RESEARCH PAPER

Antibody-Mediated "Universal" Osteoclast Targeting Platform using Calcitonin as a Model Drug

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ABSTRACT

Purpose To generate and characterize a specific monoclonal antibody (mAb) against recombinant human RANK receptor and to develop an antiresorptive strategy using this mAb as an osteoclast-targeting platform that selectively targets osteoclast cells whilst delivering an attached (i.e. chemically conjugated) active drug cargo.

Methods Using hybridoma technology, we generated a specific monoclonal antibody (mAb) against recombinant human RANK receptor and characterized by SDS PAGE, ELISA, Western Blot and immunocytochemistry, then synthesized osteoclast-targeting bioconjugates of salmon calcitonin (sCT) using this antibody by generating thiol groups on mAb using 2-Iminothiolane and subsequently reacting them with sCT-PEG-MAL synthesised from sCT and NHS-PEG-MAL. To test the efficacy of the conjugate *in vitro*, osteoclasts were generated from precursor RAW 264.7 cells by dosing with the cytokines macrophage-colony-stimulating factor (M-CSF), and RANK Ligand (RANKL) and TRAP activity assay, Resorption Pit Assay, TRAP staining were performed. Cytotoxicity of the mAb-sCT conjugate was also evaluated in RAW 264.7 cells; sCT bioactivity and CTR binding potential were evaluated by *in vitro* intracellular cAMP stimulation assay in human T47D breast cancer cells.

Results Generation of antibody against human RANK receptor was confirmed by SDS PAGE, ELISA and Western Blot. Immunocytochemistry confirmed the osteoclast targeting potential of the antibody. Successful conjugation of the antibody with sCT was confirmed by SDS PAGE and ELISA. Multinucleated osteoclast formation was confirmed by staining for tartrate-resistant acid phosphatase (TRAP). Conjugate functionality was confirmed by TRAP activity and Resorption Pit assay, showing the inhibitory effect on osteoclast differentiation. cAMP assay confirmed the retention of calcitonin bioactivity after conjugation.

Conclusions Our strategy offers the potential for a "universal" osteoclast-targeting platform—one that targets the RANK receptor on osteoclast cells by simply altering the conjugated cargo in order to affect the specific regulation of osteoclast cells.

INTRODUCTION

Osteoclasts are the cells uniquely responsible for dissolving both the organic and inorganic components of bone during

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development and are 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 (1).

Osteoporosis is a major metabolic bone disease that predisposes patients to fracture in up to 40% of aging women and 15% of aging men (2). The increase in bone resorption is due both to increased osteoclastogenesis and to decreased osteoclast apoptosis (2,3). Osteoclasts may also play a role in the etiology of post-traumatic osteoarthritis (PTOA), the most common form of arthritis. At an early stage of disease pathogenesis, there is a phase of increased bone resorption and turnover in periarticular subchondral bone volume and an increased number of osteoclasts, prior to later stage sclerosis and eburnation (4). Similarly, increased osteoclast functional activity is directly responsible for the generalized bone loss that occurs in rheumatoid arthritis (5). Thus, we hypothesized that an antiresorptive strategy that selectively targets osteoclasts and/or carries an active drug to osteoclast cells directly would be highly desirable as a therapeutic to treat bone disease involving upregulated osteoclast activity.

Osteoclast cells express the RANK receptor (receptor activator of nuclear factor Kappa B) and in effect serve as ideal molecular targets. RANK is expressed on osteoclast precursors as well as mature osteoclasts. RANK is the essential signaling receptor for osteoclast differentiation during the process of osteoclastogenesis, as triggered by the osteoclast differentiation factor known as RANK-ligand (RANKL) (6,7). RANK signaling, with additional signaling through c-Fms, the receptor for macrophage-colony-stimulating factor (M-CSF), triggers the proliferation and fusion of mononuclear cells and the formation of multinucleated, mature osteoclasts (7,8).

Mature osteoclasts will also express the calcitonin receptor upon its surface, which has been known for decades as a prominent negative controller of bone resorption (9). Calcitonin receptors are of the G protein-coupled receptor family (comprising seven transmembrane-spanning receptor domains) whose signaling will inhibit osteoclast activity both *in vitro* and *in vivo* (10). Calcitonin receptor activation upon osteoclasts, by its ligand calcitonin, will rapidly induce the loss of ruffled border and immobility followed by cell retraction and arrest of bone resorption. Calcitonin receptor signaling will also alter ion transporter distribution, impair enzyme activity (11) and inhibit the osteoclastogenic effects of RANKL (12).

Since antibodies have exquisite specificity of target recognition and, thus, generate highly selective outcomes following their systemic administration, our purpose was to develop a "universal" osteoclast-targeting platform as a drug-delivery strategy in order to deliver antiresorptive drugs using an anti-RANK mAb capable of localizing to the cells responsible for bone resorption. We have chosen salmon calcitonin as a model drug, as it acts on the calcitonin receptor also found on bone-resorbing osteoclasts.

