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RESEARCH PAPER

Synthesis, Characterization and Biodistribution Studies of ^{125}I-Radioiodinated di-PEGylated Bone Targeting Salmon Calcitonin Analogue in Healthy Rats

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ABSTRACT

Purpose The objective of this study was to prepare a bisphosphonate (BP) mediated bone targeting *di*-PEGylated salmon calcitonin analogue sCT-2(PEG-BP) as a novel bone targeting pharmaceutical.

Methods HPLC was used for isolation of sCT-2(PEG-BP) from the reaction mixture, followed by determination of possible PEGylation sites by trypsin digestion. Stability of the compound over time, bone mineral affinity using hydroxyapatite, and biodistribution in normal rats after radiolabeling of sCT-2(PEG-BP) or control sCT with ^{125}I was evaluated.

Results PEGylated sCT analogues were synthesized, and sCT-2(PEG-BP) was isolated by HPLC and confirmed by MALDI-TOF and ICP-MS. MALDI-TOF analysis of trypsinized fragments suggested Cys^{1} (or Lys^{11}) and Lys^{18} to be the two PEGylation sites. Bone mineral affinity test showed sCT-2(PEG-BP) or ^{125}I-sCT-2(PEG-BP) exhibited significantly increased bone mineral affinity over sCT or ^{125}I-sCT, respectively. sCT-2(PEG-BP) remained stable for at least 1 month. *In vivo* biodistribution study showed significantly increased bone retention and prolonged plasma circulation time for sCT-2(PEG-BP) compared to the control sCT.

Conclusion Those results support sCT-2(PEG-BP) as a promising new drug candidate for the treatment of resorptive and/or maladaptive bone conditions, such as Osteoporosis, Osteoarthritis, Rheumatoid Arthritis, Paget's disease and bone cancers.

KEY WORDS biodistribution · HPLC · osteoporosis · radio-iodination · sCT-2(PEG-BP)

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ABBREVIATIONS

%ID/g	Percentage injected dose per gram of tissue
AMW	Average Molecular Weight
BP	Bisphosphonate
BSA	Bovine Serum Albumin
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
GFR	Glomerular Filtration Rate
HA	Hydroxyapatite
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
MAL	Maleimide
MALDI-ToF	Matrix-Assisted Laser Desorption/ Ionization Time of Flight
MW	Molecular Weight
NHS	N-Hydroxysuccinimide
RTLC	Radio-Thin Layer Chromatography
sCT	Salmon calcitonin
SD	Standard Deviation
TCEP	Tris(2-carboxyethyl)phosphine

INTRODUCTION

Salmon calcitonin (sCT), a 32 amino acid peptide hormone (MW 3432 Da) has been used as an antiresorptive drug indicated in the treatment of metabolic bone diseases, such as Paget's disease, osteoporosis, and hypercalcemia (1). sCT exerts its therapeutic effect on bone-resorbing osteoclast cells which express sCT receptors on their surface. Like many other peptides, sCT has a short biological half-life (16.9–57.3 min), due to its rapid chemical and enzymatic decomposition via proteolysis in tissues, which restricts its delivery to the desired site of action (2,3).

Furthermore, calcitonin receptors not only exist on osteoclasts cells, but are also present in other organs such as

kidney, brain, lung, placenta, ovaries and in spermatozoa, and thus, the competitive uptake by these tissues may further reduce the delivery of the drug to bone-residing osteoclasts (4–6). The rapid blood clearance of sCT may be partly overcome using PEGylation, which improves sCT stability, increases circulation time in plasma, and reduces renal filtration, resulting in less frequent dosing (7–9). However, PEGylation also extends sCT retention in other tissues presenting sCT receptors. Therefore, in order to achieve the maximum therapeutic benefit, the lack of sCT affinity for bone mineral would need to be addressed, and might be achieved using a derivatization strategy capable of imparting bone mineral affinity (Fig. 1) (10,11).

BP derivatives have very high affinity for hydroxyapatite (HA, $Ca_5(OH)(PO_4)_3$), the predominant mineral of bone. The affinity of BP for calcified tissues has led to synthesis of many compounds for the purpose of both therapy and diagnosis. Alendronate (12), ^{153}Sm lexidronam (13), ^{99m}Tc-bisphosphonates (14–16) and ^{68}Ga-bisphosphonate (17) are a few notable examples. We previously reported the synthesis of a new class of sCT drugs modified with PEG and BP (10,11). The improved pharmacokinetic profiles and bone mineral affinity of sCT, achieved by conjugation with PEG and then subsequent conjugation with Thiol-BP, was considered likely to be the reason for their improved efficacy in conserving bone mass in rats with osteoporosis compared to sCT and sCT-PEG.

PEGylation of the primary amino group at the N-terminus, Lys^{11}, and Lys^{18} amino acids of sCT resulted in a mixture containing *mono*-PEGylated, *di*-PEGylated and *tri*-PEGylated analogues. Using Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry of the reaction mixture, we confirmed the identities of those three analogues, and noted that *di*-PEGylated analogue was the major product. *In vivo* evaluation of the purified conjugate mixture in osteoporotic rats showed an increased efficacy in terms of preserving bone volume, bone mineral density and trabecular micro-architecture compared to commercial sCT (10).

However, in order to further develop this bone therapeutic, it will be necessary to isolate and evaluate individual analogues from the product mixture. Hence, in this study, we aimed to isolate and accurately define the biodistribution and retention of the di-PEGylated sCT-PEG-BP, which was the major component in the product mixture, to aid in its further development as a promising osteoporosis treatment.

MATERIALS AND METHODS

Materials

Salmon Calcitonin (sCT, MW 3432 Da, purity 100.0%; peptide content: 87.0%) was purchased from CALBIOCHEM® (USA). Maleimide PEG NHS Ester (PEG_{3500}, MW 3500) was purchased from JenKem Technology (USA), 2-(3-mercaptopropylsulfanyl)-ethyl-1,1-bisphosphonic acid (Thiol-BP, MW 296) was purchased from Surfactis Technologies (France), Zebra™ Spin Desalting Columns (7 K MWCO) were purchased from Thermo Scientific (Canada), Dialysis Membrane (MWCO 1,000) was purchased from Spectra/Por® (Canada), 0.25% Trypsin-EDTA was from GIBCO® (Canada); Micro BCA™ Protein Assay Kit was from Thermo Scientific (Canada); Radio $Na^{125}I$ was purchased from PerkinElmer (Canada); Bovin Serum Albumin (BSA), HPLC solvents, DMSO,

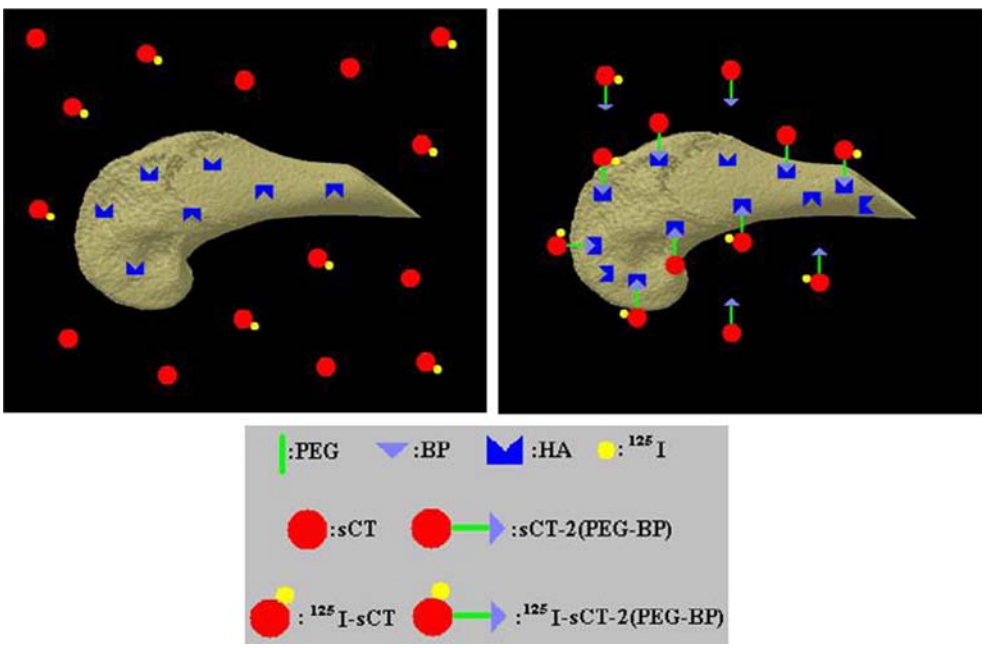

Fig. 1 Schematic picture showing the sCT delivery to bone after conjugation to BP.

