-Rowinska *et al.*, 2004; Hejdova *et al.*, 2005; Lee *et al.*, 2010; Pappa *et al.*, 2011). Since the major site of action of calcitonin is the calcitonin receptor found on osteoclasts, the desirable bone targeting strategy for effective delivery of calcitonin to the site of action would be conjugating this drug with the osteoclast targeting moiety. Clearly, an antibody with exquisite specificity towards osteoclasts may be the desirable targeting moiety for the conjugation with calcitonin to enhance specific delivery of this hormone to the site of action.

Delivery of sCT to the osteoclast cells by virtue of an antibody-mediated targeting platform would ensure that the calcitonin drug remained primarily localized to bone tissue after systemic administration. This would directly contrast with the passive interaction of calcitonin with other tissues that express calcitonin receptors, such as kidneys, liver, lungs, spleen, heart and thyroid. Hence a delivery system capable of improving sCT targeting and localization to osteoclast could have a potential to positively impact sCT therapy, whilst reducing the drug concentration in non-bone loci containing the calcitonin receptors.

PEGylation of sCT was expected to improve the major disadvantages of conventional sCT by increasing solubility, stability, efficacy while reducing the immunogenicity.

The term " universal delivery platform" is used as this platform may be employed as a drug-delivery strategy, where it directly targets and delivers various drug cargoes such as antiresorptive agents, anti-inflammatory agents, cathepsin K inhibitors, disintegrins, H^+-ATPase inhibitors, and so on, directly to osteoclast cells. Salmon calcitonin was used as a model drug for the current study as this hormone binds to the calcitonin receptors present on osteoclasts and acts as an antiresorptive agent.

The in vitro osteoclast cell culture assays showed the efficacy of the synthesised bioconjugate as well the antibody alone in inhibiting osteoclast activity. The use of the synthesised conjugate in culture significantly reduced TRAP enzyme activity as compared to the sCT alone. Use of the anti-RANK antibody alone also showed inhibition of TRAP enzyme activity. Furthermore, the resorption assay showed that both the conjugate and the mAb alone were capable of significantly reducing the resorption of mineral layer as compared to the sCT alone. The antibody itself showing an antiresorptive effect in addition to targeting was an interesting observation. The cause for this effect shown by the antibody is not clear. The antibody may be functioning by one or combined

principal modes of action as mentioned above: by blocking the action of specific molecules, by targeting specific cells or by functioning as a signalling molecule. Anti-RANK antibody generated against RANK receptor may be recognizing and occupying the same site of the receptor as recognized by the RANKL for binding and thus may be functioning by a mode of action as Anakinra which is an IL-1 antagonist. The competitive binding could be the reason for the antibody being able to antagonize the interaction of RANKL with RANK, an essential step of osteoclast differentiation. In contrast to that, there is also a possibility that this targeting antibody is functioning by the similar mode of action to Herceptin, rather than by blocking ligand-binding. Binding of this antibody to the receptor may be inhibiting receptor dimerisation and/or trimerisation, a crucial step for the functioning of RANK receptor and receptor mediated signal transduction for osteoclast activity. Nonetheless, this proof of principle led to the new research direction, focusing on valuable scale-up production and therapeutic use.

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CHAPTER 4: NEW RESEARCH DIRECTION

Expression, Characterization and Evaluation of RANK-binding Single Chain Fraction Variable: An Osteoclast Targeting Drug Delivery Strategy

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4.1. Background

Since the anti-RANK antibody by itself, without conjugation with any drug, was capable of inhibiting osteoclast activity and showed antiresorptive function, it led to a new direction of research: the generation of antibody fragments targeting osteoclasts. Antibodies represent an important category of proteins, for their ability to recognize epitopes with high affinity and specificity. Despite the potential application of the monoclonal antibodies produced by hybridoma technology, immunotherapy is limited by the immunogenicity of murine-derived antibodies. Monoclonal antibodies face several difficulties, as they are almost exclusively murine in origin thus could create human anti-mouse antibody (HAMA) reactions when introduced to humans. This limits their clinical applications (Klimka *et al.*, 2000; Watkins & Ouwehand, 2000). Added to this, monoclonal antibody production is very laborious and time consuming.

With an aim to reduce immunogenicity of mouse antibodies, genetic engineering was used to generate chimeric antibodies, that is, antibodies with human constant regions and mouse variable regions. This technique has been central to the clinical use of antibodies. Chimeric antibodies were less immunogenic, with the ability to trigger human effector functions and increased circulation half-life. Even though chimeric antibodies were perceived as less foreign, and therefore less immunogenic, human anti-

chimeric antibody responses (HACAs) have been observed (Baert *et al.*, 2003). Further minimization of the mouse component of antibodies was achieved through complementarity-determining region (CDR) grafting. In such 'humanized' antibodies, only the CDR loops that are responsible for antigen binding are inserted into the human variable-domain framework. The ability to manipulate antibodies into more human-like variants finally made antibodies in the main stream of clinical use. With the isolation of genes encoding for human variable regions, their successful expression in *Escherichia coli*, and the introduction of phage-display technology along with the developments in hybridoma technology, the task of selecting fully human variable domains has been greatly simplified (Knappik *et al.*, 2000).

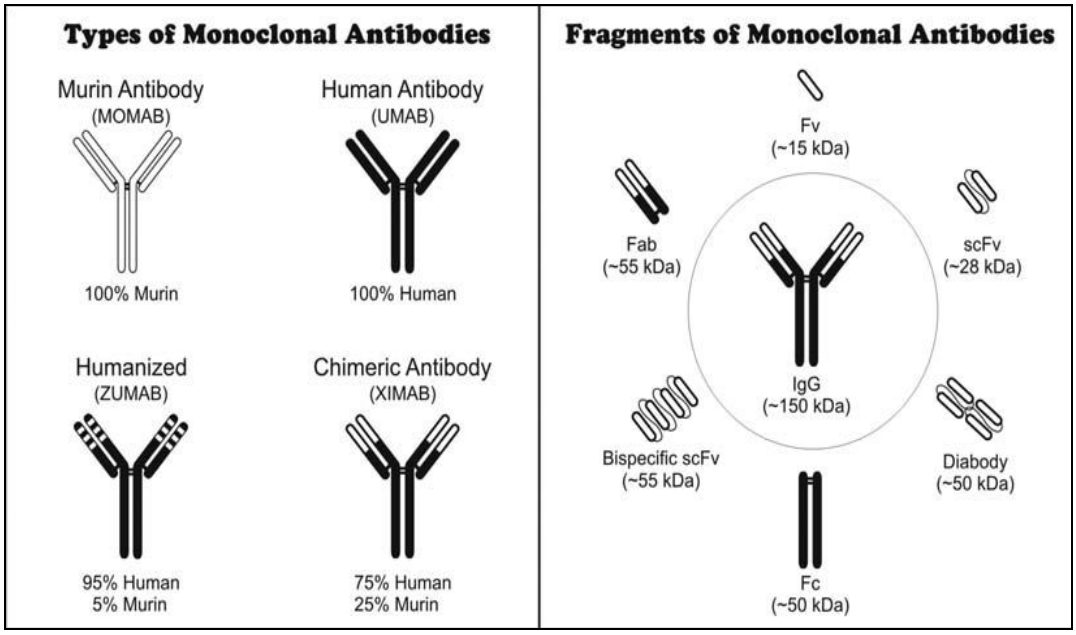

***Figure 26 A**: Types and fragments of monoclonal antibodies. (Image : taken from Pucca et al, 2011)*

4.1.1. Single-chain fragment variable (scFv)

Single-chain antibodies (scFv) were developed as an alternative system to circumvent the challenges in monoclonal antibody-based therapies. A single-chain variable fragment (scFv) is a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of immunoglobulins and this protein retains the specificity of original immunoglobulin.The heavy (V_H) and light chains (V_L) are joined together by a flexible peptide linker that can be easily expressed in functional form in E. coli, allowing protein engineering to improve the properties of scFv (single chain fragment variable) such as increase of affinity (Griffiths & Duncan, 1998). The length of the flexible DNA linker used to link both of the domains is critical in yielding the correct folding of the polypeptide chain. Previously, it has been estimated that the peptide linker must span 3.5 nm (35 Å) between the carboxy terminus of the variable domain and the amino terminus of the other domain without affecting the ability of the domains to fold and form an intact antigen-binding site (Huston *et al.*, 1991). The amino acid composition also plays an important role in the design of a viable linker peptide. They must have a hydrophilic sequence in order to avoid intercalation of the peptide within or between the variable domains throughout the protein folding (Argos, 1990). Nowadays, the most

extensively used designs have sequences comprising stretches of Glycine and Serine residues (Whitlow *et al.*, 1993).

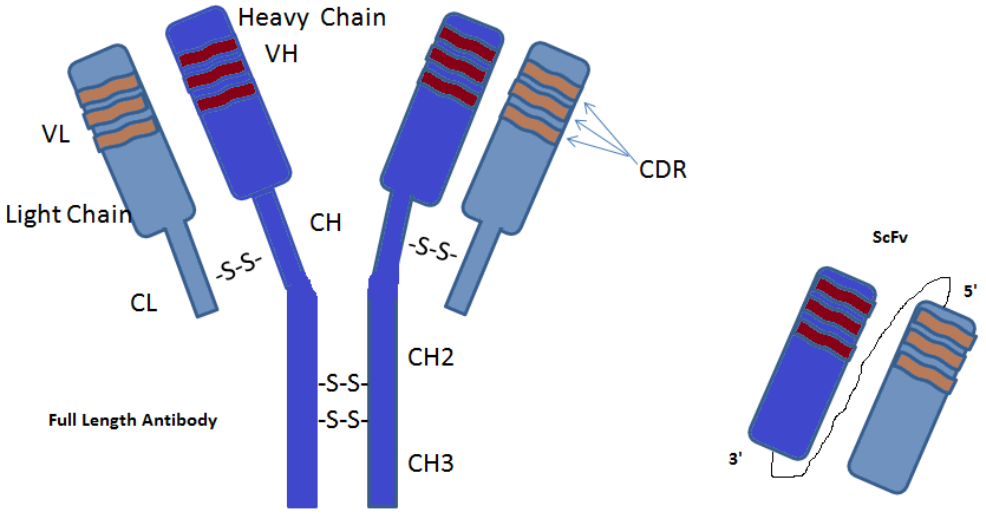

Figure 26 B: *Antibody model showing subunit composition and domain distribution along the polypeptide chains. Single chain fragment variable (scFv) is the antigen binding part of the antibody.VL: Variable Domain of Light Chain, CL: Constant Domain of Light Chain, VH: Variable Domain of Heavy Chain, CH: Constant Domain of Heavy Chain, CDR: Complementarity Determining Regions.*