MATERIALS AND METHODS

Preparation of Anti-RANK Monoclonal Hybridoma Cell Lines

Monoclonal antibody (mAb) to the RANK receptor was generated using Hybridoma technology, as previously performed in our laboratory (13,14). Briefly, 6–8-week-old female BALB/c mice were immunized intraperitoneally three times with 25 μg of recombinant human sRANK receptor (Peprotech, USA) on days 0 and 14 using complete and incomplete Freund's adjuvant, respectively, and once with 10 μg of antigen on day 28 using phosphate-buffered saline (PBS, pH 7.3). 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 (15). 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). 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 then propagated in large 175 cm² tissue culture flasks, and the conditioned supernatant collected. Purification of IgG mAbs was achieved by affinity chromatography using Protein G agarose (Sigma, USA). The IgG immunoglobulin subclass was determined using the mouse hybridoma isotyping

reagents according to instructions from the manufacturer (Sigma, USA).

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 1 µg/100 µl, overnight at 4°C. The wells were washed three times with PBS (pH 7.3) and, to avoid nonspecific binding, incubated with 3% BSA for 1 h at room temperature. After washing, the wells were incubated with 100 µl supernatant from each hybridoma clone for 1 h 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 h 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. RPMI media and positive sera from hyperimmunized mice were used as blank and positive control, respectively.

Characterization of Anti-RANK Monoclonal Antibodies

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on purified antibody under both reducing and non-reducing conditions. 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.

ELISA was performed as above, using 100 µl RANK receptor for coating at a concentration of 1 µg/100 µl, overnight at 4°C. To avoid nonspecific binding, the wells were incubated with 3% BSA for 1 h at room temperature. After washing, the wells were incubated with different concentrations of antibody for 1 h. The wells were washed with PBS, and the bound antibodies were detected using secondary antibody (goat anti-mouse IgG conjugated

with horseradish peroxidase) for 1 h. 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.

For Western blot analysis, RANK receptor (19.3 kDa) 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 PBS-T for 1 h, washed with PBS-T, cut into strips, and incubated with purified mAb solution for 1 h. After washing, strips were reacted with GAM-HRPO for 1 h, and the binding of mAbs to RANK receptor was detected using enhanced chemiluminescence. For the negative control, the primary antibody was omitted, and the antigen strip incubated with PBS.

Immunocytochemistry

To confirm the RANK receptor targeting potential of generated mAb *in vitro*, osteoclast-like cells were generated in Lab Tek II chamber slide system (Nunc) from RAW 264.7 cells, as described below for generation of osteoclasts. The MG-63 osteoblast-like cell line known to lack RANK receptor [16] was used as negative control. Likewise, osteoclast cell culture omitting primary mAb was also used as a method negative control. Cell cultures were washed with PBS, fixed in 4% paraformaldehyde in PBS (pH 7.4) for 5 min and rinsed thoroughly. After blocking with 3% BSA in PBS for 1 h, 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).

Synthesis of Anti-RANK mAb-Calcitonin Conjugate

Thiolation of Antibodies Using Traut's Reagent

In order to generate anti-RANK mAb-salmon calcitonin conjugate (mAb-sCT), free thiol groups were generated on our anti-RANK mAb by reacting it in a 10 molar excess of Traut's reagent at room temperature for 1.5 h (Fig. 1a). 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 [17,18]. No free sulfhydryl moieties were

found to be present in our IgG mAb, as determined by Ellman's assay (19).

Synthesis of Thiol-Reactive Calcitonin Analogue

To generate 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. DMSO was chosen as the reaction medium because of the instability of NHS and sCT in aqueous solutions. sCT is further highly soluble and highly stable in DMSO (20). 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 min. sCT 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 (Fig. 1b).
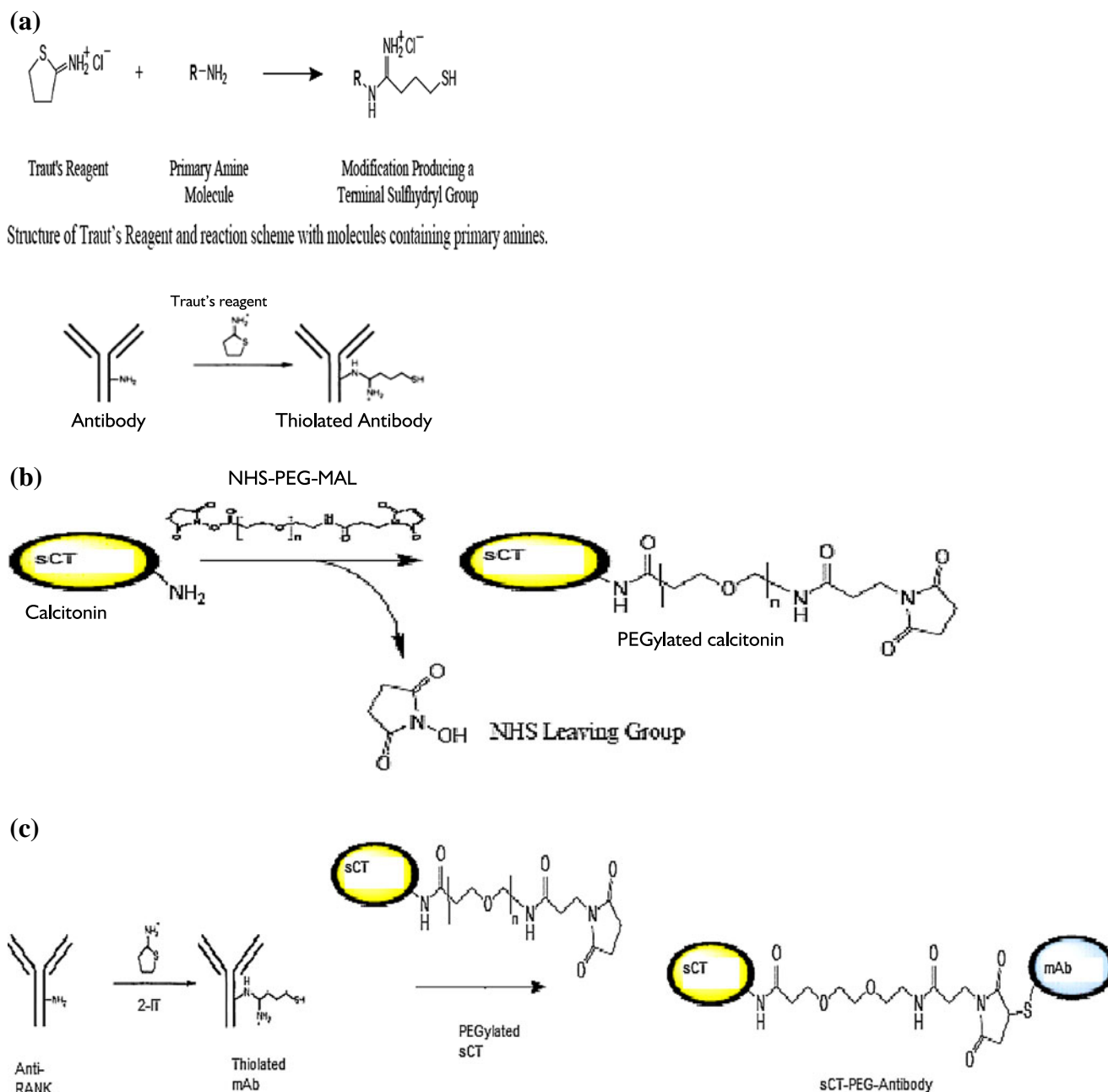
(a)