Hydroxyapatite (HA), Chloramine-T, $Na_2S_2O_5$, and other reagents were all from Sigma-Aldrich and used without further purification. 6-week-old female Sprague–Dawley rats were purchased from Biosciences (University of Alberta).

Equipment

Vortex-2, Genie® (Canada); Centrifuge, Thermo Electron Corporation (Canada); MALDI-TOF, Bruker Corporation (Canada); Savant Speed Vat concentrator, Emerston Labs, Scarborough (Canada); Spectrophotometer Fluorescence, Cary Eclipse (Canada); ICP-MS, Multicollector Nu Plasma™ (UK); Bio-Tek Instruments EL808 Microplate Reader, BioTek Instruments, Inc. (USA).

HPLC System

HPLC (SHIMADZU (Canada)) was comprised of a LC-20AT pump, SPD-M20A UV detector, SIL-20A auto-sampler, and FRC-10A Fraction Collector; Column (Analytical): Gemini C18 Column, 5 µm, 4.6×250 mm, 110 Å; Solvents: A) 0.1% TFA/H_2O, B) 0.1% TFA/ACN; Gradients: 0–5 min, 35% of B, 5–35 min, 35% to 50% of B, 35.01 min, 35% of B, 40 min, 35% of B; Flow rate: 1 mL/min; UV absorption of products was measured at 215 nm.

Synthesis of sCT-PEG-BP Conjugates

sCT-PEG-BP conjugates were synthesized according to our previous report (10). Briefly, a mixture of 123 µL sCT solution (13.72 mg/mL, DMSO) and 80 µL Maleimide PEG NHS Ester solution (55 mg/mL, DMSO) was vortexed at room temperature for 45 min, followed by the addition of 1.3 mL Thiol-BP solution (25.7 mg/mL, 100 mM phosphate buffer (pH 7.0)) and gently mixed until uniform. The mixture was then stored in a dark place at room temperature for 2 h. Unreacted sCT, PEG, Thiol-BP, and excess salts were removed by passing the reaction mixture through Zebra™ Spin Desalting Columns (7 K MWCO, 0.5 mL/each column; spin at 1,000 g for 2 min). Formation of intermediate sCT-PEG and resultant *mono*-, *di*- and *tri*-substituted sCT-PEG-BP was confirmed by MALDI-TOF and HPLC at 215 nm.

HPLC-Isolation of sCT-2(PEG-BP)

Before HPLC purification, unreacted Thiol-BP (MW 296), DMSO (MW 78.13) and other inorganic solvents were first removed from the mixture by dialysis (Dialysis Membrane MWCO 1,000) against 1 L of 20 mM acetate buffer (pH 5.0) at 4°C with two changes of buffer at 8 h and 60 h. Isolation of *di*-substituted sCT-2(PEG-BP) from the reaction mixture was achieved using HPLC. HPLC conditions are described in the "HPLC System" section of "Materials and Methods" above. Common reverse phase C18 column was employed for isolation of sCT-related analogues with 0.1% TFA/H_2O and 0.1% TFA/ACN as HPLC gradients according to reported protocol (18). The major peak at 20.6 min corresponding to the sCT-2(PEG-BP) was collected for further evaluation. The HPLC solvents (0.1% TFA/H_2O and 0.1% TFA/ACN), were immediately evaporated by speed concentrator under vacuum at room temperature, resulting in a white sCT-2(PEG-BP) powder. This powder was re-dissolved in 20 mM acetate buffer (pH 5.0) and stored in −30°C for further studies. Since analytical reverse phase C18 column was used, isolation only resulted in approximately 20 µg of sCT-2(PEG-BP), therefore the procedure was repeated for ~30 times in order to obtain an adequate amount of sCT-2(PEG-BP). Each HPLC purification step required about 35 min in total.

sCT-2(PEG-BP) Conjugate Characterization

HPLC-purified fraction was concentrated using a centrifuge vacuum concentrator to obtain a white powder. It was dissolved in 20 mM Acetate buffer (pH 5.0). sCT concentration of the conjugate solution was determined using Micro BCA™ protein assay using sCT standard curve constructed as follows. In a 96-well plate, 150 µL of sCT solution (PBS, pH 7.4) of concentrations ranging from 0 to 50 µg/mL was incubated with equal volume of freshly prepared BCA reagent (50:48:2 of reagents A:B:C) at 37°C for 2 h, cooled down to room temperature and the absorbance at 562 nm was measured using microplate reader. Absorbance was plotted against the concentration and the concentration of unknown was determined based on its absorbance value. MALDI-TOF was then used to determine molecular weight of HPLC-isolated fraction. The percentage of BP in each fraction was monitored by ICP-MS based on the phosphorus concentration.

Identification of PEGylation Sites

PEGylation sites of sCT were determined according to previously reported method (18,19). In brief, 20 µL of sCT-2(PEG-BP) at a concentration of 1.5 mg/mL was digested by 20 µL trypsin (0.25% EDTA) in 50 µL of PBS (pH 7.4) at 37°C for 12 h, followed by MALDI-TOF analysis of the digested fragments.

Radio-Iodination of sCT and sCT-2(PEG-BP)

Approximately 16 µg sCT (~4.2 nmol) [or 32 µg, ~2.7 nmol, sCT-2(PEG-BP)] in 70 µL of 20 mM acetate buffer (pH 5.0) was taken in a tube and ~20 µCi (~0.01 nmol) $Na^{125}I$ (mixed with equal volume of 0.1 M AcOH) was added into it followed

by addition of 10 μL Chloramine-T solution (1 mg/mL). The reaction mixture was incubated at room temperature for 30 min, and quenched by adding 400 μL of 0.1 M $Na_2S_2O_5$ solution. Yield was checked using RTLC on Whatman filter paper (grade #1) using Acetonitrile: water (1:1) as mobile phase. Free radioactive iodine was removed from the sample by dialysis using 1 L of 20 mM acetate buffer (pH 5.0) for 60 h with two buffer changes.

In Vitro HA Binding Studies of sCT, sCT-2(PEG-BP), ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP)

In order to evaluate the bone mineral affinity of these compounds, HA binding studies were used according to published protocol (10,20–22). Briefly, 20 μg of sCT-2(PEG-BP) was mixed with 5 mg of HA powder in 750 μL of binding buffer of various concentrations (double-distilled water, 10 mM PBS [pH 7.4], 50 mM PBS [pH 7.4]) and shaken gently at room temperature for 1 h. Similarly, 20 μg of sCT-2(PEG-BP) in corresponding buffers without HA was used as negative control. After 1 h, the suspension was centrifuged at 10,000 g for 5 min, supernatant was transferred to a fresh tube and the HA pellets were washed three times using 750 μL of above corresponding incubation buffers and centrifuged again to separate the supernatant form the pellet.