The small antigen-binding molecule of scFv antibodies could offer several advantages over a whole antibody molecule in therapeutic applications (Colcher *et al.*, 1998; Hudson, 1999). The smaller fragments allow these molecules to penetrate more rapidly and evenly to tumors and other tissue in comparison to the whole antibodies (Yokota *et al.*, 1992; Colcher *et al.*, 1998). As these fragments have more rapid clearance from

blood, they can be coupled with drugs and radionuclides in order to result in low exposure of the healthy tissue (Marasco & Dana Jones, 1998; Oriuchi *et al.*, 2005; Stipsanelli & Valsamaki, 2005). Human scFvs that specifically targets arthritis cartilage was developed using phage display technology (Hughes *et al.*, 2010). Likewise, numerous single chain fragment variable have been constructed against tumor antigens (Shadidi & Sioud, 2001; He *et al.*, 2002), proteins (Dai *et al.*, 2003; Guo *et al.*, 2003), carbohydrates (Ravn *et al.*, 2004; Sakai *et al.*, 2007), haptens (Kobayashi *et al.*, 2005), viruses (Sheikholvaezin *et al.*, 2006) and receptors (Galeffi *et al.*, 2006). The scFv conjugated to a chemotherapeutic drug mitomycin was reported to inhibit growth, and induce apoptosis, of cancer cells in vitro (Chen *et al.*, 2007). There have been reports on delivery of toxic drugs by Ab-directed enzyme prodrug therapy (ADEPT), where scFvs are fused to enzymes (e.g. β-lactamase) that selectively convert a subsequently administered non-toxic prodrugs into cytotoxic drugs at the target site (Sharma *et al.*, 2005). Inhibition of cancer cell growth in vitro and in vivo was observed following the treatment with tumor cell specific scFv fused to protein toxins (Schmidt *et al.*, 2001).

There are several advantages of phage-displayed scFv over monoclonal antibodies. Phages are more stable and can be stored up to several years at 4˚C (Burritt *et al.*, 1996). They can be produced rapidly

and inexpensively just by infecting the E. coli *(Wang et al., 1995)*. Also, their genes can be easily manipulated and lastly they can be produced by circumventing hybridomas and immunization (Marks *et al.*, 1991).

Antibody-like fragments have both pros and cons as therapeutics compared with full-size mAb therapeutics. Fragments demonstrate short circulating half-lives in humans (Larson *et al.*, 1983), likely due to kidney clearance (Arend & Silverblatt, 1975; Schott *et al.*, 1992). They are often rapidly eliminated from the circulation by renal filtration because their size is below 65 kDa cut-off size of filtration in the kidneys. ScFvs generally have short in vivo half-lives due to their low molecular weight and the absence of Fc region, thus negating binding to the FcRn. FcRn receptors are expressed on the surface of phagocytic cells of the reticuloendothelial system and prevent the rapid elimination of IgG. Following nonspecific pinocytosis, the binding of IgG to FcRn in endosomes allows salvage from lysosomal degradation and recycling to circulation. IgG binds to the FcRn with nanomolar affinity under the acidic conditions of the endosomes (pH less than 6.5) and is released into the extracellular space due to the neutral pH (Liu & Lv, 2008). Since rapid elimination of these protein fragments is a problem in therapeutic applications that require high doses and repeated administration, numerous studies have been directed at increasing the molecular size of the protein (e.g., via PEGylation)(Krinner *et al.*, 2006) or at engineering the fusion to heavy chain fragments (i.e. to

CH3 or Fc) (Hu *et al.*, 1996; Kenanova *et al.*, 2005). PEGylation involves the chemical coupling of polyethylene glycol chains to the protein fragments to increase their apparent molecular weight and thus extends their circulating half-lives.

The conjugation of small proteins, peptides, and oligonucleotides with polyethylene glycol or PEGylation, has become an increasingly common method of improving the half-life of biological products, mainly through reducing the urinary excretion of the molecule (Yang *et al.*, 2004), but also by reducing the enzymatic degradation due to the increased steric bulk (Veronese & Pasut, 2005b). Several PEGylated scFv molecules have been constructed and have shown a prolonged in vivo half-life (Yang *et al.*, 2003; Krinner *et al.*, 2006; Xiong *et al.*, 2006).

4.1.2. Generation and expression of scFv

Molecular engineering technologies have enabled the structure of antibodies to be fine tuned for specific therapeutic actions and to minimise immunogenicity. So far, antibody fragments (scFv) have been successfully isolated and displayed as fragments in various expression systems such as mammalian cells and yeast (Ho *et al.*, 2006), plants (Galeffi *et al.*, 2006), and also insect cells (Choo *et al.*, 2002).

Single-chain fragment variable antibodies can be expressed as correctly folded and directly active proteins or as aggregates requiring *in vitro* refolding to become active. Depending on the expression system, their ability to fold and secrete tends to vary. There are some general regulations to consider on the design of vectors and expression systems used with the different hosts and each host has certain advantages and disadvantages for the production of active scFv antibody (Verma *et al.*, 1998). Nevertheless, bacterial expression systems are most often used for the production of scFv antibody fragments.Recent progress in both the perceptive of both genetics and biochemistry of *E. coli* makes this organism an ideal tool as an scFv expression host (Baneyx, 1999).

4.1.3. Phage display techniques

The phage display technique was discovered in 1985. Smith was the first to reveal that foreign DNA fragments can be fused to the gene encoded for pIII coat protein of a nonlytic filamentous phage and expressed as a fusion protein on the virion surface without disturbing the infectivity of the phage (Smith, 1985).

It was later successfully demonstrated that a scFv fragment can be displayed on the phage surfaces as a functional protein which retains an active antigen-binding domain capability (McCafferty *et al.*, 1990) **(Figure**

27). It is a widely used and well-established technique for the selection and production of antibodies expressed in libraries (Maynard & Georgiou, 2000). Using this technique, it is possible to mimetize the strategy used by the humoral immune system to produce completely human antibodies or fragment antibodies while waiving the immunization process or the construction of hybridomas (Roque *et al.*, 2004).

The main component of this in vitro technique, the M13 filamentous phage, has the same property as all other bacteriophages. These phages are viruses capable of infecting various Gram-negative bacteria which use pili as receptors. The M13 filamentous phage contains a single-stranded DNA as its genetic material, encapsulated by several different coat proteins. Each M13 phage is encoded by the gene 8 protein as the major capsid coat and capped with 2 minor coat proteins on each end – the gene 3 protein and the gene 6 protein on one end, with the gene 7 protein and gene 9 protein on the other (Dunn, 1996; Azzazy & Highsmith, 2002; Kehoe & Kay, 2005). The M13 filamentous phage initiates its life cycle by first infecting E.coli by attaching to the bacterium's pilus. Single stranded DNA from the phage is inserted into the bacterium after the attachment.

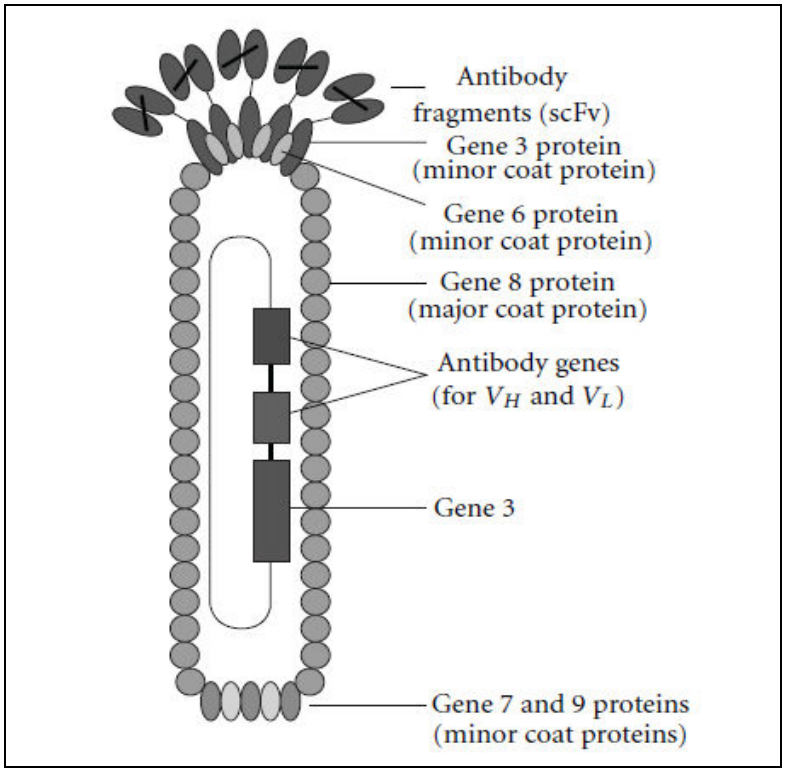

Figure 27: *Structure of a filamentous phage displaying scFv fragments on its surface. Image taken from (McCafferty et al., 1990).*

For the M13 filamentous phage used in phage display, the single-stranded DNA within the virus does not contain the entire phage genome, but only single-stranded hybrid genetic material, called a phagemid, containing only a target fragment and a coating protein. The engineered phagemid is a cloning vector that contains the replication origins of both M13 phage and E.coli, an antibiotic resistant gene, and a multiple cloning site for sub-cloning the desired insert. These phagemid-containing clones can only replicate the single-stranded DNA as typical plasmid, but cannot

package into a functional phage without the presence of a “helper phage”. Phagemids by themselves lack the essential machinery to replicate and package viral particles. Therefore, a “helper phage” is required to provide the necessary replication and packaging proteins in order to form phages that display the scFv. After the infection of the helper phage to a phagemid-containing *E.coli*, the helper phage supplies all required proteins for phage packaging, thus allowing “phage rescue” (Smith, 1985; Azzazy & Highsmith, 2002). The phagemid is constructed such that the scFv DNA and the phage coating protein DNA are separated by an amber stop codon TAG. When phage is being packaged in amber suppressor strain such as TG1, the *E.coli* will translate the amber stop codon into a glutamine residue, in turn synthesizing a scFv-fusion protein.

