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Research Paper

Design, synthesis, characterization and in-vivo activity of a novel salmon calcitonin conjugate containing a novel PEG-lipid moiety

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Abstract

Objectives The aim of the study was to explore (1) the synthesis of a novel poly(ethylene glycol) modified lipid (PEG-lipid, PL) containing a chemically active tri-block linker, ε-maleimido lysine (Mal), and its conjugation with salmon calcitonin (sCT), and (2) the biophysical properties and activity of the resulting conjugate, Mal-PL-sCT, relative to the control, 2PEG-Mal-sCT, which comprises sCT conjugated with α-palmitoyl-*N*-ε-maleimido-L-lysine at cysteine 1 and cysteine 7, and PEG moieties at lysine 11 and lysine 18 via a conventional stepwise method.

Methods The PEG-lipid was obtained by condensing palmitic acid derivative of ε-maleimido lysine with methoxy poly(ethylene glycol) amine. Under reductive conditions, the PEG-lipid readily reacted with sCT to yield the resultant compound, Mal-PL-sCT.

Key findings Dynamic light scattering analyses suggested that Mal-PL-sCT and 2PEG-Mal-sCT exhibited robust helical structures with a high tendency to aggregate in water. Both compounds were more stable against intestinal degradation than sCT, although Mal-PL-sCT was less stable than 2PEG-Mal-sCT. However, 2PEG-Mal-sCT did not possess hypocalcaemic activity while Mal-PL-sCT retained the hypocalcaemic activity of sCT when it was subcutaneously injected in the rat model. Multiple functional groups may be conjugated to a peptide via a tri-block linker without the risk of obliterating the intrinsic bioactivity of the peptide.

Conclusions The resultant novel PEG-lipid has a potential role to optimize protein and peptide delivery.

Keywords bioconjugation; calcitonin; lipidization; PEGylation; tri-block

Introduction

PEGylation has evolved to become an established method for enhancing the enzyme stability of peptide drugs.[1] The PEG chains emanating from a peptide molecule have been shown to bind strongly with water molecules to form an effective shield against enzyme approach. PEGylation is also employed to prolong the systemic circulating time of a peptide, as the significant increase in molecular size brought about by PEGylation impeded excretion by renal filtration.[2] More recently, lipidation was also found to potentiate both the in-vivo[3,4] and in-vitro activities of peptide drugs.[5,6] Lipid conjugated to biopharmaceutical molecules may facilitate access across absorptive membranes by hydrophobic interactions,[7] and create a depot effect by non-specific binding to membrane lipids at the local administration site and to circulating serum proteins.[7,8]

Several publications have combined strategies to develop PEG-lipid conjugated systems for the delivery of pharmaceuticals. PEG-lipids have been widely applied in stealth liposomes to prolong systemic circulation,[9,10] and are found to be particularly useful for the encapsulation of poorly water-soluble hydrophobic bioactives.[11,12] Immunological PEG-lipids, in which a lipid is conjugated to an antibody via a PEG spacer, have been designed for incorporation into stealth liposomes for targeting to cancer cells[13] and across the blood–brain barrier.[14] More recently, small amphiphilic oligo-PEG fatty acid peptide conjugates, which use a fatty acid spacer to link an oligo(ethylene glycol) group to peptide drugs via a non-reversible amide bond, were shown to enhance the oral absorption of insulin,[15,16] human brain natriuretic peptide[17,18] and salmon calcitonin.[19]

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Despite the many publications extolling the benefits of PEGylation, lipidation and combined PEG-lipid systems for drug delivery, there is not as yet a published study on the benefit of PEG-lipid on peptide drug delivery. In addition, most studies on PEG-lipid systems have used either the PEG[14] or lipid[18] chain as a linker to tether the other components of the system. There has been no discussion on the possibility and general methods of synthesis of PEG-lipid peptides in which both the PEG and lipid moieties are exposed to synergize their respective potentials for optimizing peptide delivery.

We have previously synthesized several novel lipid conjugates of salmon calcitonin (sCT), a 32-amino acid peptide with a proline amide at residue 32 and an N-terminal disulfide bridge between cysteine 1 and cysteine 7.[20–22] Mal-sCT, consisting of sCT conjugated with palmitic acid via non-reversible thioether bonds at cysteine 1 and cysteine 7, was shown to produce comparable hypocalcaemic activity to sCT *in vivo*.[20] Further studies led to the development of 2PEG-Mal-sCT, which comprises Mal-sCT co-conjugated with PEG 5000 at Lys11 and Lys18.[23] 2PEG-Mal-sCT showed significant stability against intestinal enzymes, but the multiple conjugation sites in the peptide also resulted in a complete loss of hypocalcaemic activity, in line with the high risk of compromised peptide bioactivity associated with multiple conjugation sites observed in other studies.[24–26] On this basis, we hypothesized that sCT would maintain its bioactivity if the PEG and lipid conjugations were restricted to solely the cysteine 1 and cysteine 7 residues.