Supernatants were assayed for unbound drug using fluorescence spectrometer (λ_{Ex} 215 nm, λ_{Em} 305 nm). The percentage of HA binding was calculated as: (Intensity of control − cumulative intensity of supernatants)/Intensity of control × 100%. Similar experiments were carried for equimolar sCT, ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP).

To carry out the HA binding of ^{125}I-sCT or ^{125}I-sCT-2(PEG-BP), 10,000 to 20,000 cpm of ^{125}I-sCT or ^{125}I-sCT-2(PEG-BP) was mixed with 5 mg HA powder and 50 mg bovine serum albumin in 750 μL of binding buffers. The supernatants were separated as previously described and the radioactivity in the supernatant and pellet were determined using Gamma Counter. Percentage of HA binding was calculated as: (HA pellet cpm)/(HA pellet cpm + supernatant cpm) × 100%.

Stability of sCT-2(PEG-BP), ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP)

An aliquot of approximately 0.5 mg/mL of sCT-2(PEG-BP) in 20 mM acetate buffer (pH 5.0) was stored at −30°C. At 3, 7, 10, 15, 30 days; 5 μL of the solution was sampled and analyzed by MALDI-TOF to monitor the stability of the conjugate over time. For the ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP), dialyzed radio-labelled samples were stored at 4°C in dark in 20 mM acetate buffer (pH 5.0) for further stability monitoring and quality control.

Biodistribution Studies

Six-week-old (~275 g) female Sprague–Dawley rats were randomly allocated into ^{125}I-sCT ($n = 9$) and ^{125}I-sCT-2(PEG-BP) groups ($n = 9$). ^{125}I-sCT or ^{125}I-sCT-2(PEG-BP) in an amount containing approximately 10^5 to 10^6 cpm radioactivity was injected via tail vein. Following 3 h, 6 h and 24 h post administration, three rats from each treatment group were euthanized and tissues like bone (diaphysis and epiphysis), kidney, liver, spleen, lung, heart, blood, thyroid, muscle, and a segment of the tail were collected, weighed, and the amount of radioactivity in them was determined as %ID/g. In order to compensate for the radioactive decay of ^{125}I, standard ^{125}I solutions were counted at the same time and included in the calculations.

Statistical Analysis

All data are presented as mean ± SD. Statistical analysis was performed using the two-sided *t*-test by analysis of variance and a P value of less than 0.05 ($P < 0.05$) was considered significant.

RESULTS

Synthesis of sCT-PEG-BP Conjugates and HPLC-Isolation

sCT-PEG-BP mixture was synthesized as previously described (10) and *di*-substituted conjugate sCT-2(PEG-BP) was separated using HPLC (Fig. 2). Unreacted PEG (retention time 6.5 min) and *mono-*, *di*, *and tri-*substituted sCT-PEG-BP analogues (retention time from 17.0 to 24.0 min) were identified. During dialysis procedure, unreacted Thiol-BP and DMSO were both removed from reaction mixture, and un-reacted sCT was washed quickly within 4 min with HPLC solvent front (this was consistent to the previous report (18). The main fraction representing sCT-2(PEG-BP) at 20.6 min was collected and characterized by MALDI-TOF. MALDI-TOF results confirmed the successful synthesis and separation of sCT-2(PEG-BP). Three primary amines at Lys^{11}, Lys^{18} and N-terminal in Cys^1 in sCT (molecular weight 3432) could react with PEG with an average molecular weight (AMW) of approximately 3957 to form *mono* (sCT-1PEG, AMW ~7274), *di* (sCT-2PEG, AMW ~11116), and *tri* (sCT-3PEG, AMW ~14958) substituted sCT-PEG intermediates analogues. In sCT-1PEG, sCT-2PEG and sCT-3PEG, the AMW will be less than sum of MW of sCT and PEG(s) by approximately 115, 230 and 345 D, respectively, when subtracting the MW of the leaving NHS group from the MW obtained by adding the individual MWs of sCT and PEGs. MALDI-TOF spectra of the sCT-PEG reaction mixture are shown in Fig. 3a. The Reaction of these sCT-PEG intermediates with BP (MW 296) resulted in *mono*

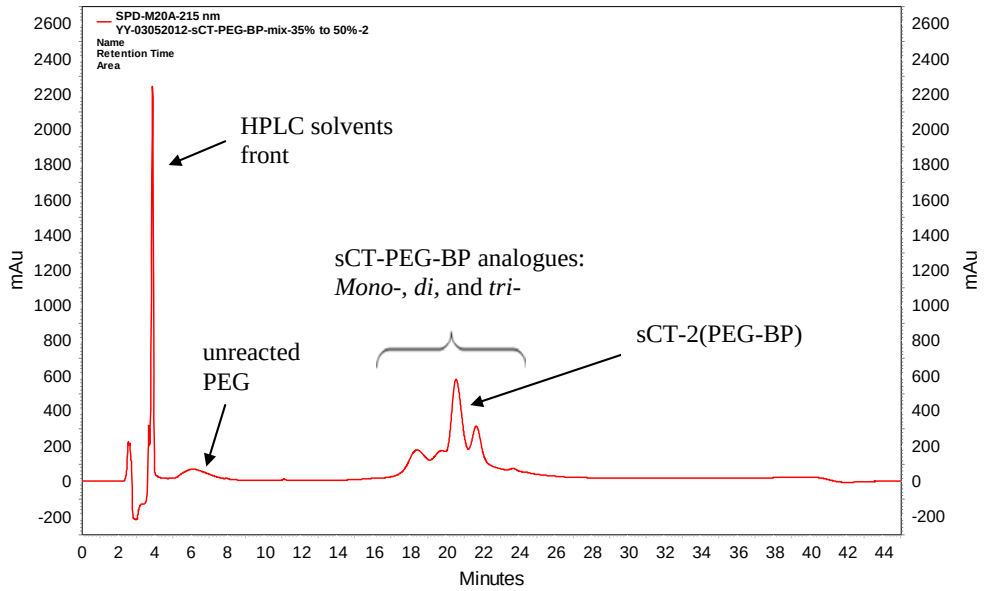

Fig. 2 HPLC peaks of sCT-PEG-BP analogues.

[sCT-1(PEG-BP), AMW ~7570], *di* [sCT-2(PEG-BP), AMW ~11708], and *tri* [sCT-3(PEG-BP), AMW ~15846] substituted bone targeting sCT-PEG-BP analogues. These peaks are shown in Fig. 3b. The peaks close to theoretical values are seen in MALDI-TOF spectra. However, the exact MW was varied by few 100 s D. It was due to the polydispersed nature of PEG. Figure 3c, represents the MALDI-TOF spectra of HPLC isolated *di*-substituted PEGylated bone targeting sCT analogue [sCT-2(PEG-BP)] with an AMW of 11816 (*m/z*). As shown in Fig. 4 sCT-2(PEG-BP) was formed by 2-step reactions: first, two amines in sCT reacted with NHS (leaving group) terminus of NHS-PEG-MAL via nucleophilic substitution, resulting in sCT-2PEG; followed by nucleophilic addition reaction (thiol-ene) with Thiol-BP on MAL terminus as second step. As a result, 3 theoretical species would be expected: sCT-2PEG (i.e. sCT-2PEG did not react with thiol-BP), sCT-2PEG-1BP (i.e. sCT-2PEG reacted with only one thiol-BP), and sCT-2(PEG-BP) (i.e. sCT-2PEG reacted with two thiol-BP). Quantitative ICP-MS was used to confirm mass of phosphorus in each specific species. Calculations showed 2 thiol-BP molecules incorporated onto MAL terminus of the product "sCT-2(PEG-BP)".