Following the screening and selection of phage displaying the protein against desired antigen, the relevant phage is then infected into an amber codon non suppressing bacterial strain (HB2151) during the expression phase, where the translation will halt at the amber codon and generate phage-free scFv. For a large scale scFv expression, Isopropyl β-D-1-thiogalactopyranoside (IPTG) is used in the culture. It is a commonly used inducer for β-Galactosidase activity in many bacterial gene sequences controlled by the lac operon. Generally, induction is done at an OD600 of 0.7 as the cells at that OD have reached exponential growth. In other words, the vast majority of cells are alive and healthy, which makes them ideal for protein expression. At OD of 1.0 or higher, the culture will

start collecting dead cells, which cannot express protein. An OD that is too low is also not good (<0.4) because most of the culture is media and so there aren't enough cells to make enough protein. For induction, a sterile 1 M solution of IPTG is typically added by 1:1000 dilutions into a logarithmically growing bacterial culture. Expressed scFv is then extracted using extraction buffer followed by the purification of the desired protein.

4.1.4. New research specific aims

As single chain Fraction variable (scFv) employ antibody- like target recognition specificity, the scFvs against RANK found on OC may serve as an excellent platform to selectively target osteoclasts and find utility as “a universal osteoclast targeting platform”. Specific aims for this study were to express scFvs against soluble human RANK receptor using phage display technology and to characterize and evaluate in vitro efficacy of this anti-RANK scFv using osteoclast specific assays.

4. 2. Experimental procedures

4.2.1. Materials

The phage display library used for this work was the Tomlinson Human scFv Library purchased from Source BioScience, UK.

Recombinant human soluble RANK protein was from Peprotech, USA. Trypsin, 50mM NaH_2PO_4 buffer, 300mM NaCl, 10mM imidazole, 3, 3′, 5, 5′-tetramethylbenzidine (TMB), Leukocyte Acid Phosphatase (TRAP) Kit and 4-6-diamidino-2 phenylindole (DAPI) was purchased from Sigma-Aldrich (St.Louis, MO, USA). The QIAprep Spin Miniprep Kit was from Qiagen Sciences, MD USA. DNA sequencing using Big Dye v3.1 was purchased from the Molecular Biology Service Unit at the University of Alberta. The Micro BCA protein assay kit and Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Thermo Fisher Scientific, Canada. The Bug Buster Master Mix extraction buffer was from EMD Chemicals, NJ USA. Bradford Assay was Bio-Rad, CA USA. Anti-M13 HRP was kindly provided by Dr. Suresh's Lab, University of Alberta. TNF I receptor, TNF II receptor, osteoprotegerin, macrophage colony stimulating factor (M-CSF) and RANKL was from PeproTech, NJ, USA. Anti-myc HRP antibody was from Invitrogen, CA USA. Western Blotting Reagent and Blotting film was from GE Healthcare, UK. Light seal cassette and developing and fixing solution (Kodak, NY USA). RAW 264.7 cells (a transformed murine monocytic cell line) were purchased from the American type culture collection (ATCC, VA, USA). Alexa Fluor® 488 conjugate was from Millipore, USA and the Acid Phosphatase Assay Kit was from Cayman chemical, USA. QuantiChromTM Calcium Assay Kit (DICA-500) was from Bio Assay systems.

4.2.2. Generation of Anti-RANK scFv using phage display technique

The phage display library used for this work contained both a myc tag and a 6xHis tag. Anti-RANK scFv was expressed by lab colleagues Michael Lam and Biwen Xu. Briefly, the commercial human scFv-phage library was incubated in wells containing 1μg of recombinant human soluble RANK protein. Unbound phage were rinsed away whilst RANK-binding phage were eluted with trypsin and amplified in E.coli TG1 (provided along with the purchase of the Tomlinson library). The Tomlinson scFv phage display library requires the use of two E.coli strains – TG1 and HB2151, where TG1 was used entirely for phage screening and HB2151 was used entirely for scFv expression. The amplified phages were screened for two additional rounds with the same procedure and the RANK-binding phage was used to infect *E.coli* HB2151 for scFv expression. By infecting the amber codon non suppressing strain HB2151 with the scFv phagemid, translation would be halted at the amber stop codon and only the soluble antigen-binding scFv were expressed. ELISA was performed on RANK receptor coated wells to identify individual RANK-binding scFv clones.

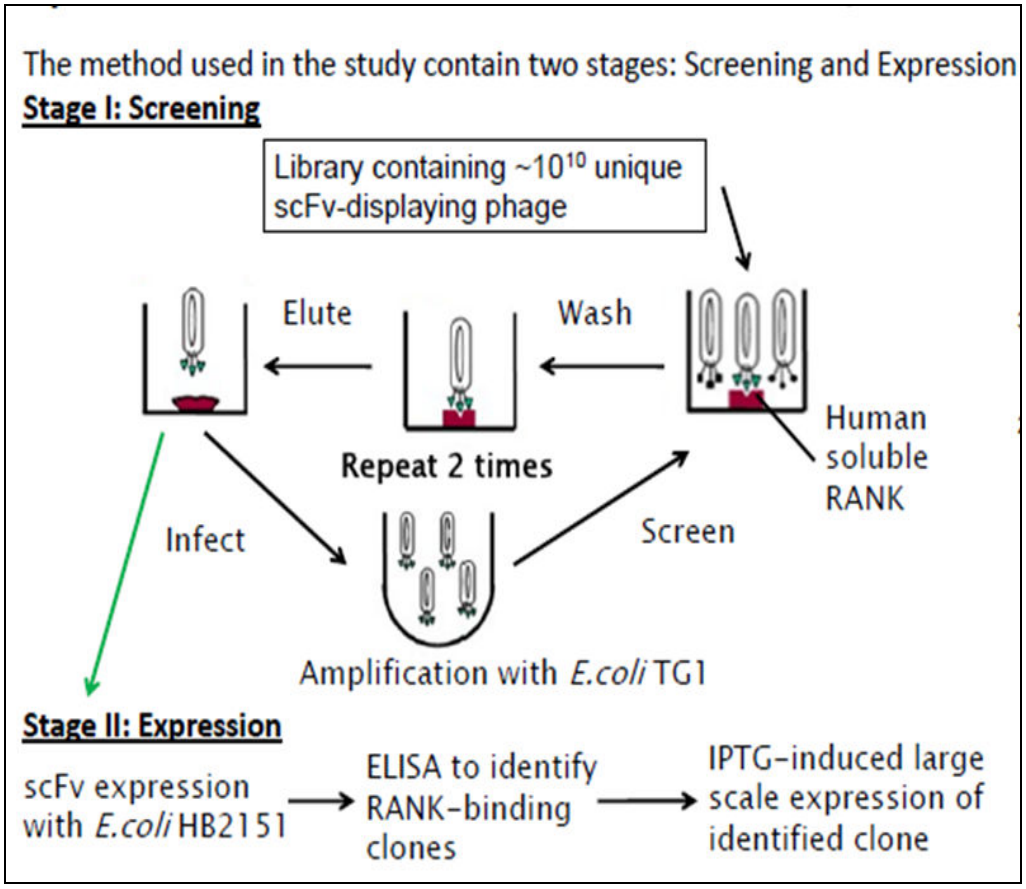

Figure 28: *Diagrammatic display of overall concept of Phage display. First stage consists of screening of scFv-phage with high affinity to RANK receptor and second stage is large scale expression of RANK binding scFv clone.*

4.2.3. DNA sequencing

QIAprep Spin Miniprep Kit was used to isolate DNA template from RANK antigen-binding clone. 3mL of overnight culture was pelleted by centrifugation and the pellet was resuspended in the resuspension buffer

provided. Subsequently, lysis buffer was added to the suspension, followed by addition of the provided neutralization buffer. The resulting mixture was centrifuged to remove all precipitates. The supernatant was then applied to the provided spin column to allow binding of DNA to column. Wash buffer 1 was added to the column and spun to remove endonucleases, and wash buffer 2 was added to the column and spun to remove all salts. Finally, DNA was isolated by adding 50 µl of the provided elution buffer to the column.

For DNA sequencing, 2 µl of the BigDye, 3 µl of the BigDye buffer, 4 µl of the isolated DNA, 1 µl of 5 µM primer and 10 µl DNAse and RNAse free water were mixed to create a 20 µl reaction mixture. The reaction mixture was left to react in a thermocycler for 25 cycles with the following settings: i. Denaturing temperature: 96°C, 30 seconds; Annealing temperature: 50°C, 15 seconds; Extension temperature: 60°C, 2 minutes. All reactions were carried out using the primer 5'- CAG GAA ACA GCT ATG AC -3'. After the reaction was completed, 20 µl of the reaction mixture was added to 2 µl of the provided ethyl-acetate and 80 µl 95% ethanol. The resulting mixture was mixed by vortex and incubated on ice for 15 minutes for DNA precipitation. After incubation, DNA was pelleted by centrifugation the supernatant was aspirated. DNA pellet was resuspended in 70% ethanol. The mixture was again mixed by vortex and pelleted by centrifugation at 10800 rcf, 4°C for 5 minutes. After aspirating

the supernatant, the dried product was submitted to the Molecular Biology Service Unit DNA sequencing facility at the University of Alberta.

4.2.4. Large scale scFv expression and purification

Once phage selection had been completed and the antigen-binding clones identified and cross infected to the expression strain HB2151, large scale amplification was conducted by Isopropyl β-D-1-thiogalactopyranoside (IPTG) - induced overnight expression. After centrifugation to pellet the cell bodies, scFv was extracted by adding BugBuster MasterMix extraction buffer. The extract was passed through a Ni-NTA column. The fraction collected was labeled as “flow through (FT)”. After the flow through fraction was collected, wash buffer 1(W1), which consisted of 50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole and pH 8.0, was added to the column until minimal protein was detected using the Bradford Assay. Subsequently, wash buffer 2(W2), which consisted of identical NaH_2PO_4 and NaCl concentration and pH except with 50mM Imidazole, was added to wash the column until minimal protein was detected using the Bradford Assay.