Structure of Traut's Reagent and reaction scheme with molecules containing primary amines.

(b)

(c)

Fig. 1 (**a**) Thiolation of antibodies using Traut's reagent. (**b**) Synthesis of thiol reactive sCT analogue by reacting sCT with NHS-PEG-MAL. **c** Coupling free thiol containing mAb with functionalized thiol reactive sCT analogue.

Coupling Free Thiol-Containing mAb with Functionalized Thiol-Reactive sCT Analogue

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

Characterization of Anti-RANK mAb-sCT Conjugate

Anti-RANK mAb-sCT conjugate was examined under both reducing and non-reducing conditions of SDS-PAGE analysis, as described above. ELISA was performed, using 100 µl RANK receptor for coating at a concentration of 1 µg/100 µl, overnight at 4°C. To avoid nonspecific binding, the wells were incubated with 3% BSA for 1 h at room temperature. The wells were incubated in triplicate with either mAb alone, functionalized sCT-PEG, or mAb-sCT conjugate for 1 h. The wells were washed with PBST followed by incubation with rabbit anti-salmon calcitonin antibody (US Biologicals) for 1 h. The wells were washed with PBST three times and incubated with HRPO conjugated goat anti-rabbit IgG (R&D Systems) for 1 h. After final washing with PBST, TMB substrate was added to each well, and the OD measured at 650 nm.

In Vitro Testing of mAb-sCT Conjugate Efficacy

Generation of Osteoclasts

Osteoclast-like cells were generated in culture from RAW 264.7 cells (transformed murine monocytic cell line), purchased from the American type culture collection (ATCC, VA, USA) (21). RAW 264.7 cells were cultured to confluence in a 75 cm^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 (10,000 U/mL; Invitrogen, USA). Confluent cells were harvested by scraping, centrifuged at 1,500 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 2×10^3 cells/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 h, 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 h 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.

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 2×10^3 cells/well in 96-well culture plates and incubated for 24 h. Peptide factors 50 ng/ml RANKL and MCSF 25 ng/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 h. 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.

In Vitro Mineral Resorption Assay

To quantify the mineral resorptive activity of osteoclast-like cells in culture, RAW264.7 cells were suspended in cell culture medium and seeded in a 16-well BD Biosciences Osteologic® Slide (BD Biosciences, MA, USA) at the density of 2×10^3 cells/well and incubated for 24 h. Osteoclast-like cells were successfully generated by dosing with 50 ng/ml RANKL and 25 ng/ml M-CSF every 48 h 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 h. 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 javabased image-processing program ImageJ (NIH, USA).

Cytotoxicity Test for mAb-sCT Conjugate

To ensure that the mAb-sCT conjugate was not cytotoxic to cells other than osteoclasts, we employed the MTT assay, in which 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 2×10^3 cells/well and incubated for 72 h at 37°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 min. 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 (in quadruplicate). The cells were incubated for 4 h 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 h 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 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.

In Vitro Bioactivity of mAb-sCT and Calcitonin Receptor Binding Affinity

To confirm that calcitonin bioactivity was not lost after conjugation to mAb and to confirm the ability for mAb-sCT conjugate to trigger the calcitonin receptor, T47D human breast cancer cells (known to contain calcitonin receptors) were used, as previously described (22). Briefly, T47D cells (ATCC, VA, USA) were cultured in triplicate in RPMI-1,640 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 dosed with the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, 1 mM) and incubated at 37°C for 30 min. One hundred 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 min 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).

STATISTICAL ANALYSIS

Statistical analysis was conducted using Graph Pad Prism 5 (GraphPad Software Inc., CA). 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 the MTT assay.