PEGylation Sites

In order to determine the PEGylation sites in sCT, trypsin digestion of sCT-2(PEG-BP) was performed. MALDI-TOF analysis of trypsinized fragments revealed two major peaks at 4528 and 9213 (Fig. 5 and Table I); both of the two peaks represented digested "PEG-fragments", due to the polydispersity witnessed on the MALDI-TOF profile. Compared to MW of PEG, higher MWs of fragments implied that conjugation bonds between PEG and amines of sCT were not cleaved by trypsin. Moreover, since (A)MWs of sCT and PEG are 3432 and 3500 respectively, and the MW of fragment (if without PEG) was smaller than sCT, thus MW of single-PEGylated must be no more than 6932, as a result, one fragment with MW of 9213 was double-PEGylated species.

Radio-Iodination

As determined by RTLC technique, radiolabeling yield of ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP) were ~30% and ~20%, respectively. Unbound ^{125}I was removed using dialysis, and purified radiolabeled peptides (^{125}I-sCT and ^{125}I-sCT-2(PEG-BP)) both showed>95% of radiochemical purity (by RTLC), which was qualified for *in vivo* biodistribution studies. The R_f of free ^{125}I, ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP) were 1.0, 0.6, 0.8, respectively.

In Vitro HA Binding Studies

Prior to *in vivo* experiments, *in vitro* bone mineral affinity of radioiodinated sCT-2(PEG-BP) was determined and compared with uniodinated sCT-2(PEG-BP). As shown in Fig. 6a & b, bone targeting sCT analogues had significantly higher bone mineral affinity compared to corresponding non bone targeting sCT. ^{125}I-sCT-2(PEG-BP) had the highest bone mineral affinity at low strength buffer. As determined by the radioactivity measurement, about 40% of ^{125}I-sCT-2(PEG-BP) was bound to HA in 10 mM buffer. However, it had lowest affinity for bone mineral in 50 mM buffer. Also, in case of sCT-2(PEG-BP), bone mineral affinity decreased with increasing buffer concentration.

Stability

To determine the long term storage stability and to ensure that the compounds were not degrading during the course of

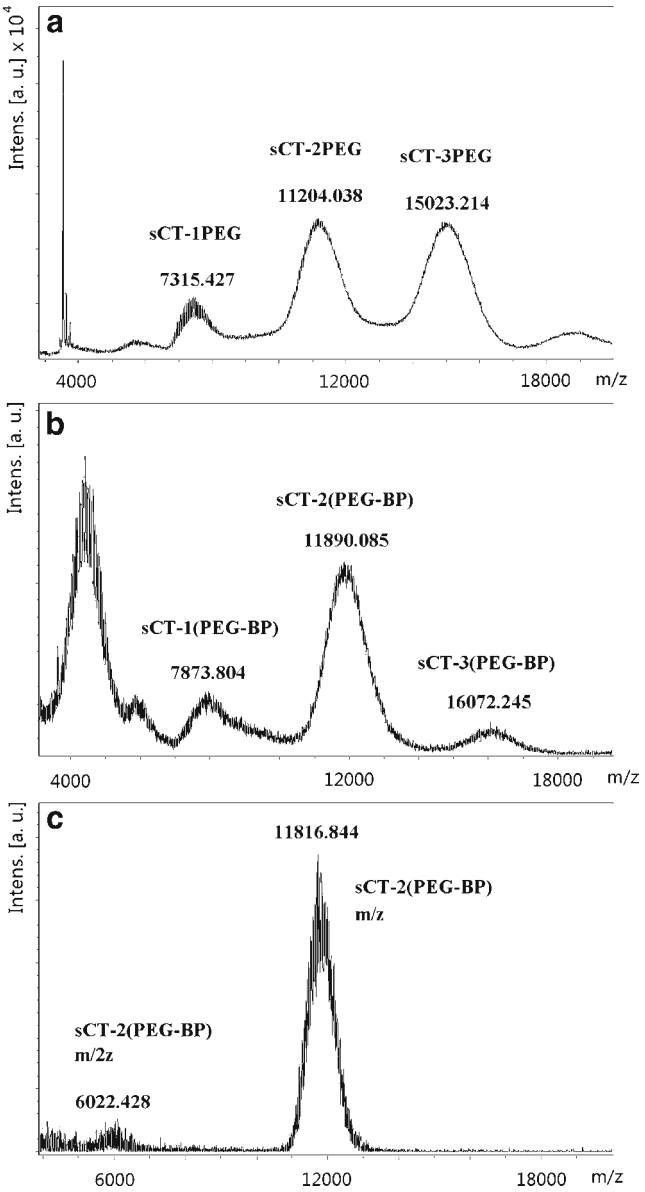

Fig. 3 MALDI-TOF spectra of (**a**) sCT-PEG analogues, (**b**) sCT-PEG-BP analogues, and (**c**) sCT-2(PEG-BP) after HPLC separation.

studies, storage stability of sCT-2(PEG-BP) in 20 mM Acetate buffer pH 5 at −30°C was monitored by taking aliquots from a sample at different intervals up to 1 month post synthesis and purification, and subsequent analysis by MALDI-TOF. There were no degradation peaks observed and all the peaks matched with the initial peaks at the beginning, indicating the stability of these compounds in this storage buffer and conditions (Fig. 7a). The radiolabeling stability of ^{125}I-sCT & ^{125}I-sCT-2(PEG-BP) in the same buffer was monitored by RTLC after radiolabelling, and as shown in Fig. 7b, the high (> 95%) radiochemical purities suggested these two radio-iodinated peptides were stable over time.

Biodistribution

As shown in Fig. 8, *in vivo* biodistribution profile of ^{125}I-sCT in normal rats was altered due to PEGylation and imparted bone mineral affinity. In blood, ^{125}I-sCT-2(PEG-BP) showed significantly higher activity at all time-points compared to ^{125}I-sCT suggesting the longer circulation time and plasma stability of *di*-PEGylated bone targeting sCT. The decrease in the plasma radioactivity over time was due to tissue distribution and partly to elimination from body. However, more than 7 fold higher radioactivities were determined in blood at 24 h for ^{125}I-sCT-2(PEG-BP). Regardless of BP conjugation, both ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP), showed high retentions in thyroid glands and lungs. Significantly, higher radioactivity was seen for ^{125}I-sCT-2(PEG-BP) at 3 and 6 h in lungs and at 24 h in thyroid. As the plasma concentration of ^{125}I-sCT-2(PEG-BP) was higher due to improved blood circulation time, more of it would have reached to highly perfused lung.

Important to mention, kidney accumulation of ^{125}I-sCT was significantly higher than ^{125}I-sCT-2(PEG-BP) for all time points. Increased accumulation of ^{125}I-sCT was found in kidney after 3 h (%ID/g: 145.6±19.5), and 6 h (%ID/g: 152.7±18.1), whereas, kidney retention of ^{125}I-sCT-2PEG-BP was dramatically decreased after BP conjugation to less than half of ^{125}I-sCT at 3 h (%ID/g: 60.5±6.8) and 6 h (%ID/g: 62.2±10.1).

As expected, bone accumulation of *di*-PEGylated bone targeting sCT was higher than sCT, as shown in Fig. 9a & b. The femur was dissected from the muscle, and the epiphysis and diaphysis were separated and counted separately. Bone accumulation was correlated with plasma concentration. Decreased accumulation with time was due to decreased plasma concentration. The most significant difference in bone absorption appeared at 3 h post administration with almost 2 times more bone uptake for ^{125}I-sCT-2(PEG-BP). Higher bone accumulation of ^{125}I-sCT-2(PEG-BP) than ^{125}I-sCT for the same time points indicated successful delivery of the sCT to bone by improving its stability, increasing the circulation time and also by reducing kidney elimination as a result of PEGylation and imparting bone mineral affinity through BP conjugation.