To elute the expressed scFv, the column was loaded with 10mL of elution buffer, which consisted of identical NaH_2PO_4 and NaCl concentration and pH as the wash buffers except with 250mM imidazole,

and sample was collected in two fractions (E1 and E2). Then the resin was resuspended with the remaining elution buffer. The eluted samples were analysed by SDS-PAGE and subsequent dialysis (MWCO = 13,000) against PBS pH 7.2 overnight at 4°C. The protein concentration was then determined by micro BCA protein assay.

4.3. Characterization of anti-RANK scFv

4.3.1. SDS-PAGE

SDS-PAGE analysis was performed for the preliminary characterisation of anti-RANK scFv. The various fractions collected during purification process and labelled as “flow through (FT)”, wash 1(W1), wash 2 (W2), Elution1 (E1), Elution2 (E2) and resin were mixed with loading buffer and run in 10% polyacrylamide gel under non-reducing condition. The gel was then stained with Coomassie blue to detect the scFv.

4.3.2. Enzyme linked immunosorbent assay

For phage ELISA, an indirect ELISA was performed in flat bottomed 96 well plates. 100 µl of antigen (human sRANK receptor) was used for coating at a concentration of 1µg/100 µl, overnight at 4^{o}C. The wells were washed three times with PBS (pH 7.2), and to avoid nonspecific binding,

incubated with 2% Bovine Serum Albumin (BSA) in PBS for 2 hours at room temperature with agitation. The blocking solution was then discarded and the wells were further washed 3 times with 200 µl of PBS. At the same time, the overnight culture was pelleted by centrifugation at 2000RPM for 10 minutes at room temperature, and 50 µl of the phage-containing supernatant was used for phage ELISA. The supernatant was incubated at room temperature with agitation for 1 hour. After thorough washing, 100 µl of 1:10000 dilution of anti-M13 HRP in PBS was added to each well and incubation was carried out for 1 hour at room temperature with agitation. The wells were then washed and 100 µl of substrate solution 3,3′,5,5′-Tetramethylbenzidine, or TMB, was added to each well and incubated at room temperature for 10 – 15 minutes. The optical density was measured at 650nm using a plate reader (BioTek EL808) and analyzed using the software KC Junior.

For the characterisation of expressed scFv by ELISA, the 96 well plate was coated with 100 µl /well of 1 µg/ 100 µl of antigen (human sRANK receptor) overnight at 4°C. After blocking with BSA, the wells were incubated with different concentration of scFv (0.5 µg, 1 µg, 2 µg, 4 µg) in PBS for 1 hr at room temperature, followed by the incubation with 100 µl of anti-myc HRP antibody (1:5000) at room temperature for 1 hour. Anti-myc antibody was used for the detection purpose as the library used for this work contained myc tag. After final washing, 100 µl of TMB substrate was

added to each well and incubated for 15 min. The optical density (OD) was measured at 650 nm.

4.3.3. ELISA to check for the cross reactivity of scFv with other members of TNFR super family

An ELISA was conducted to check for the cross reactivity of scFv with other members of TNFR superfamily. Wells were coated with 1µg of sRANK receptor, 1µg of TNF I receptor, 1 µg of TNF II receptor and 1 µg osteoprotegerin. After blocking with BSA, the wells were incubated with 2 µg of scFv for 1 hr, followed by the incubation with 100µL of 1:5000 anti-myc HRP antibodies at room temperature for 1 hour. After final washing, 100 µl of TMB substrate was added to each well and incubated for 15 min. The optical density (OD) was measured at 650 nm.

4.3.4. Confirmation of RANK receptor binding ability of the generated scFv by Western blot

A sample of purified scFv (1 µg) was electrophoresed on SDS-PAGE using 10% acrylamide gel and transferred onto nitrocellulose membrane using mini trans-blot apparatus. The membrane was blocked with 5% skim milk in PBST overnight at 4°C. After washing with PBST, it

was incubated with 1:5000 anti-myc HRP for 1 hr at room temperature, followed by detection of protein band using enhanced chemiluminescence.

To confirm the RANK receptor binding ability of the expressed scFv, 1µg of RANK receptor (19.3 kDa) was electrophoresed on SDS-PAGE gel and transferred onto nitrocellulose membrane as mentioned above. After blocking with 5% skim milk in PBST overnight at 4°C, the membrane was incubated with 40 µg anti-RANK scFv followed by the incubation with anti-myc HRP at a dilution of 1:5000 for 1 hr at room temperature. The binding of scFv to RANK receptor was detected using enhanced chemiluminescence.

4.3.5. Matrix-assisted laser desorption/ionization-time of flight

The accurate mass of the expressed protein was determined by MALDI-TOF. The sample was diluted twenty fold in 50% acetonitrile/water. One µl of each sample was mixed with 1µl of sinapic acid (10 mg/ml in 50% acetonitrile/water + 0.1% trifluoroacetic acid). One µl of the sample/matrix solution was then spotted onto a steel target plate and allowed to air dry. All MALDI-MS experiments were carried out using a Bruker Ultraflex MALDI-ToF/ToF (Bruker Daltonic GmbH) in positive mode. Data analysis was carried out using the flexAnalysis software (Bruker Daltonic GmbH) at the Institute for Biomolecular design.

4.4. In-vitro evaluation

4.4.1. Osteoclast generation and TRAP staining

RAW 264.7 cells were seeded in culture plates and incubated for 24 hrs. Peptide factors RANKL (50 ng/ml) and M-CSF (25ng/ml) were added to the culture and the medium was replaced every 48 hr. Tartrate Resistant Acid Phosphatase (TRAP) being an osteoclast marker, the cultured cells were stained for the enzyme tartrate-resistant acid phosphatase using the, Leukocyte Acid Phosphatase Kit according to the manufacturer's instructions. TRAP-positive multinucleated osteoclasts were visualized by light microscopy.

4.4.2. Osteoclast targeting ability of anti-RANK scFv

Immunocytochemistry was conducted to check for the osteoclast targeting potential of the generated scFvs. Osteoclast-like cells were generated in a Lab Tek II chamber slide system (Nunc) from RAW 264.7 cells (transformed murine monocytic cell line) by dosing them with 25 ng/ml macrophage colony stimulating factor (M-CSF) and 50 ng/mL RANKL every 48 hrs for 7 days. The osteoblast-like MG-63 cell line was used as negative control. Likewise, osteoclast cell culture omitting primary

mAb was also used as negative control. Cell cultures were washed with PBS, fixed in 4% paraformaldehyde in PBS (pH 7.4) for 5 minutes and rinsed thoroughly with PBS. After blocking with 3% BSA in PBS for 1hr, the cells were incubated with our anti-RANK scFv (4µg/ml) for 2 hrs at 4°C, followed by incubation with Anti-Myc Tag, clone 9E10, Alexa Fluor® 488 conjugate for 60 min at 4°C. To visualize cell nuclei, slides were counterstained with 1 µg/ml 4-6-diamidino-2 phenylindole (DAPI) diluted in PBS. Culture slides were separated from their chambers mounted and photographed using confocal microscopy (Zeiss LSM 710 with ZEN software and the microscope the Observer.Z1).

4.4.3. Spectrophotometric assay of In vitro TRAP activity

To see the effect of the generated scFv osteoclast culture and TRAP activity, RAW 264.7 cells were seeded in culture plates and incubated for 24 hrs. Peptide factors RANKL (50 ng/ml) and M-CSF (25ng/ml) were added to the culture, with or without the addition of 1nM scFv or 100nM scFv and was replaced with the fresh treatment every 48 hr. Osteoclastogenesis was assessed by the spectrophotometric measurement of TRAP activity on day 7. TRAP activity in the cell lysate was determined using an Acid Phosphatase Assay Kit. The absorbance was measured at 405 nm using a microplate reader.

4.4.4. Resorption assay

The bone resorption assay, being one of the most important assays for osteoclast functionality was performed using RAW 264.7 cells. The cells were suspended in cell culture medium and seeded in Corning® Osteo Assay Surface Stripwells and incubated for 24 hrs. Cells were treated with 50 ng/ml RANKL and 25ng/ml M-CSF, with or without the addition of 1nM scFv or 100nM scFv every 48 hr over the course of 7 days. After 7 days of culture, the medium was aspirated and washed with double-distilled water. To remove the adherent cells, 100 µl of 10% bleach solution was added for 5 min at room temperature. Bleach solution was aspirated and the wells were washed twice with 150µl/well of double-distilled water. The wells were allowed to air dry completely at room temperature. To quantify the amount of calcium phosphate remained after the culture, 50 µl of 1N HCl was added to each well and QuantiChromTM Calcium Assay Kit (DICA-500) was used. The absorbance was measured at 630 nm using a microplate reader.

4.4.5. Effect of scFv on viability of different cell line

To ensure that the scFv did not have any adverse effect on the proliferation of other cells, we used UMR-106 (Rat osteosarcoma cell line: ATCC) cells for the cytotoxicity test. Cell culture was treated with 1nM and

100nM of scFv every 48 hours over a period of 6 days. Observations were made to check if there was any difference in the proliferation of cells treated with testing agent and the cells treated with media alone. For quantitative analysis, Cell viability was assayed by using Thiazolyl Blue Tetrazolium Blue (MTT; Sigma). UMR-106 cells were seeded on well plates in DMEM medium supplemented with 10% FBS and 1% Penicillin-Streptomycin. The culture was treated with media, 100nM and 1nM scFv. Media and factors were replaced every 48 hours. On day 6, the media was aspirated and MTT was added to each well and incubated further for four hours at 37ºC. The medium was then discarded and the formazan crystals were dissolved in 200 µl solubilization solution using an *in vitro* toxicology assay kit (TOX-1, Sigma) and the absorbance was measured at 570nm using a microplate reader.

4.5. Statistics

All experiments were performed in triplicate. Statistical analysis was conducted using Microsoft Excel 2007. Results are presented as the mean ± standard deviation. Unpaired t-tests of two samples assuming equal variances were used to assign significance between groups, with a P-value of less than 0.05 as the threshold for significance.