RESULTS

SDS-PAGE Analysis

A band at 150 kDa was seen for unconjugated anti-RANK IgG, with separate bands for heavy chain at 50 kDa and light chain at 25 kDa observed under reducing condition. For mAb-sCT conjugate, a band above 150 kDa was observed, confirming the conjugation to have taken place. Under reducing condition of SDS-PAGE for conjugate, some bands above 50 kDa and 25 kDa were observed, suggestive of conjugation of PEGylated calcitonin to the heavy and light chains of the antibody. (Fig. 2)

ELISA

The optical density was seen to linearly increase with the concentration of mAb (Fig. 3a). 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.

ELISA carried out for the conjugate showed the binding of the conjugated antibody to the coated RANK receptor. 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 anti-calcitonin antibody. Similarly, 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 (Fig. 3b). ELISA-derived spectrophotometric absorbance values for mAb, sCT-PEG and mAb-sCT conjugate were 0.002, 0.025 and 0.153, respectively.

Western Blot Analysis

Recombinant human sRANK receptor is a 19.3 kDa polypeptide. It was electrophoresed on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. On incubation with generated mAb, a band was detected between 15 and 20 kDa, after the chemiluminescent detection of anti-RANK IgG (Fig. 4).

Osteoclast Generation and Confirmation by TRAP Staining

Osteoclasts were generated by dosing the RAW264.7 cells with RANKL and MCSF. 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 fast garnet GBC salt, resulting in the
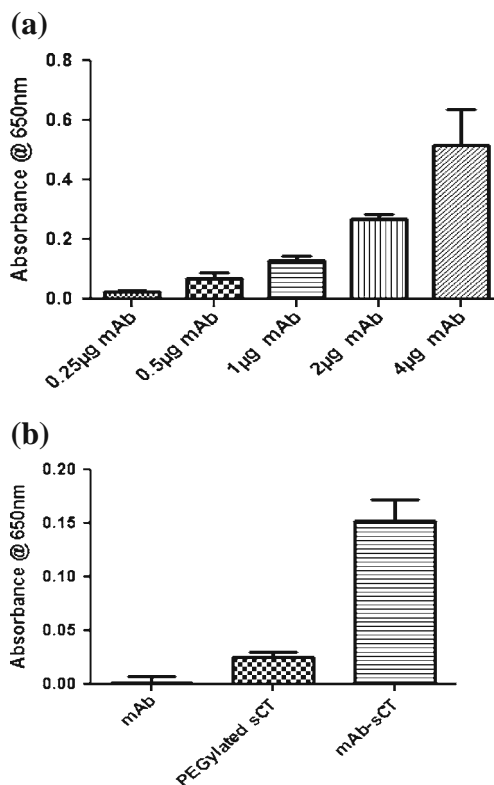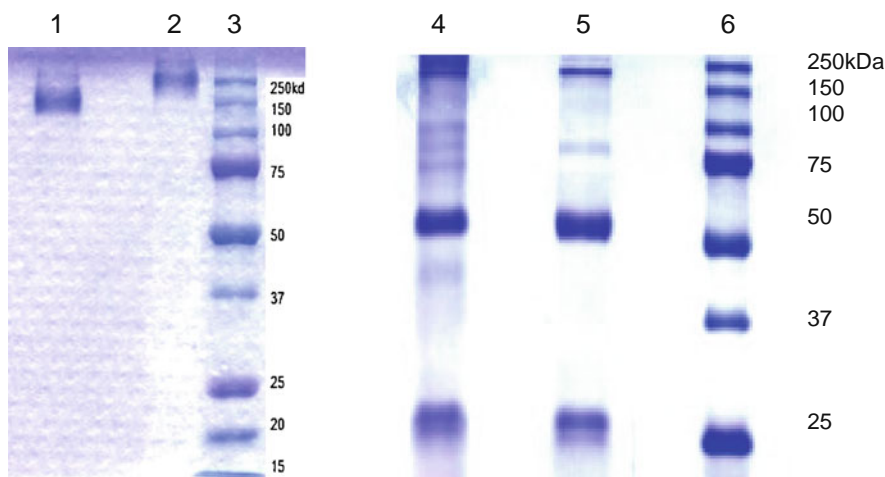
Fig. 3 (a) ELISA conducted on RANK receptor-coated plate. The optical density was seen to be proportional with the concentration of anti-RANK antibody. (b) ELISA of the mAb-sCT conjugate where anti-Calcitonin secondary antibody reagent was used to detect immobilized Calcitonin residues that were part of the primary anti-RANK conjugates and that were binding to the RANK-coated wells. Optical density for mAb alone was negligible as it did not have calcitonin to be detected by anti-calcitonin antibody. Similarly, OD for sCT-PEG was also significantly low as it did not have anti-RANK antibody to bind to RANK-coated plate.
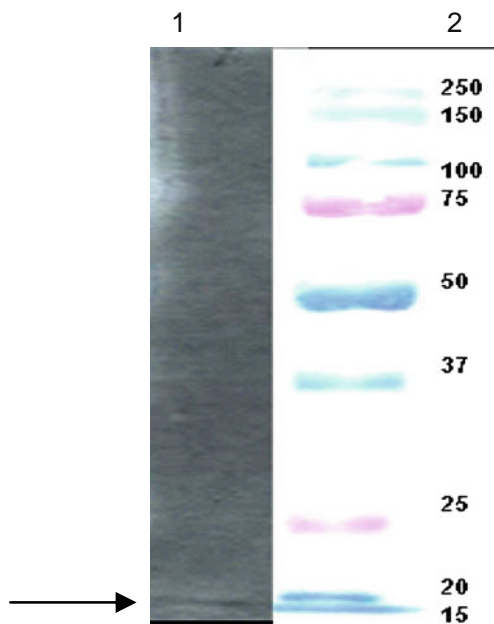
Fig. 2 SDS-PAGE analysis of mAb-sCT conjugate and unconjugated antibody. A protein band above 150 kDa was seen for mAb-sCT conjugate in non-reduced SDS-PAGE analysis. Some bands above 50 kDa as well as 25 kDa were observed for the conjugate in reduced SDS PAGE. Lane 1: unconjugated antibody (non-reducing condition), Lane 2: mAb-sCT conjugate (non-reducing), Lane 3, 6: standard marker, Lane 4: mAb-sCT conjugate (reducing), Lane 5: unconjugated antibody (reducing).