DISCUSSION

The synthesis and isolation of *di*-PEGylated bone targeting sCT analogues was successfully achieved. Our previous *in vivo* efficacy studies of sCT, sCT-BP and sCT-PEG-BP analogues in ovariectomized rats showed that both sCT-BP (without PEGylation) and sCT-PEG-BP preserved trabecular bone, however, that effect was more pronounced in the case of sCT-PEG-BP (10). Therefore, the major focus of the current article was to accurately define the biodistribution of the PEGylated

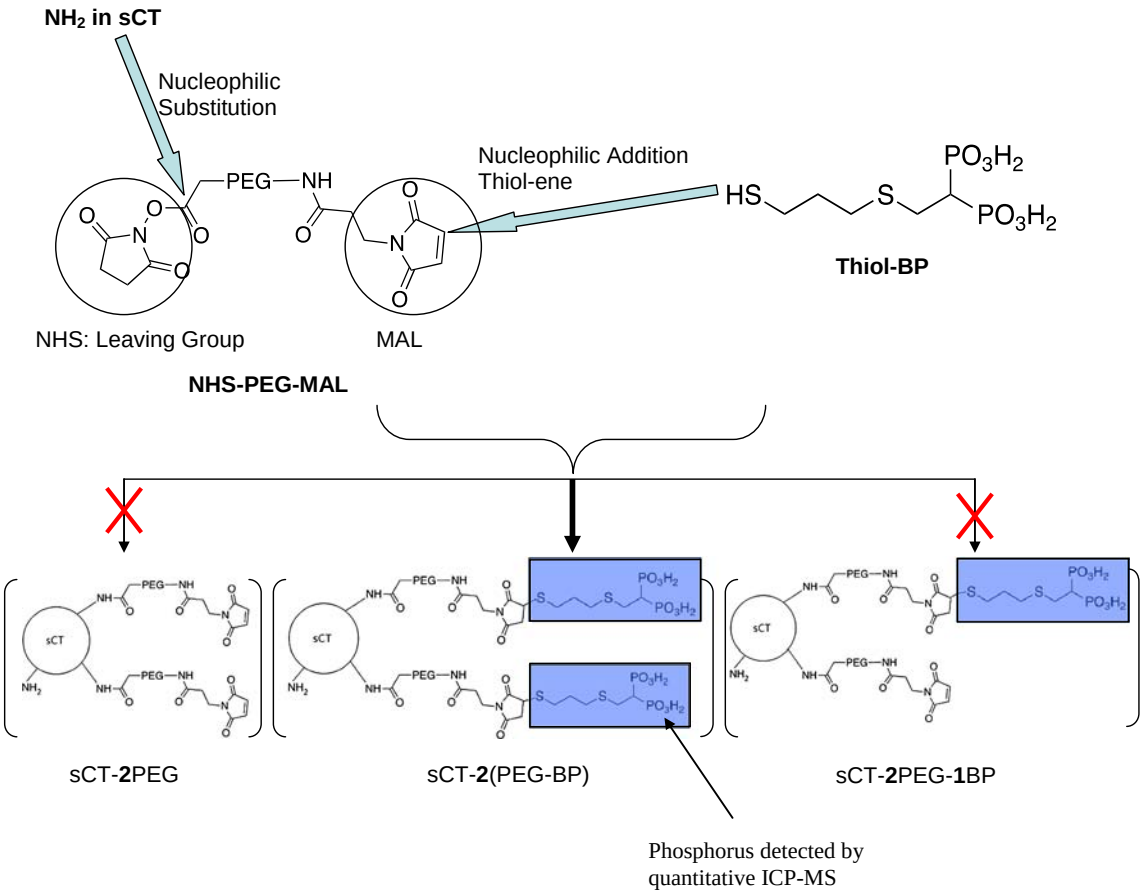

Fig. 4 Chemical reaction scheme of sCT-2(PEG-BP): bifunctional NHS-PEG-MAL first reacted with amines of sCT (on NHS) and then with Thiol-BP (on MAL), resulting in 3 possible species (sCT-**2**PEG, sCT-**2**(PEG-BP), sCT-**2**PEG-**1** BP), which could be distinguished directly by quantitative ICP-MS analysis on detection of phosphorus element, since these species had different phosphorus content.

principal conjugate fraction sCT-2(PEG-BP), to aid in its further development as a promising osteoporosis treatment. As shown on HPLC spectra, sCT-PEG-BP conjugates had similar retention times, which could be due to their similar structures and lipophilicity. As previously reported (23), sCT has a disulfide bridge in Cys^{1}-Cys^{7}, and under certain conditions with reducing agent such as TCEP or DTT, that disulfide bridge could be opened for thiol-ene reaction (24,25). However, under mild reaction conditions without reducing agent, disulfide bridge in Cys^{1}-Cys^{7} could not be cleaved, and hence there is little possibility for another disulfide bridge to form between Cys and Thiol-BP. As a result, after the initial substitution reaction between sCT and NHS of PEG (resulting in sCT-PEG), the sulfur on Cys did not compete with sulfur on Thiol-BP for Thiol-ene reaction with MAL of sCT-PEG (10). Moreover, in order to avoid possible competition between Cys and Thiol-BP on reaction with MAL of sCT-PEG at the second step, a large amount of Thiol-BP (molar ratio: Thiol-BP to sCT 230:1) was added to reaction solution. If Cys of sCT had reacted with MAL of sCT-PEG, a feasible by-product of "sCT-PEG-sCT" should have been formed which does not contain any phosphorus element. However, that was not the case as phosphorus was detected by ICP-MS in the product.

We have previously reported this sCT-PEG-BP conjugate (as a heterogeneous mixture of mono-, di- and tri-PEGylated products), and showed the significantly improved efficacy of the PEGylated mixture in osteoporotic rats (10). Clearly, it

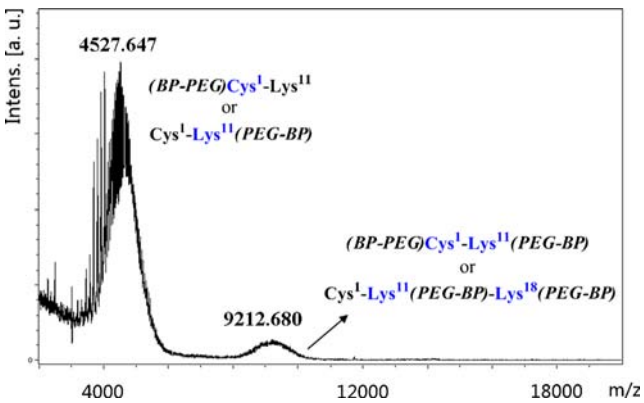

Fig. 5 MALDI-TOF spectra of trypsin digested sCT-2(PEG-BP).

Table I Amino Acid Sequence of sCT and Structure of sCT-2(PEG-BP). Blue Colored Amino Acids ("Cys^{1}", "Lys^{11}" and "Lys^{18}") are the Possible Attachment Sites of PEG

Fragments	Calculated Mass (Da)	MALDI-TOF Mass (Da)
Cys^{1}-Lys^{11}	1122	N/A
Leu^{12}-Lys^{18}	854	N/A
Leu^{19}-Arg^{24}	777	N/A
Thr^{25}-Pro^{32}	733	N/A
(BP-PEG)-Cys^{1}-Lys^{18}-***(PEG-BP)*** or Cys^{1}-Lys^{11}***(PEG-BP)***-Lys^{18}-***(PEG-BP)***	9270	9213
(BP-PEG)-Cys^{1}-Lys^{11} or Cys^{1}-Lys^{11}-***(PEG-BP)***	4622	4528

would be interesting to examine efficacy between the 3 bone-targeting analogues of sCT-PEG-BP. However, as the conjugation reaction resulted in predominantly sCT-2(PEG-BP), we elected to isolate that fraction in an attempt to clearly define its biodistribution and excretion following radiolabeling and bolus intravenous injection. Due to the poor yields of the other 2 analogues, they would likely not be pursued for pharmaceutical production further, and detailed pharmacokinetic analysis would likely only show different retention at the bone (marginally longer retention with tri-conjugates and shorter retention with mono-conjugates), although that remains to be determined.