4.6. Results

4.6.1. DNA sequencing

DNA template from individual antigen-binding clone was isolated using QIAprep Spin Miniprep Kit and was submitted to the Molecular Biology Service Unit DNA sequencing facility of University of Alberta. As reported by Michael Lam, ten clones with highest absorbance readings indicating strong binders to RANK receptor were selected. Plasmid DNA for each clone was isolated and DNA sequencing was performed in order to deduce the amino acid sequence. Two of the ten clones failed to be sequenced after numerous attempts and the remaining eight picked clones all possessed identical DNA, and so the amino acid sequence.It was interesting to find all eight picked clones to possess identical DNA sequence. This may have occurred due to stringent washings during the first round of phage screening, leaving the RANK-binding and high abundance scFv behind that got amplified exponentially over round 2 and 3 of the screening.

The DNA sequence was then analyzed by an online translation tool ExPASy Translate tool (http://expasy.org/tools/dna.html). A full detail of sequencing is shown in below in **Figure 29**. Molecular weight was calculated using Protein Molecular Weight tool (http://www.bioinformatics.org/sms/prot_mw.html). The protein weighed 28.1 kDa as per the result for 268 residue sequence.

Amino Acid Sequence of RANK-binding scFv

Figure 29: *Amino Acid Sequence of RANK-binding scFv. Amino acid sequence of anti-RANK scFv clone was deduced from obtained DNA sequencing result using primer 5'-CAG GAA ACA GCT ATG AC-3'. DNA sequence was translated to amino acid sequence using an online translation tool ExPASy Translation Tool (http://expasy.org/tools/dna.html). Each letter represents the letter abbreviation of individual amino acid sequence. Bolded letters denote the heavy chain of the scFv. Underlined letters denote the light chain of the scFv. Italicized letters denote the poly-glycine serine linker. 6xHis and c-myc tag are highlighted in black. Asterisk (*) represents translation termination due to the amber (TAG) stop codon.*

4.6.2. Characterization of anti-RANK scFv by SDS-PAGE

Taking advantage of the 6xHis tag within the scFv construct, the desired RANK-binding scFv was purified from the overnight culture using

the Ni-NTA column. **Figure 30** shows the SDS-PAGE image of the purification process. While some impurities were found present in the elution fractions, the percentage was deemed lower than 5% judging at the intensity of the desired scFv band in comparison. A protein band at 28 kDa was seen for the Anti-RANK scFv.

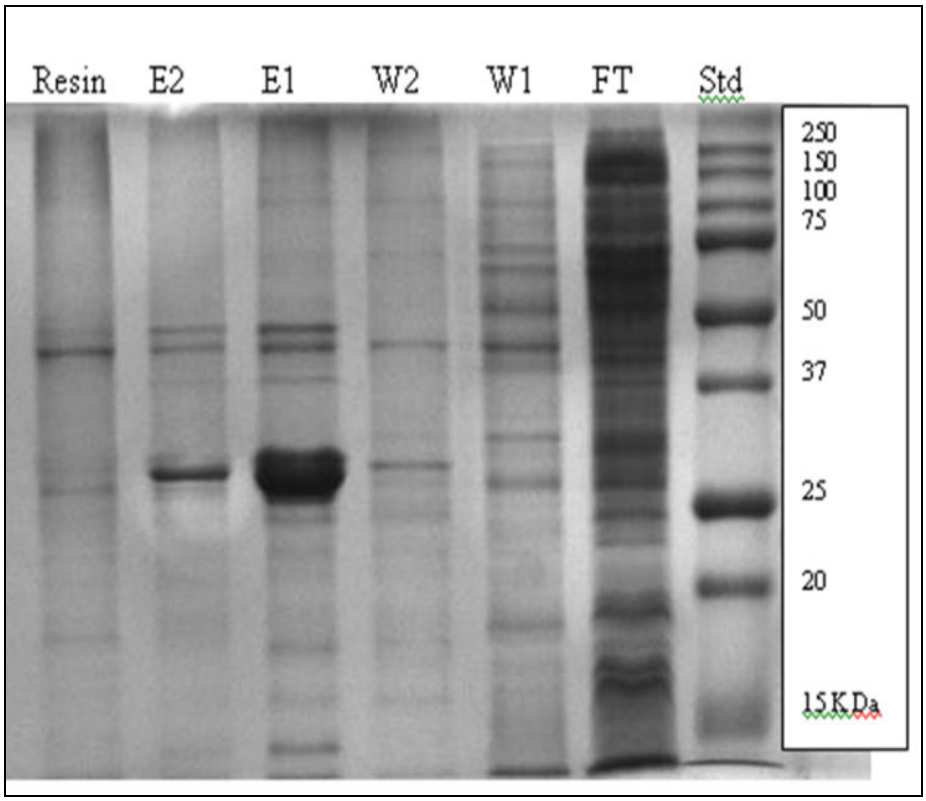

Figure 30: *SDS-PAGE of Ni-NTA Column Purification of anti-RANK scFv. Purification process was competitive affinity chromatography. Wash 1 (W1) and Wash 2 (W2) were performed using Imidazole with concentration 10mM and 50mM respectively. Elution 1(E1) and Elution 2 (E2) used imidazole with concentration 250mM: An intense protein band was observed above 25 KDa for the expressed anti-RANK scFv and Elution fraction 1 contained significantly more scFv than elution fraction2.*

4.6.3. Characterization of anti-RANK scFv by ELISA

After purification of RANK-binding scFv and determination of protein concentration by BCA assay, a serial dilution scFv ELISA was performed. The wells coated with same amount of RANK receptor were incubated with different concentrations of scFv.The optical density was seen to linearly increase with the concentration of scFv **(Figure 31).**

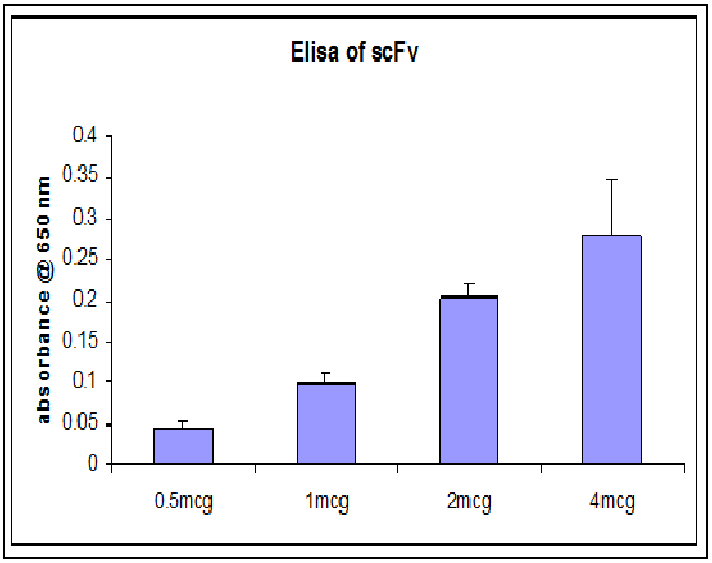

Figure 31: *ELISA of the generated anti-RANK scFv using commercially available recombinant soluble human RANK receptor.*

No cross reactivity was observed for the anti-RANK scFv with other TNFR members when Elisa was conducted with wells coated with TNFR I, TNFR II and OPG receptors **(Figure 32).**

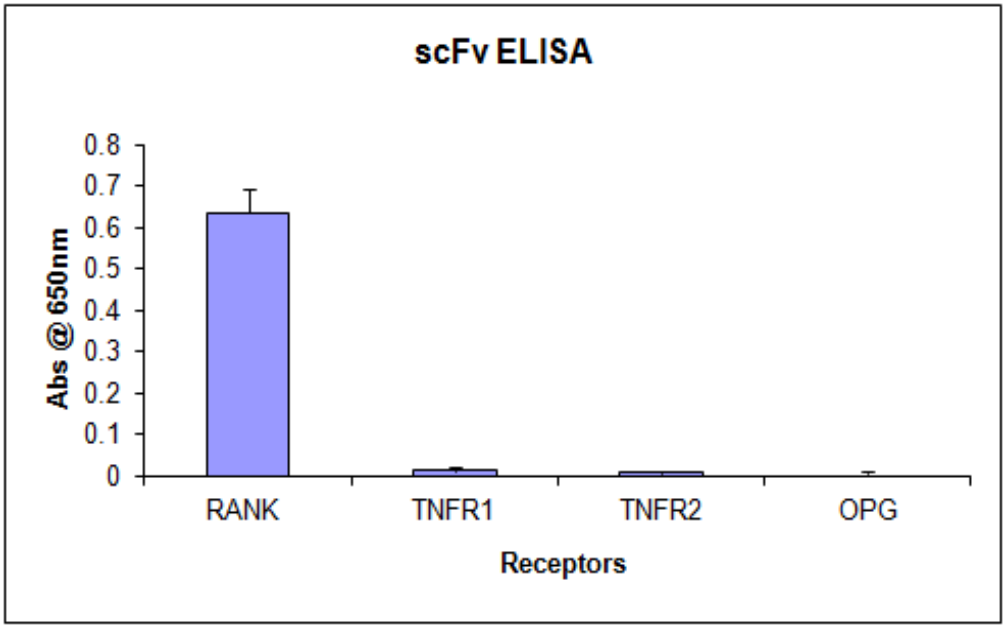

Figure 32: *Elisa to check for the cross reactivity of scFv with other members of TNFR super family.*

4.6.4. Characterization of anti-RANK scFv by Western blot

RANK receptor binding ability of the generated scFv was confirmed by Western blot analysis. ScFv has myc epitope and when it was electrophoresed on SDS gel and transferred to nitrocellulose, a band at 28 kDa, which is the molecular weight of scFv, was detected by anti-myc antibody. For the more sensitive method to confirm the binding of scFv with RANK receptor, recombinant human sRANK receptor, a 19.3 kDa polypeptide, electrophoresed on a 10% polyacrylamide gel and transferred

to nitrocellulose membrane. On incubation with generated scFv and followed by incubation with anti-myc antibody, a band was observed between 15 to 20 kDa **(Figure 33).**