Fig. 4 Western Blot Analysis: Lane 1: a band seen between 20 kDa and 15 kDa as a result of chemiluminescent detection of anti-RANK IgG. Lane 2: standard marker. 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 mAb. Reaction was detected by using GAM-HRPO, and the membrane was developed using ECL reagent and exposure to X-ray film.

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 (Fig. 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 (Fig. 6a). Counterstaining with DAPI (blue color) confirmed the multinucleated phenotype of osteoclast-like giant cells. Nucleoli were also witnessed in the images. There was no demonstrable fluorescent fluorescein isothiocyanate (FITC) staining in negative control slides (Fig. 6b and c). 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.

TRAP Activity Assay

RAW 264.7 cell cultures treated with exogenous RANKL and MCSF showed a 9.9-fold increase of TRAP activity compared to controls without cytokine treatment. We found that the release of this osteoclast-associated enzyme was potently inhibited by our mAb-sCT conjugate (and by our unconjugated mAb alone), as compared to sCT alone (Fig. 7). We measured a 5.47-fold decrease in TRAP activity after treatment with mAb-sCT conjugate. Also, the conjugate showed a 2.82-fold greater inhibition of TRAP compared to sCT treatment alone.

***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. 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 (Fig. 8).
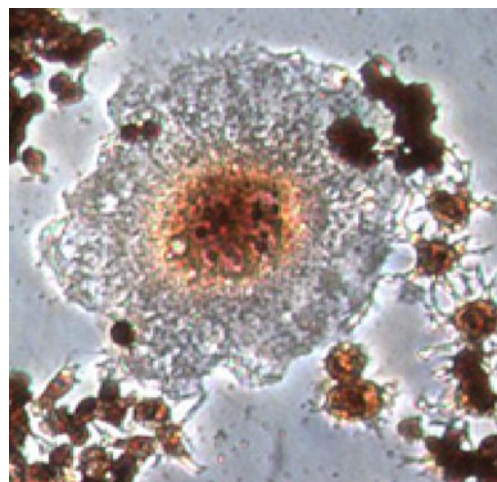

Fig. 5 Multinucleated TRAP-positive osteoclast was observed by TRAP staining. RAW 264.7cells, a murine macrophage-like osteoclast precursor line, were stimulated with 50 ng/ml RANKL and 25 ng/ml MCSF for osteoclast generation.
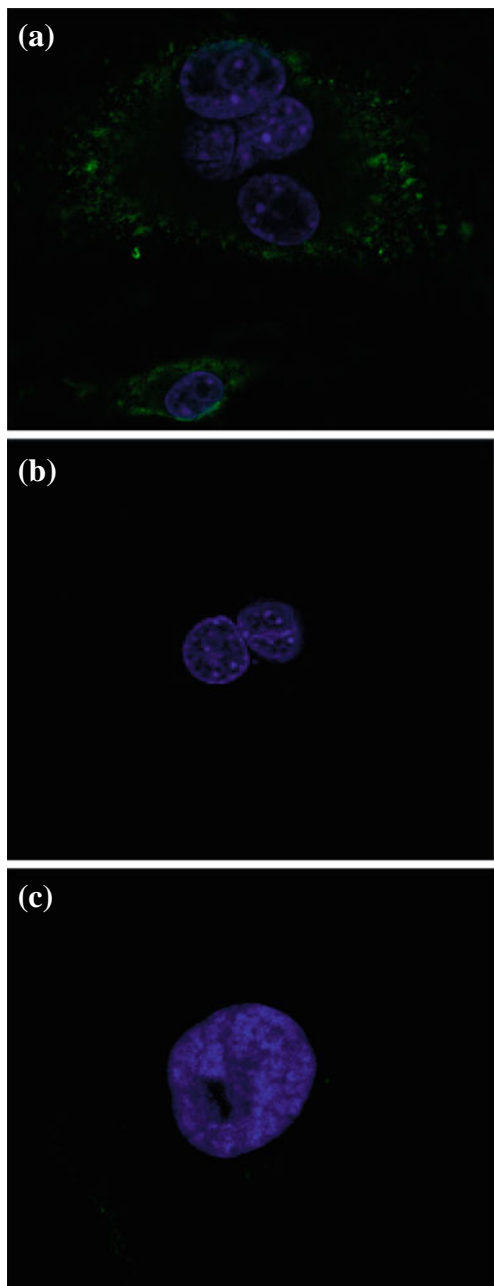
Fig. 6 (**a**) Confocal images of immunocytochemistry performed with anti-RANK mAb which detected RANK receptors, the surface receptors present on the osteoclasts as well as osteoclast precursors. Counterstaining with DAPI showed the multinucleation in osteoclasts. (**b**) Confocal image of immunocytochemistry for osteoclast cell culture omitting primary antibody, as a negative control. (**c**) Confocal image of immunocytochemistry for osteoblast-like MG-63 cell line as a negative control. The MG-63 cell line does not express RANK receptors.