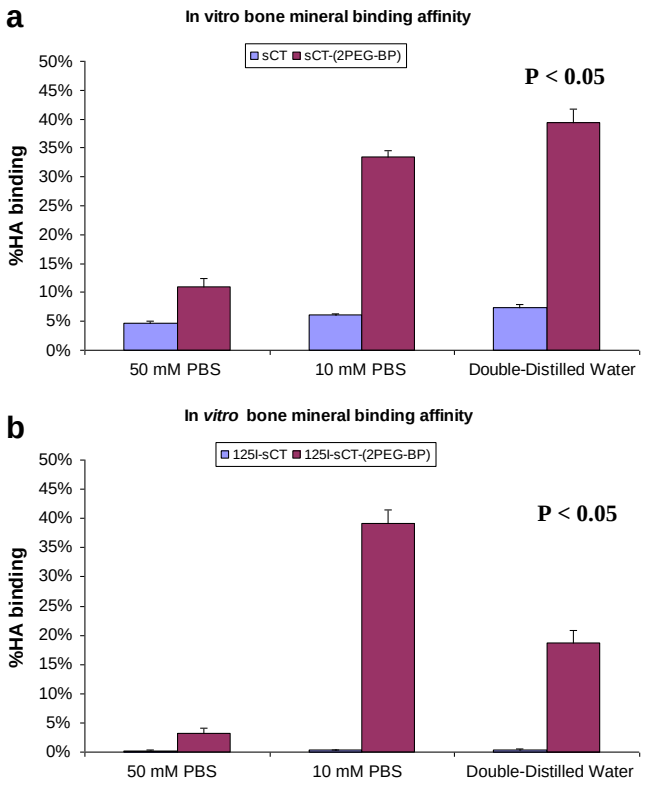

Fig. 6 *In vitro* bone mineral binding affinity of (**a**) sCT and sCT-2(PEG-BP) (**b**) ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP) measured in double-distilled water, 10 mM and 50 mM of PBS, respectively; For binding comparison of ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP), BSA was added as protein background.

According to the report (18), three potential sites for PEGylation exist in sCT: the N-terminus (Cys^{1}), Lys^{11} and Lys^{18}. Thus, there should be three possibilities for *di*-substituted products naming sCT(Cys^{1}, Lys^{11})-2(PEG-BP), sCT(Cys^{1}, Lys^{18})-2(PEG-BP), sCT(Lys^{11}, Lys^{18})-2(PEG-BP). Trypsin digestion was performed to determine PEG-BP conjugation sites. Trypsin selectively cleaves the peptide chains at the carboxyl side of Lys or Arg as shown below. Hence, for sCT the cleavage sites are the bonds between **Lys^{11}**−/−Leu^{12}, **Lys^{18}**−/−Leu^{19} and Arg^{24}−/−Thr^{25}. For clarity, the amino acid sequence of sCT is shown below:

H_2N-**Cys^{1}**-Ser^{2}-Asn^{3}-Leu^{4}-Ser^{5}-Thr^{6}-Cys^{7}-Val^{8}-Leu^{9}-Gly^{10}-**Lys^{11}−/−Leu^{12}**-Ser^{13}-Gln^{14}-Glu^{15}-Leu^{16}-His^{17}-**Lys^{18}−/−Leu^{19}**-Gln^{20}-Thr^{21}-Tyr^{22}-Pro^{23}-**Arg^{24}−/−Thr^{25}**-Asn^{26}-Thr^{27}-Gly^{28}-Ser^{29}-Gly^{30}-Thr^{31}-Pro^{32}-COOH

Trypsin digestion of sCT-2(PEG-BP) was performed to determine the PEGylation sites in sCT. As Fig. 5 and Table I showed, peak at 4528m/z approximately equals to the sum of MWs of ***(BP-PEG)-Cys^{1}***-Lys^{11} or Cys^{1}-***Lys^{11}-(PEG-BP)*** (4622), thus one of the PEGylation site could be either Cys^{1} or Lys^{11}, but not both. This information was very important as it confirmed that the PEGylation had not happened in both Cys^{1} and Lys^{11}. However, the other peak at 9213m/z could represent the fragment of ***(BP-PEG)-Cys^{1}***-Lys^{11}-***Lys^{18}-(PEG-BP)*** (9270) or Cys^{1}-***Lys^{11}(BP-PEG)-Lys^{18}-(PEG-BP)***, confirming that the second PEGylation site was Lys^{18}. In any case, a *di*-substituted product was the major product of the conjugation reaction. Although, the higher reactivity and selectivity of Lys^{11} and Lys^{18} primary amines ($-NH_2$) towards NHS in NHS-PEG-MAL, strongly suggests di-substitution at these amino acids, this does not preclude the possible reaction of Cys^{1}. Hence, to confirm Cys^{1} or Lys^{11} substitution, a method has to be devised to isolate the ***(BP-PEG)-Cys^{1}***-Lys^{11} or Cys^{1}-***Lys^{11}-(PEG-BP)*** peptides and to further fragment them, so that the resultant PEGylated chain would have either Cys^{1} or Lys^{11} but not both. MALDI-TOF analysis of these chains would then help to determine the actual PEGylation site. However, this was out of our objective for this paper.