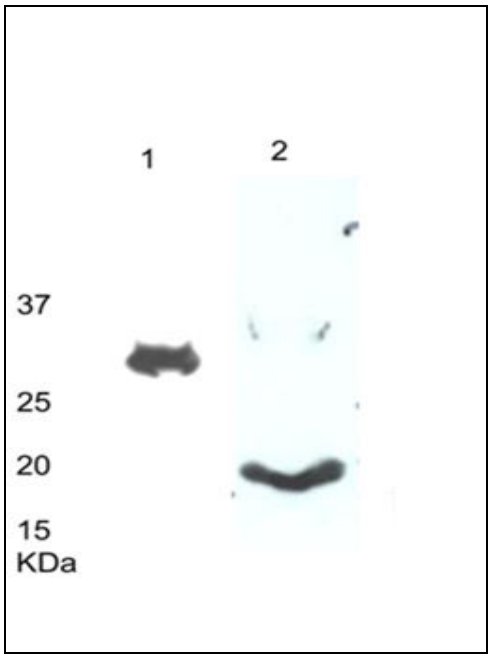

Figure 33: *Confirmation of RANK receptor binding ability of the generated scFv by Western blot analysis. Lane 1: A band seen at 28 kDa as a result of chemiluminescent detection of epitope c-myc of anti-RANK scFv using anti myc HRP antibody. Lane 2: RANK receptor(19.3 kDa) was electrophoresed on 10% SDS-PAGE. Protein was transferred to nitrocellulose membrane which was blocked with 5% skim milk, followed by the incubation with anti-RANK scFv. Reaction was detected by using anti-myc HRP antibody and the membrane was developed using ECL reagent and exposure to Xray film.*

4.6.5. Characterization of anti-RANK scFv by MALDI-TOF

Molecular weight of the generated scFv was confirmed by MALDI-TOF analysis. MALDI-TOF result of scfv showed a sharp peak at 28011.008 m/z. Other peaks represent either impurities or degradation products during analysis **(Figure 34).**

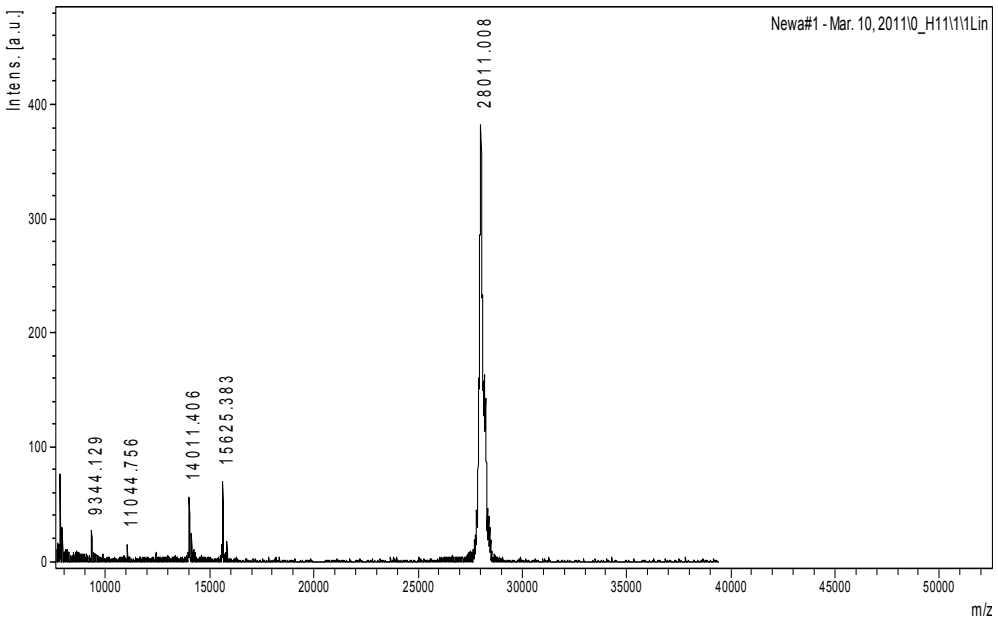

Figure 34: *Confirmation of molecular weight of the generated scFv by MALDI-TOF*

4.6.6. Osteoclast targeting ability of anti-RANK scFv

Immunocytochemistry was conducted to check for the osteoclast targeting potential of the generated scFv. The expressed scFvs possess myc tag. Hence, anti-myc Alexa fluor 488 was used to detect the scFv with

myc tag. DAPI was used for nuclear staining. Confocal images confirmed the osteoclast receptor targeting potential of generated scFv. Fluorescent staining of anti-myc Alexa fluor 488 was observed in the slides treated with anti-RANK scFv **(Figure 35 A).** No demonstrable fluorescent Alexa 488 staining was seen in the negative control slides **(Figure 35 B and 35 C).**

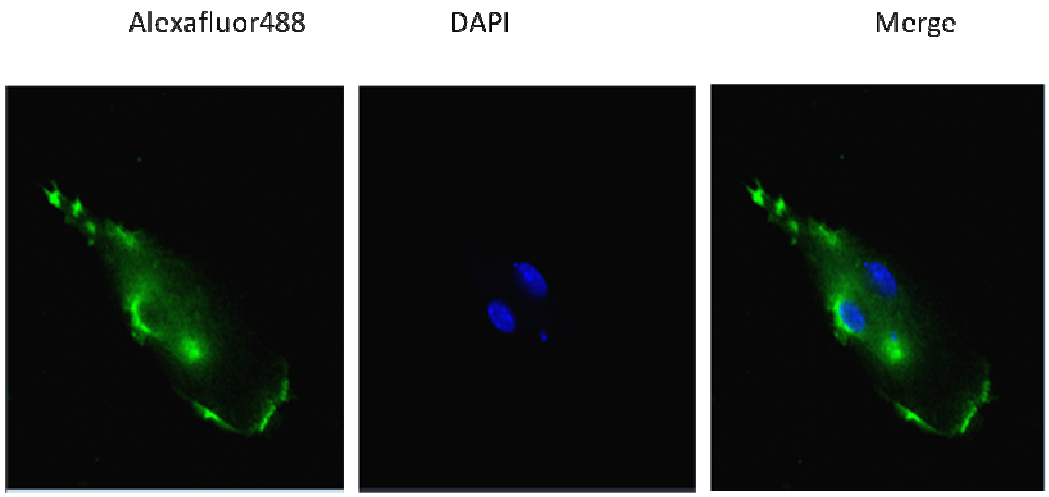

Figure 35 A: *Osteoclast targeting ability of anti-RANK scFv. Immunocytochemistry was conducted to check for the osteoclast targeting potential of the generated scFv. Confocal images of immunocytochemistry for the osteoclast cell culture treated with anti-RANK scFv with myc tag detected by anti-myc Alexa fluor 488.*

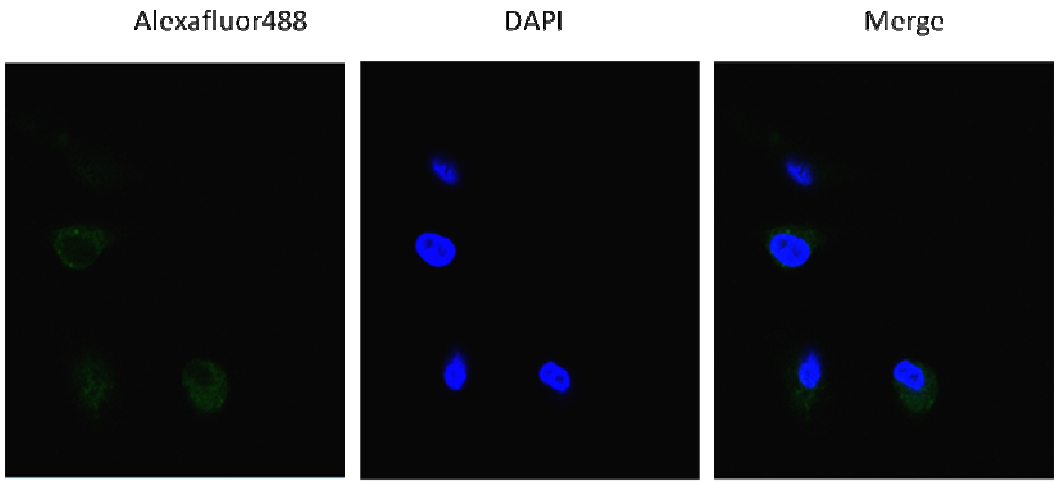

Figure 35 B: *Confocal image of immunocytochemistry for osteoclast cell culture omitting anti-RANK scFv treatment, as a negative control. No demonstrable fluorescent staining of anti-myc Alexa fluor was seen.*

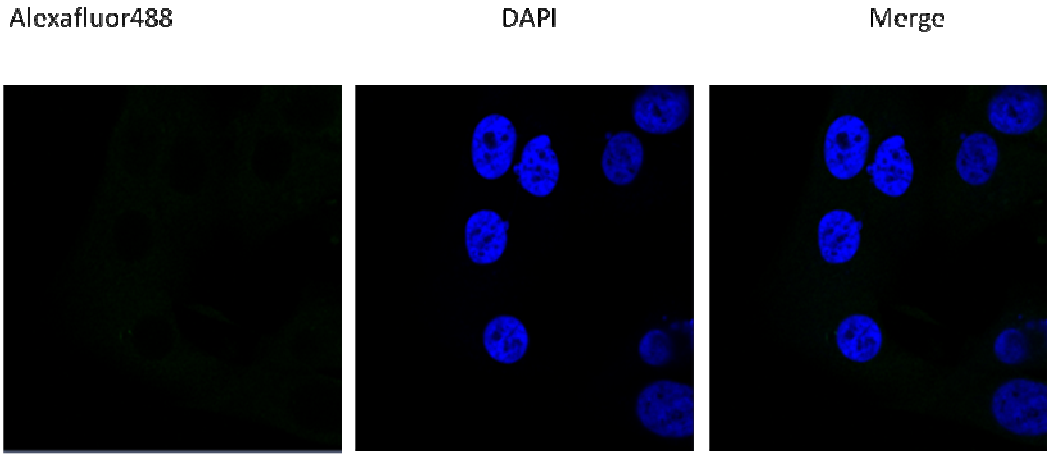

Figure 35 C: *Confocal image of immunocytochemistry for osteoblast-like cell MG-63 cell line treated with anti-RANK scFv. No demonstrable fluorescent staining of anti-myc Alexa fluor was seen.*