Cytotoxicity of mAb-sCT Conjugate

To test for potential cytotoxic effects of our mAb and/or mAb-sCT conjugate, we tested RAW 264.7 cell viability using the MTT assay. Anti-RANK monoclonal antibody, mAb-sCT conjugate and sCT showed no demonstrable cytotoxicity

at the concentrations tested (up to 200 nM), as measured by the absorbance of formazan solution formed after 4 h incubation (Fig. 9). Thus, the viability of these cells was not perturbed by our mAb and/or conjugate treatments.

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 and the mAb-sCT conjugate were capable of stimulating cAMP production after binding the calcitonin receptor. 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 (Fig. 10).

DISCUSSION

Osteoclasts are the only cells capable of resorbing mineralized bone. However, current antiresorptive therapies do not specifically target the osteoclast cell and elicit systemic side effects in cells other than osteoclasts. For example, the development of duodenal ulcers, gastric erosions and potentially even osteonecrosis of the jaw or catastrophic low energy fracture of the femoral shaft have all been
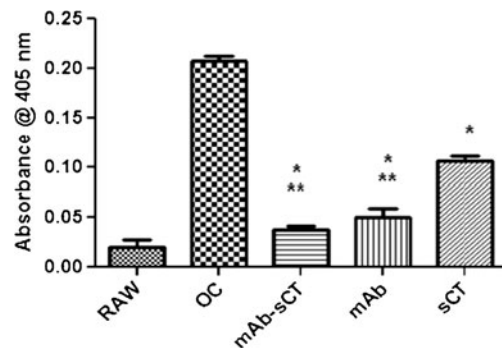

Fig. 7 Tartrate-resistant acid phosphatase (TRAP) activity assay. mAb-sCT conjugate showed an inhibitory effect on TRAP activity, an important marker of osteoclasts. RAW: cells cultured in media with 0 ng/ml RANKL + 0 ng/ml MCSF; OC: cells cultured in media with 50 ng/ml RANKL + 25 ng/ml MCSF; mAb-sCT: cells cultured in media with 50 ng/ml RANKL + 25 ng/ml MCSF + 100 nM conjugate; mAb: cells cultured in media with 50 ng/ml RANKL + 25 ng/ml MCSF + 100 nM mAb; sCT: cells cultured in media with 50 ng/ml RANKL + 25 ng/ml MCSF + 100 nM sCT . * $P < 0.05$ *versus* OC; ** $P < 0.05$ *versus* sCT. No statistically significant difference was observed between naked antibody and the conjugate.
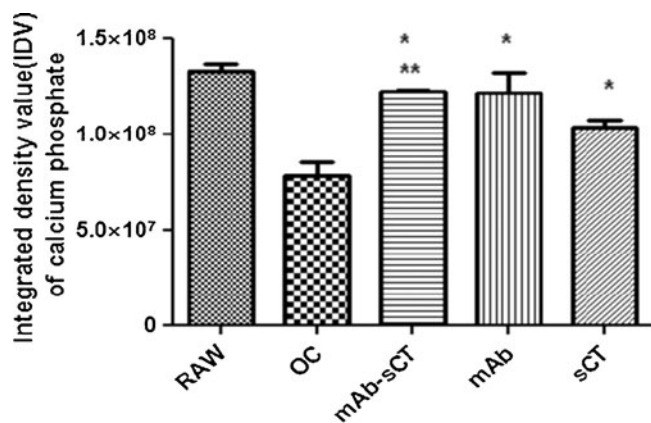
Fig. 8 Demonstration of resorption pit formation on osteologic substrate plate. After 7 days of culture, adherent cells were removed, and integrated density value of the remaining osteologic layer was calculated using alphaimager densitometer. RAW: cells cultured in media with 0 ng/ml RANKL + 0 ng/ml MCSF; OC: cells cultured in media with 50 ng/ml RANKL + 25 ng/ml MCSF; mAb-sCT: cells cultured in media with 50 ng/ml RANKL + 25 ng/ml MCSF + 100 nM conjugate; mAb: cells cultured in media with 50 ng/ml RANKL + 25 ng/ml MCSF + 100 nM mAb; sCT: cells cultured in media with 50 ng/ml RANKL + 25 ng/ml MCSF + 100 nM 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.