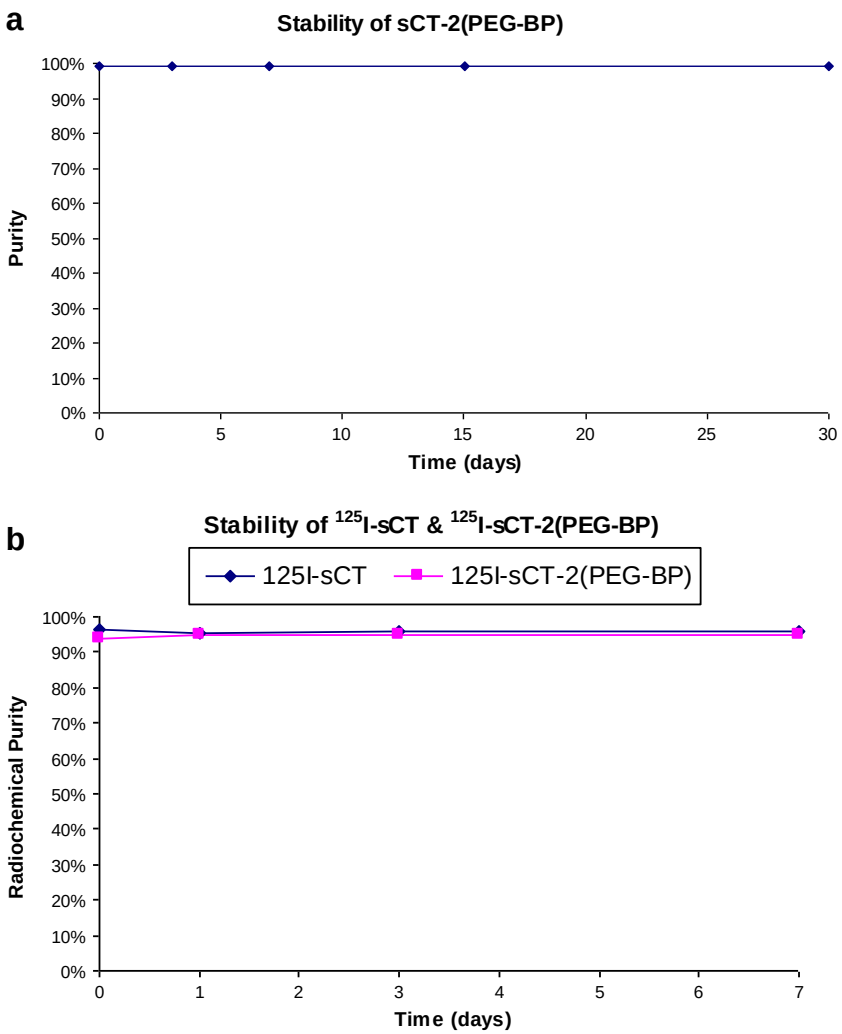

Fig. 7 Stability studies in 20 mM acetate buffer (pH 5.0): (**a**) MALDI-TOF spectra of sCT-2(PEG-BP) at 3, 7, 15, 30 days; (**b**) radiochemical purity (RTLC) of ^{125}I-sCT & ^{125}I-sCT-2(PEG-BP): at 0, 1, 3, 7 days in 20 mM acetate buffer (pH 5.0).

There is only one tyrosine (Tyr^{22}) available in sCT-2(PEG-BP) for radio-iodination, which resulted in a low yield of ^{125}I-sCT-2(PEG-BP). The addition of one iodine atom in sCT-2(PEG-BP) was not differentiated by MALDI-TOF due to

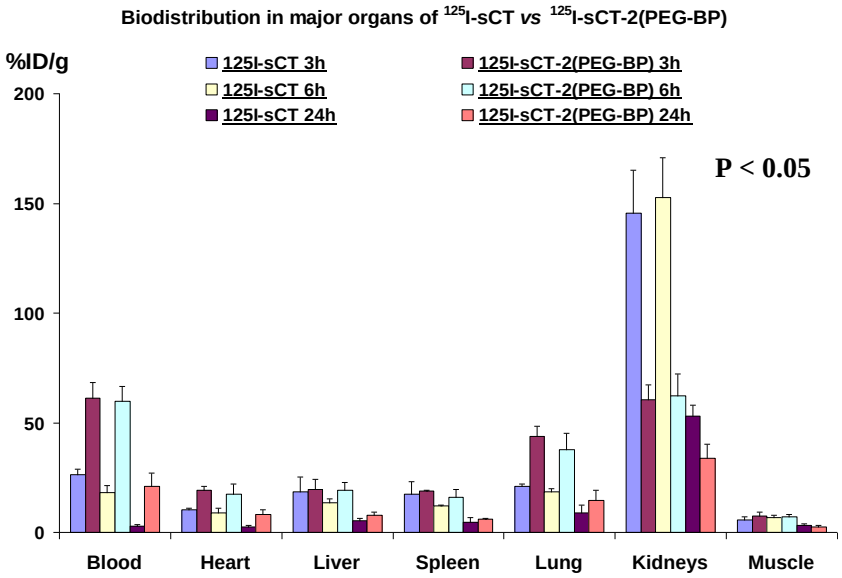

Fig. 8 Biodistribution of ^{125}I-sCT & ^{125}I-sCT-2(PEG-BP) in major tissues of normal rats, at different time points (3 h, 6 h, 24 h), respectively.

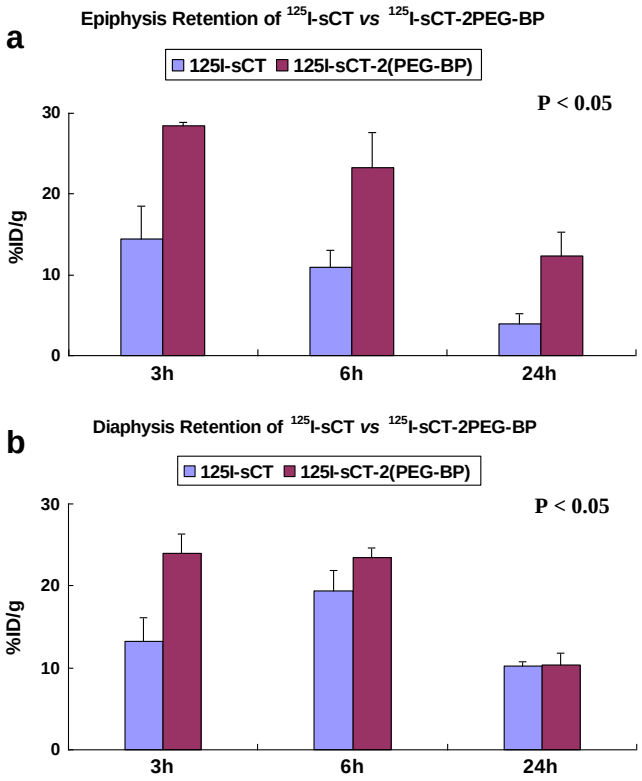

Fig. 9 Bone retention at different time points (3 h, 6 h, 24 h): (**a**) epiphysis retention of ^{125}I-sCT & ^{125}I-sCT-2(PEG-BP); (**b**), diaphysis retention of ^{125}I-sCT & ^{125}I-sCT-2(PEG-BP).

the polydispersed nature of the NHS-PEG-MAL, which contained mixtures of different lengths of -$[CH_2$-CH_2-$O]_n$-.

The HA binding affinity of sCT-2(PEG-BP) in 10 mM PBS and double distilled water was very similar. However, it was significantly decreased by increasing the buffer concentration to 50 mM. On the other hand sCT had a similar non-specific binding to HA in all media. Significant bone mineral affinity of ^{125}I-sCT-2(PEG-BP) and sCT-2(PEG-BP) in 10 mM pH 7.4 PBS (i.e., with the same osmolality as blood) indicated that *di*-PEGylated bone targeting sCT had a potential to bind to bone mineral surface *in vivo*. In HA binding affinity studies of ^{125}I-sCT-2(PEG-BP) and ^{125}I-sCT, BSA was used to block the non-specific binding of ^{125}I-sCT-2(PEG-BP) or ^{125}I-sCT to HA. As seen from Fig. 6a and b, by using BSA, non-specific HA binding of sCT decreased from ~5% to <0.5% in the case of ^{125}I-sCT. Although, the presence of BSA may influence interaction of BP in ^{125}I-sCT-2(PEG-BP) with HA, binding affinity of ^{125}I-sCT-2(PEG-BP) was significantly higher (more than 78 times compared to ^{125}I-sCT) in 10 mM PBS solution, indicating that a specific bone mineral affinity was imparted to *di*-PEGylated sCT through BP conjugation. Since 10 mM PBS with BSA mimics plasma better than 10 mM PBS alone, it is highly probable that sCT-2(PEG-BP) would exhibit bone mineral affinity *in vivo*.

Further, in all three types of buffers (*dd*H_2O, 10 mM PBS, 50 mM PBS) sCT/^{125}I-sCT demonstrated non-specific binding to HA, especially with BSA as protein background; HA binding affinities of sCT-2(PEG-BP) and ^{125}I-sCT-2(PEG-BP) were similar in 50 mM and 10 mM of PBS, respectively; they had the same trend in graphs that higher concentration of buffer led to weaker binding affinity; however, in double-distilled water, binding affinity of ^{125}I-sCT-2(PEG-BP) decreased compared to sCT-2(PEG-BP), that might be due to addition of BSA: HA-BP interaction was a charge-based binding, in double-distilled water, sCT-2(PEG-BP) showed highest binding affinity without interference of phosphate from PBS; however, once BSA (mg/mL) was added in, it might change electrical properties of micro-environment around ^{125}I-sCT-2(PEG-BP) (pg/mL) without PBS, as a result, in double-distilled water, ^{125}I-sCT-2(PEG-BP) did not show as high binding affinity as in PBS solutions. For the use of BSA to simulate *in vivo* HA binding behaviors of sCT and sCT-2(PEG-BP), it could only be added to radiolabelled samples since BSA was not radioactive and once it was added to sCT or sCT-2(PEG-BP) solutions, HA binding affinity of peptides by fluorescence spectra (21) or BCA assay (10) or other methods based on their spectroscopic properties (BSA and sCT had a lot of same amino acids fragments) would be influenced significantly.