4.6.7. Spectrophotometric assay of *In vitro* TRAP activity

The effect of scFv on TRAP release from osteoclast cell culture was determined using a spectrophotometric assay of *In vitro* TRAP activity. TRAP has long been used as a histochemical marker of the osteoclast (Burstone, 1959). The enzyme has been shown to be a specific and sensitive indicator of bone resorption (Halleen *et al.*, 1999) and contributes to the intracellular processing of primary bone matrix degradation products prior to its final release through the basolateral membrane of resorbing osteoclast cells (Vaaraniemi *et al.*, 2004; Vaananen, 2005). The effect of scFv on osteoclast TRAP enzyme activity was quantified by an enzyme assay kit on the cell lysate. Osteoclast cell culture treated with scFv showed decrease in TRAP release **(Figure 36).**

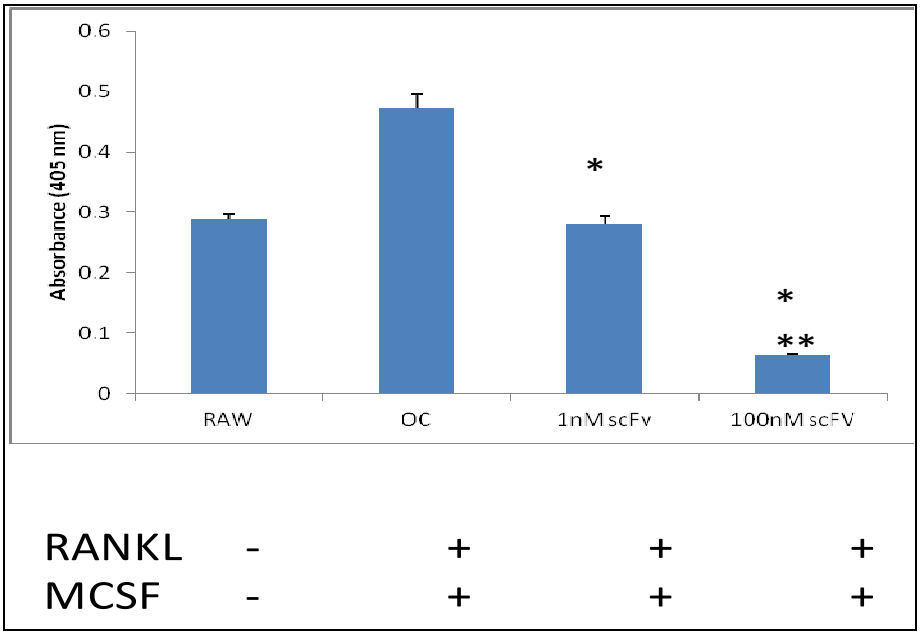

Figure 36: *In vitro TRAP activity assay to determine the effect of scFv on TRAP release from osteoclast (OC) cell culture. Osteoclasts were generated from RAW 264.7 cells in media treated with 50ng/ml RANKL and 25 ng/ml of M-CSF. Treatment with 100nM as well as 1nM of scFv showed significant inhibition of TRAP release from osteoclast culture. * P < 0.05 versus OC. ** P<0.05 versus 1nM scFv.*

4. 6. 8. Resorption assay

Anti-resorptive effect of scFv on osteo assay surface is shown below. Osteoclasts, as the bone-resorbing cells of the body, show the ability to dissolve the mineralized inorganic phase of bone matrix known as hydroxyapatite (Vaaraniemi *et al.*, 2004). To evaluate if this antibody fragment has any effect on the ability of osteoclasts to resorb bone, we cultured osteoclast-like cells on calcium phosphate coated culture wells. The wells treated with RANKL and M-CSF showed a decrease in the remaining calcium when measured by the calcium assay kit **(Figure 37).** The scFv treated osteo assay surface showed more calcium as compared to that of the osteoclast culture. The greater absorbance was observed for 100nM scFv treatment.

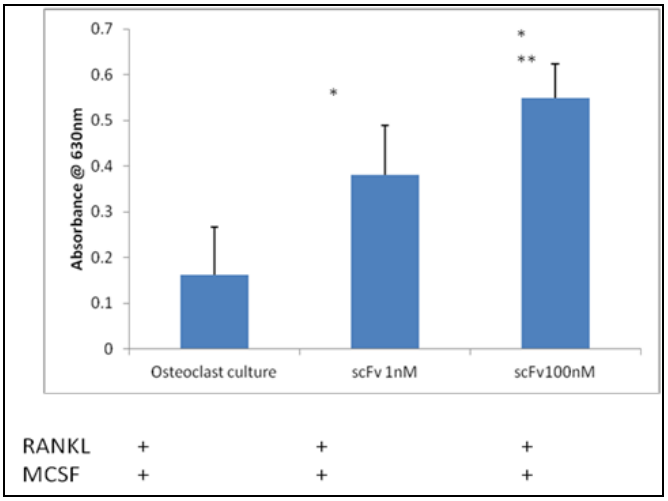

Figure 37: *Anti-resorptive effect of scFv on osteo assay surface. Osteoclasts were generated on osteologic well plate using RAW 264.7 cells in media treated with 50ng/ml RANKL and 25 ng/ml of M-CSF. Treatment with 100nM as well as 1nM of scFv showed significant conservation of osteologic surface. * P < 0.05 versus OC. ** P<0.05 versus 1nM scFv.*

4. 6. 9. Effect of scFv on proliferation of other cell line

No noticeable difference was observed in the growth of UMR-106 cells on treatment with scFv every 48 hours over a period of 6 days. When observed under a microscope, the same degree of cell confluency was observed for all the groups. The MTT assay further confirmed that this antibody like fragment did not have any adverse effect on the proliferation of UMR-106.

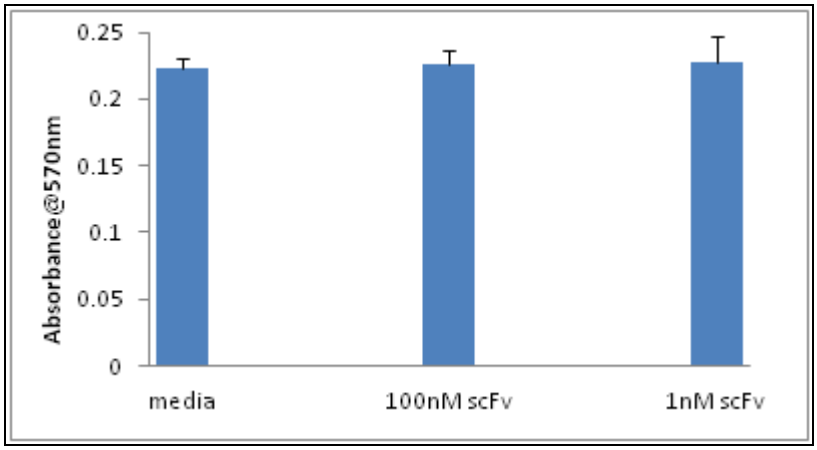

Figure 38: *Cell viability test using MTT assay on UMR-106 cells.*

No demonstrable cytotoxicity was seen as measured by the absorbance of formazan solution formed after incubation with scFv, compared to that seen with untreated media. No statistically significant difference was observed with scFv treated culture as compared to media treated culture.

4. 7. Discussion

This study details about the generation of antibody fragment against the RANK receptor of osteoclasts by phage display technology. Our previous work was on RANK targeting monoclonal antibody generated by hybridoma technique. Monoclonal antibodies represent a new strategy of antiresorptive therapy that was made possible by recent advances in antibody biologics. The Fc effector function of whole antibodies becomes largely redundant in immunocompromised patients due to a limited supply of lymphocytes and macrophages (Badger *et al.*, 1987). Another concern

with the Fc domain is potential cross-reactivity with normal tissues, which can also lead to unwanted side effects, particularly when cytotoxin loaded antibodies are used. In order to address these characteristics, antibody formats, devoid of Fc-regions such as scFvs and Fabs have been engineered. Molecular engineering technologies have made it possible to remove domains from antibodies resulting in miniaturized antibodies (Holliger & Hudson, 2005). Only the antigen-binding sequences can be selected for 'humanization', these sequences can be expressed as a miniaturized scFv, thus to be fine tuned for specific therapeutic actions and to minimize immunogenicity.

The advantage of antibody phage display is the direct isolation of human antibodies in comparison to antibodies generated by mouse hybridoma technology where a laborious humanisation of the lead candidates is necessary. When using transgenic mice comprising the human antibody repertoire human antibodies are also isolated, but antibody generation is limited by the immune system, whereas antibody phage display circumvents this limitation (Schirrmann *et al.*, 2011).

RANK binding scFv clone was successfully screened and expressed from Tomlinson human single fold scFv library using Phage Display Techniques. SDS PAGE analysis and MALDI TOF analysis characterised scFv showing protein band at 28kDa and a peak at 28011.008 m/z respectively. The increase in optical density with increase in concentration of scFv when ELISA was performed on the RANK

receptor coated wells also confirmed the generated scFv to be RANK binding ones. It did not show cross reactivity with other members of TNFR superfamily. Western Blots also confirmed the RANK binding scFv. Immunocytochemistry further confirmed the osteoclast targeting potential of scFv. The fluorescent staining of Anti-Myc Tag, clone 9E10, Alexa Fluor 488 conjugate detected the myc epitope of scFv and hence binding of scFv on osteoclast receptor. The effect of scFv on inhibition in the osteoclast enzyme TRAP and conservation of osteo surface suggest this antibody fragment could serve as an antiresorptive agent rather than just a targeting agent.

Recently, osteoclasts have drawn attention as a therapeutic target in various bone disorders (Akiyama *et al.*, 2008). The osteoclast is the sole cell that resorbs bone and is central in pathologic situations, where bone destruction is highly involved. The increase in development trends for therapeutic fragments (Nelson & Reichert, 2009) and several studies on the importance of RANK in osteoclast formation and activity is the prime impetus for this research work.