Fig. 10 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 in RPMI 1,640 containing insulin, and the phosphodiasterase activity was blocked using 3-IBMX. Cells were then 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.

associated with differing classes of bisphosphonates, the most commonly prescribed medication for osteoporosis (23). Estrogen hormone replacement therapy stimulates uterine and breast tissue, which predisposes the patient to neoplastic disease (24). Leg muscle cramps, joint aches and deep vein thrombosis has been associated with selective estrogen receptor modulator therapy (25). Similarly, conventional salmon calcitonin has shown limitations in antiresorptive efficacy, likely in part because of poor bioavailability to bone cells and the undesired uptake of
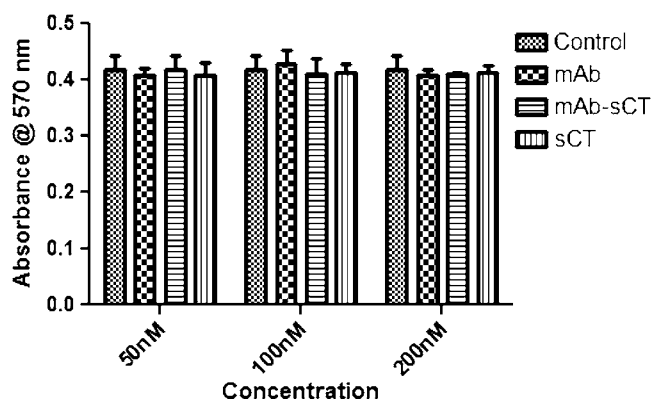

Fig. 9 *In vitro* cytotoxicity of mAb, mAb-sCT & sCT on RAW 264.7 cells was determined by MTT assay. No demonstrable cytotoxicity was seen as measured by the absorbance of formazan solution formed after 4 h incubation with compounds, compared to that seen with untreated media. Samples were not statistically significant compared to the control at all concentrations.
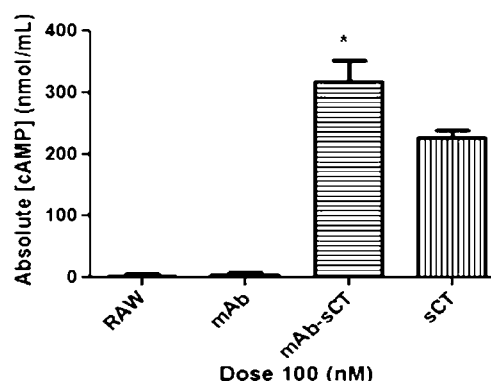
the drug by calcitonin receptors present in tissues other than bone (26,27).

Since the pharmacological arrest of the osteoclast is the mainstay of treating systemic bone loss, drug delivery strategies that target the osteoclast directly would provide a potential therapeutic advantage at reducing systemic bone loss by selectively delivering an antiresorptive drug "cargo" to the osteoclast. Antibodies are renowned for their exquisite specificity of target recognition and generate highly selective outcomes following their systemic administration. As the RANK receptor is predominantly expressed on the surface of mature osteoclasts, we hypothesized that an anti-RANK antibody would facilitate an effective osteoclast targeting platform for the delivery of antiresorptive drug directly to the resorbing bone cells.

Salmon calcitonin, a single chain polypeptide hormone consisting of 32 amino acids, was chosen as a "model" drug for this study, as sCT therapy is known to inhibit and/or slow osteoclast-mediated resorptive bone loss, whilst positively influencing osteoinduction and bone formation (28). Hence, we designed a conjugative strategy 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." 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 the calcitonin receptor, 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.

Using hybridoma technology, we generated and characterized a specific monoclonal antibody against recombinant human RANK receptor, and chemically coupled synthetic sCT to that anti-RANK mAb carrier. We further PEGylated sCT in the process of conjugation by using the polymer polyethylene glycol, which is known to improve the major disadvantages of conventional sCT by increasing solubility, stability, and efficacy and reducing the immunogenecity. It has also been established that PEGylation enhances the enzymatic stability of calcitonin (29) against proteolysis, by forming an effective shield against degradative enzymes (30) whilst improving its efficacy *in vivo* by increasing molecular size, thus contributing to reduced renal filtration (31) allowing less frequent administration (32).

The synthesized bio-conjugate was initially characterized using SDS-PAGE analysis and ELISA. Protein band above 150 kDa was observed for the conjugate under non-reducing condition of SDS-PAGE analysis, and some bands were seen above 50 kDa and 25 kDa under reducing condition, 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 we assume that 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 conjugate, such MAL groups were utilized in the reaction with free thiol groups generated in antibody.

In order to verify the efficacy of mAb-sCT conjugates, we employed *in vitro* osteoclast-like cell cultures. It is well established that osteoclasts are derived from cells of the monocyte/macrophage lineage and are formed after the cellular fusion of their mononuclear precursors (33). Hence, we utilized the RAW 264.7 cell line that can be differentiated *in vitro* into osteoclast-like cells after exposure to M-CSF and RANKL cytokines. RANKL is the essential factor necessary for osteoclast differentiation, whilst M-CSF (also known as CSF-1) has also been identified as an essential factor necessary to initiate osteoclastogenesis (34). The main role of M-CSF is to act as a survival factor for osteoclast precursor cells and to induce the expression of the RANK receptor for stimulation by the RANKL.

Immunocytochemistry confirmed the osteoclast targeting potential of our generated anti-RANK mAb *in vitro*. Fluorescent staining of our anti-RANK mAb was specifi-cally localized to cellular surface receptors. DAPI staining showed the successful generation of multinucleated osteoclast-like cells. No demonstrable FITC staining was seen for negative controls. These results support the evidence that osteoclast-like cells generated from RAW 264.7 precursor cells express the RANK receptor, and our generated anti-RANK antibody can be used as a potential osteoclast targeting platform. The successful generation of osteoclast-like cells was also confirmed by TRAP staining, which confirmed multinucleated cells with maroon-colored dye deposits due to the presence of TRAP, the enzyme that has been used as a marker of osteoclast function for more than 20 years (35).