With respect to the conjugate stability, MALDI-TOF spectra of samples of sCT-2PEG-BP and ^{125}I-sCT-2PEG-BP were eluted at different time points and compared to their respective spectra at time zero. That suggested the conjugates were stable in 20 mM Acetate buffer pH 5 at 4°C for at least 1 month, likely due to the improved stability imparted through PEGylation.

Biodistribution studies were performed in normal rats to minimize the effect of adaptive bone pathology on the bone mineral affinity of ^{125}I-sCT and ^{125}I-sCT-2(PEG-BP). Significantly higher bone accumulation of ^{125}I-sCT-2(PEG-BP) compared to ^{125}I-sCT confirmed the imparted bone mineral affinity of *di*-substituted sCT. Various tissues were collected at 3, 6, 24 h post IV injection of the compounds. Higher uptake by bone may potentially be the result of: 1) BP conjugation, which imparted bone mineral affinity, and 2) PEGylation, which improved plasma circulation and stability. After PEGylation, sCT-PEG-BP was shown to be more stable than sCT by circular dichroism (10), and since sCT as a peptide hormone has adequate stability in plasma to elicit biological effect, we did not test plasma stability further in this study.

In our study, unmodified sCT showed no specificity for long bone epiphyses over the diaphyses. A similar finding was also reported for bone in an imaging study, following the administration of radio-iodinated sCT (26). Since BP moieties readily accumulate at regions of mineralizing bone, imparting BP mediated bone mineral affinity to *di*-PEGylated sCT resulted in the higher affinity of ^{125}I-sCT-2(PEG-BP) for the

epiphyses, that region of bone undergoing active remodeling at growth plates. In addition to BP conjugation, PEGylation played a role in delivering sCT to bone by prolonging its blood circulation. As a result, the blood clearance of sCT was retarded, and thus, a higher concentration of BP conjugate was made bioavailable for bone targeting.

Since sCT receptors are also present in kidneys, and as sCT is primarily excreted by glomerular filtration, a very high concentration of non-targeting ^{125}I-sCT was observed in kidneys at all the time points, compared to the bone-targeting ^{125}I-sCT-2(PEG-BP) analogue. That strongly suggested enhanced bone uptake as well as stability, improved plasma circulation time and reduced kidney accumulation of sCT conjugates following conjugation with bone mineral targeting agent BP and PEGylation, which may find utility in patients with kidney disease and poor GFR. As previously reported (27), kidney accumulation of ^{125}I-sCT-PEG without BP conjugation was even higher than ^{125}I-sCT, indicating PEGylation alone could not inhibit sCT from reacting with sCT receptors on kidneys. However, our results showed, at 3 h and 6 h, retention of ^{125}I-sCT-(PEG-BP) in kidneys were less than half of ^{125}I-sCT, suggesting improved bone accumulation. Although Shin *et al.* did not report bone-targeting affinity or *in vivo* bone uptake, it has been reported by our group that sCT-PEG did not improve bone binding affinity of sCT (10).

Elemental Iodine normally accumulates in the thyroid, for the biosynthesis of tri-iodothyronine and thyroxine (28–30). On the other hand, during circulation of ^{125}I-labelled compounds *in vivo*, de-iodination would occur and unbound ^{125}I could be absorbed by the thyroid. Thus, a higher radioactivity for ^{125}I-sCT-2(PEG-BP) in thyroid compared to ^{125}I-sCT was likely due to improved blood circulation time, subsequently higher opportunity of accumulation of unbound ^{125}I in thyroid, which is a very common phenomenon.

Muscle was collected and counted for radioactivity to confirm that the radioactivity source was bone and not muscle, as shown in Fig. 10a & b, bone (epiphysis and diaphysis) to muscle ratios of ^{125}I-sCT-2(PEG-BP) were all higher than ^{125}I-sCT, especially for epiphysis/muscle ratio, at 6 h and 24 h ^{125}I-sCT-2(PEG-BP) indicated approximate 2 and 3 times of ^{125}I-sCT, respectively. Since tail vein injection was used for this study, tail segments were collected to confirm that radiolabelled peptides were successfully administrated to rats, but not retained in tissues at the injection site alone.

Bisphosphonates have been reported to cause BP related osteonecrosis (BRON) of the jaw in contemporary clinical applications (31,32). However, the thiol-BP in our study was used only as a vehicle for delivery of sCT to bone, with little to no efficacy as an anti-resorptive drug as this quite small amount of BP was much lower (~1000 times) than an effective clinical dose. Accordingly, we believe there is little chance our BP conjugates will result in BRON of the jaw, as the BP moiety used are simple non-nitrogen containing compounds. Most reports of BRON have occurred with powerful intravenous nitrogen-containing BPs, such as zoledronate and pamidronate.

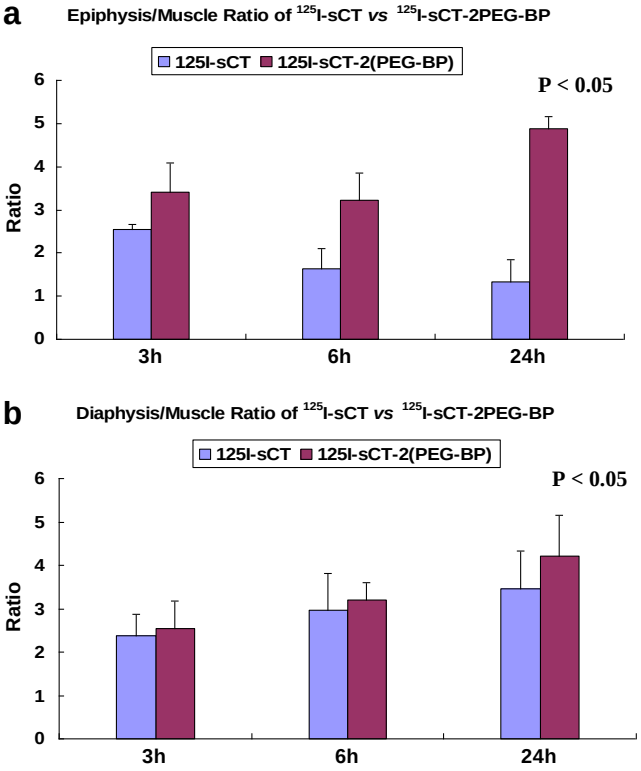

Fig. 10 Bone/Muscle ratio at different time points (3 h, 6 h, 24 h): (**a**) epiphysis/muscle ratio of ^{125}I-sCT & ^{125}I-sCT-2(PEG-BP); (**b**) diaphysis/muscle ratio of ^{125}I-sCT & ^{125}I-sCT-2(PEG-BP).

CONCLUSIONS

In summary, BP mediated bone targeting *di*-PEGylated sCT analogue was synthesized and isolated. That sCT analogue showed significantly higher plasma circulation and *in vitro*/*in vivo* bone mineral affinity, confirming enhanced bone targeting and retention. Our results support sCT-2(PEG-BP) use as a potential new drug candidate for further evaluation to determine its utility in resorptive bone disorders such as Osteoporosis and Paget's disease.

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