RANK is a member of the TNF receptor superfamily of proteins (Anderson *et al.*, 1997) and is designated as member 11a (TNFRSF11a). The only known ligand for RANK is RANKL (Hsu *et al.*, 1999), which is a member of the TNF ligand superfamily member 11 (TNFS11). The TNFRSF member RANK probably is the only functional receptor for RANK-L, because *RANK* -/- and *RANK-L* -/- mice display similar

phenotypes (Dougall *et al.*, 1999; Li *et al.*, 2000). Interactions between RANKL and RANK are essential for osteoclastogenesis. According to Boyle et al., 2003, RANK is now recognized as an important receptor for RANKL in the context of osteoclast (OC) differentiation, the cell type responsible for the resorption of bone during bone remodeling (Boyle *et al.*, 2003). Hence, the possible reason for the effect of anti-RANK scFv on osteoclast activity could be due to interference in binding of RANKL on RANK receptor, the indispensible step for osteoclast formation and its activity. Understanding the mechanisms underlying the interaction of anti-RANK scFv and RANK receptor may contribute to the development of new potential approaches in the treatment of bone metabolic diseases.

Therapeutic targeting of the RANKL--RANK interaction could be a concern as it not only plays a pivotal role in osteoclast formation and function, but is also involved in other biological processes like lymph node development (Kong *et al.*, 1999b; Kim *et al.*, 2000), mammary gland development (Fata *et al.*, 2000), DC survival and activation (Wong *et al.*, 1997; Josien *et al.*, 2000). It may cause adverse side effects on patient immune responses (Toulis & Anastasilakis, 2010; Kyrgidis & Toulis, 2011). However, therapeutics targeting the RANKL-RANK interaction is unlikely to affect on mammary gland development since this biological process is largely an irrelevant issue in most patients with bone disorders such as postmenopausal women (Fata *et al.*, 2000) and may not affect lymph node organogenesis since this process is complete in adults (Hoorweg &

Cupedo, 2008). RANK signaling is indispensable for the lymph node formation in the developmental stage but not for adult lymph node function (Yoshida *et al.*, 1990). Denosumab, a new drug for treating osteoporosis, targets the RANK-RANKL interaction and shows the great potential to serve as a therapeutic target for various bone disorders. With a growing knowledge of importance of the RANK receptor in the differentiation and activity of osteoclast, scFvs targeting RANK receptor seems a meaningful strategy in the treatment of various bone diseases. Future studies involving animal models can further address the potency and specificity of these antibody-like fragments.

Osteoclast targeted therapy has good potential in the management of various bone disease. The increased bone resorption in osteoporosis is due both to increased osteoclastogenesis and to decreased osteoclast apoptosis (Hanley & Josse, 1996; Jones *et al.*, 1996; Sturtridge *et al.*, 1996). Osteoclast-targeted therapies show a great potential for treating conditions in which excess resorption of bone is a key pathological process (Broadhead *et al.*, 2011a; Broadhead *et al.*, 2011b). Many researchers have shown that subchondral bone modifications occur early in the development of osteoarthritis (OA). At an early stage of osteoarthritis pathogenesis, there is a phase of increased bone resorption and an increased number of osteoclasts (Pelletier *et al.*, 2004). Increased bone resorption results in increased articular damage (Ham & Carlson, 2004; Ham *et al.*, 2004; Hoegh-Andersen *et al.*, 2004). Since osteoclasts

are the sole bone cells responsible for resorption, targeting osteoclasts has a potential in OA therapy as well. Paget's disease of bone and multiple myelomas are characterized by increased numbers of osteoclasts and markedly increased bone resorption at the sites of the disease (Roodman & Windle, 2005).

With the current therapeutics, there is an unmet need of development of therapeutics targeting the desired site of action. We believe that this development, which would allow for targeting the sole cell responsible for bone resorption, has the potential to revolutionize the treatment of osteoporosis, although further studies are needed to optimize the pharmacokinetics of this scFv. The development may also have a significant impact on the treatment of osteoarthritis as well as other osteoclast related bone disorders.

4.8. Problems, limitations and future direction

This research showed the potential of the use of antibody molecule in targeting the osteoclast as well as inhibiting the osteoclast activity. The in vitro efficacy was observed on osteoclast cell culture generated from murine monocytic RAW 264.7 cell line. Equal number of cells were seeded on well plates and treated with same concentration of cytokines every alternate days assuming to get equal number of osteoclasts in all the wells. However, not all the cells may fuse to develop multinucleated giant

osteoclasts and also the size of the osteoclasts may not be the same. Some osteoclasts seemed to have less than 4 nuclei and some had more than 10 nuclei. Since all the wells were treated under the same condition, it was assumed that degree of variation in the size and number of osteoclasts in each well remained the same. The in vitro efficacy study was performed on murine osteoclast cell culture and further study on Human Primary osteoclasts cell culture may provide more information. Also, the MTT assay on primary cell line may provide more information on the cell viability study.

To generate free thiol group on monoclonal antibody, Traut's reagent was used in 10 molar excess. It is reported that on reacting with 10 fold molar excess of Traut's reagent, all IgG molecule is modified with at least 3-7 sulfhydryl groups. The limitation with this reaction is that there is no exact control on the level of thiolation. Further issues with the reaction are the use of polydispersed PEG. The reported molecular weight of this polymer is based on its average molecular weight. There is a possibility of having variation in pharmacokinetic parameters of the compounds using such polydispersed PEG if the average molecular weight is variable by a huge range. Use of monodispersed PEG is desirable from a reaction efficiency point of view and also from a regulatory point of view. But monodispersed products are not commercially available for the entire PEG molecular weight ranges. Also,

the formation of mono, di and tri substituted PEGylated calcitonin products is not desirable as this eventually lead to variable and unpredictable pharmacodynamic parameters. Hence, a new reaction technique is needed that allows the generation of exact number of free thiols on IgG. Likewise, the use of monodispersed PEG and control in the PEGylation by blocking specific amine group in calcitonin is required for the therapeutic use.

The antibody and antibody-like fragment generated against RANK receptor showed osteoclast targeting potential as well the inhibition of osteoclast activity. The anti- RANK antibody generated against the RANK receptor could be recognizing and occupying the same site of the receptor as recognized by RANKL for binding. The competitive binding could be the reason for the antibody being able to antagonize the interaction of RANKL with RANK, an essential step of osteoclast differentiation. Since a RANK trimer is required for complete osteoclastogenesis, it could be that the antibody/ antibody-like fragment is disrupting the trimerization process. However, the cause for this effect has not been studied yet. Understanding the mechanisms underlying the interaction of the antibody and osteoclast receptor may contribute to the development of new potential approaches in the treatment of such bone metabolic diseases.

The anti-RANK antibody/ fragment has shown good efficacy in inhibiting osteoclast activity in in vitro assays. Future studies involving animal models can further address the potency and specificity of these antibody fragments. Biodistribution of any therapeutic agent is a key consideration for getting the desirable in vivo pharmacological effect. Selective localisation of such agent in desired site of action is important in achieving the efficacy. RANK receptor is highly expressed on osteoclasts. However, they are expressed in dendritic cells, liver, colon and small intestine. Hence, the biodistribution study for the RANK targeting antibody should be conducted to compare the level of localisation at the desired site of action with other RANK bearing cells other than osteoclasts. Furthermore, it is important to study if it leads to adverse effects by binding of this antibody to those cells. The effective targeting of therapeutic agents to skeletal tissues may greatly improve the efficacy of a variety of drug molecules while potentially greatly reducing undesired side-effects.

Studies need to be conducted regarding the formulation of this antibody. Antibody pharmaceuticals are delicate molecules susceptible to different stresses during normal pharmaceutical operations such as manufacturing, storage, transportation, and delivery. Extensive study is required to check and confirm the physical and chemical stability of protein formulation. Liquid formulations are generally preferred over lyophilised products due to low cost and ease of use. However, lyophilized

formulations are more stable in case of certain proteins. Formulation development should be based on a sound understanding of the stability of the antibody. Proteins are prone to aggregation, precipitation, denaturation, and adsorption to surfaces and exhibit chemical instability. Methods need to be developed to stabilize the formulation by addition of inactive ingredients like surfactants, solubilizers, stabilizers, buffers, tonicity modifiers, bulking agents and viscosity enhancers/reducers. PEGylation may be carried out to improve stability and half life of the antibody fragment. As with all pharmaceutical products, development of this antibody formulation requires continuous comparison of the cellular and in vitro studies with in vivo studies.

The route of administration is another aspect that needs to be considered for the successful development of the pharmaceutical product. Since biologics have become an important class of pharmaceutical products, there has been a continuous development in finding delivery methods for protein pharmaceuticals. Prerequisite for the successful delivery of peptides and proteins is the stabilization of the biologicals at all stages before they reach their target. Considering high levels of patient acceptance and long term compliance, oral route would be preferred to any other route. However, due to poor bioavailability and unfavorable physicochemical properties, designing and formulating an oral protein drug is a big challenge and might be possible in the future with innovative

delivery systems. Injections are the most common means for administering therapeutic proteins and peptides. Injection systems like pen injectors and autoinjectors are developed to meet both the bioavailability and efficiency requirements of the therapy. Although most therapeutic mAbs today are developed for IV delivery and subcutaneous delivery, interest has grown in finding more efficient delivery routes that improve patient compliance and response.Patient compliance being one of the important factors for the success of the pharmaceutical product, non-invasive and comfortable self-administration options are developed focusing on the need of patient. Microstructured Transdermal System is a drug delivery platform offering the characteristics of both a syringe and a transdermal patch to meet the therapeutic and patient needs. This transdermal system is designed for the efficient targeted delivery of a wide range of biopharmaceutical therapeutics, from peptides and antibodies.

While still in its infancy, antibody mediated osteoclast-targeted strategy has tremendous potential as a novel management option for various bone diseases. However, it is still a challenge for widespread acceptance by clinicians and regulatory bodies. Careful examinations of the effect of this antibody therapy on bone biology and intense studies uncovering its potential advantages and disadvantages are needed to establish this novel therapy.

4.9. Conclusion

Since the pharmacological arrest of the osteoclast is the mainstay of treating various bone disorders, targeting osteoclasts represents a viable option for the development of new therapeutics. The generated antibody fragment targeting the osteoclast receptor may find utility as “a universal osteoclast targeting platform” to directly target as well as to deliver drug cargoes to osteoclasts, thereby treating or controlling the progression of osteoclast related bone disorders. With the advances in the development of therapeutic scFv fragments and a growing knowledge on the importance of osteoclast targeted therapy, scFv targeting of osteoclasts seems to be a meaningful strategy for the treatment of bone diseases.

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