To determine the *in vitro* efficacy of the anti-RANK mAb-sCT conjugate on osteoclast activity, we quantified osteoclast activity using a spectrophotometric total culture TRAP activity assay and a mineral resorption assay. TRAP has been shown to be a specific and sensitive indicator of bone resorption (36) 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 (37,38). Osteoclasts, as the bone-resorbing cells of the body, further show the ability to dissolve the mineralized inorganic phase of bone matrix known as hydroxyapatite (39). We found that the use of our conjugate in culture significantly reduced TRAP enzyme activity as compared to sCT alone, and use of the mAb alone also showed inhibition of TRAP enzyme activity. Although the cause for this effect shown by our anti-RANK antibody alone has not been tested, we speculate that in binding the RANK receptor, the antibody may be functioning as a receptor antagonist. That competitive binding would interfere with the interaction of RANKL with RANK, an essential step of osteoclast differentiation. Nonetheless, further antiresorptive and/or anabolic effector functions could be attributed with the development of a targeted delivery strategy to osteoclast cell receptors. We further evaluated if the mAb-sCT conjugate had an effect on the ability of osteoclasts to resorb bone. We cultured osteoclast-like cells on calcium phosphate-coated culture plates in the presence of conjugate, antibody or sCT. We found that both the conjugate and the mAb alone were capable of significantly reducing the resorption of mineral layer as compared to the sCT alone. Then, we performed cyclic AMP assay to confirm that bioactivity of conjugated calcitonin. Human breast carcinoma T47D cells known to express the calcitonin receptor were used for this purpose. Binding of sCT with its receptor activates adenylyl cyclase, an enzyme responsible for the generation of cAMP. Our *in vitro* cAMP assay results confirmed that the modification of sCT by conjugation did not interfere with its ability to bind the calcitonin receptor and trigger its biological activity. We measured the

conjugate to have increased activity over calcitonin alone, and the exact reason for that effect is still not clear. It is likely our conjugation strategy allowed one mole of antibody to become conjugated with greater than one mole of calcitonin. It has been reported that when IgG is treated with a 10 molar excess of Traut's reagent, 3-7 thiol groups are generated, which may result in the attachment of 3-7 PEGylated calcitonin molecules per molecule of IgG.

The results obtained so far have been quite encouraging from the generation of antibody and to the synthesis and *in vitro* evaluation of the conjugate. The conjugate and the antibody alone showed good efficacy in TRAP activity inhibition and resorption pit assay. However, cAMP assay showed that the conjugate served the purpose of delivering active drug cargo. The conjugate showed the ability to bind the calcitonin receptors and generate intracellular cyclic AMP, thus showing an advantage over the antibody alone.

With the advent of novel RANKL-scavenging antibody-based antiresorptive strategies (such as Denosumab– Prolia®, Amgen Inc.), it may further prove efficacious to target the RANK receptor directly with an antibody, in order to successfully antagonize receptor signaling and osteoclast function. However, neither the "scavenging" nor "antagonist" motives of those antiresorptive strategies focus on the targeted delivery of a given drug cargo (such as our anti-RANK mAb-conjugate design) which would function as a universal osteoclast targeting platform.

One potential limitation of our osteoclast targeting platform remains that RANKL-RANK signaling is not entirely restricted to osteoclasts and their precursors, but is known to be involved in the regulation of other cell types. Studies have that RANKL-RANK signaling controls the development of lactating mammary glands in pregnancy, with the highest levels being at day 15.5 in mice (40,41). Several studies that looked at transgenic Rankl-/- and Rank-/- mice found that there was a complete absence of lymph nodes (42). This led to further studies involving patients with an osteoclast-poor form of the autosomal recessive osteopetrosis (ARO), where they found the cause of the disease to be various mutations in RANKL (43). The same research then found that these patients showed no palpable signs of lymph nodes, further strengthening the theory that RANKL-RANK signaling is involved in lymph node formation in humans. RANK is further known to be expressed by monocyte/macrophages and dendritic cells, specialized immune cells programmed to capture and process antigens in the body and present them to naïve T-cells (44). However, the targeting of drug "cargo" to the RANK receptors of those cells may not necessarily result in drug action with those cells if the cargo is carefully chosen not to affect those cell types. For example, it is highly unlikely for any cell (other than the mature osteoclast) to express both the RANK receptor and calcitonin receptor at the same time. Thus, the Boolean "and/or" logic can be applied to the selection of the differential cargo targeting to cellular phenotypes expressing the RANK receptor at any given time. The extent of this limitation remains to be fully determined, however, should not necessarily preclude the further exploration of RANK receptor targeting strategies, which may in turn provide a more comprehensive understanding of RANK signaling and crosstalk with other signaling pathways.

CONCLUSION

This study details the generation of an antibody-mediated osteoclast-targeting platform. This drug-delivery strategy may find utility as "a universal osteoclast-targeting platform" in order to directly target and deliver antiresorptive agents, anti-inflammatory agents, cathepsin K inhibitors, disintegrins, H^+-ATPase inhibitors, and so on, directly to osteoclast cells. We have shown this platform is capable of being employed as an antiresorptive strategy, and our efforts will now center on the *in vivo* evaluation of this targeting strategy.

ACKNOWLEDGMENTS

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