In consideration of this, a novel strategy was developed, which might also be applicable to other PEGylated or lipidized drug molecules. This strategy employed the ε-maleimido-lysine molecule as an anchor linker to simultaneously bond the sCT, PEG and lipid together to form a tri-block system (Figure 1). The anchor linker ensured all three molecules remained exposed for biological interactions so that their individual pharmacological advantages could continue to be realized in the final compound. This paper explores the feasibility of synthesis, followed by the evaluation of the characteristics and bioactivity of sCT conjugated with the novel PEG-lipid, with 2PEG-Mal-sCT (Figure 2b) as the control sCT conjugate. To our knowledge, this is the first report of a drug co-conjugated with both PEG and lipid simultaneously by a single bond. This conjugation strategy will expand further the current area of polymer therapeutics in drug delivery.

Materials and Methods

sCT was purchased from Unigene Laboratory (Boonton, NJ, USA). mPEG propylamine (mPEG-amine) (MW 5000, SUNBRIGHT MEPA-50H) was from NOF Corporation (Tokyo, Japan). *N*-α-(tert-butoxycarbonyl)-L-lysine (α-Boc-lysine), palmitic acid *N*-succinimidyl ester (Pal-Suc), methylpyrrolecarboxylate, tris(2-carboxyethyl) phosphine (TCEP), *N*,*N*′-methylenebis(acrylamide) (electrophorosis grade), trifluoroethanol (TFE), dicyclohexylcarbodiimide (DCC) and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St Louis, MO, USA). Tris(hydroxymethyl)methyl-glycine (Tricine) was from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Polyacrylamide gel (40% stock solution with 38 : 1 w : w ratio of acrylamide to *N*,*N*′-methylene bis(acrylamide)) was from Bio-Rad (Hercules, CA, USA). MicroBCA Protein Assay Kit was from Pierce Biotechnology (Rockford, IL, USA). Acetonitrile and iso-propanol of high pressure liquid chromatography (HPLC) grade were supplied by Fisher Scientific (Irvine, CA, USA). Milli-Q water was used in the mobile phase for HPLC analysis.

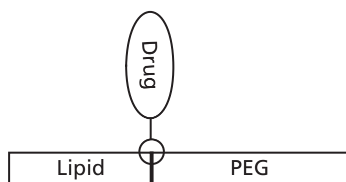

Figure 1 Schematic representation showing a novel strategy to conjugate PEG and lipid to a drug

Synthesis

Mal-PL-sCT

Mal-PL-sCT was synthesized according to the scheme shown in Figure 2a. Preparation of the ε-maleimido lysine derivative of palmitic acid (Pal-Lys-Mal, **I**) has been described in our previous publication.[20] Briefly, Pal-Lys-Mal was synthesized by reacting palmitic acid *N*-succinimidyl ester with ε-maleimido lysine, which in turn was generated from a reaction of α-Boc-lysine and methylpyrrolecarboxylate, with subsequent deprotection of the Boc group.

To synthesize the PEGylated Mal-Lys-Pal (PEG-lipid) (**II**) (Figure 2a), α-palmitoyl-ε-maleimido lysine (11.95 mg, 25.7 μmol), *N*-hydroxy succinimide (1.14 mg, 9.91 μmol) and mPEG-amine (117.56 mg, 22.6 μmol based on an average molecular weight (MW) of 5197) were stirred vigorously with 131.5 mg (637 μmol) of DCC in 1.5 ml of CH_2Cl_2 for 18 h under argon protection. The reaction mixture was filtered and the clear solution was eluted in a silica-gel column (10 ∞ 250 mm) with $CHCl_3/CH_3OH$ in stepwise gradients from 100 : 1 to 100 : 5. Those fractions containing the pure target were pooled together for solvent evaporation. The yield was 96.74 mg (17.1 μmol; 75.8%). A sample from the pooled fractions was developed in silica-gel thin layer chromatography (TLC) (Merck, Darmstadt, Germany), with $CHCl_3$: CH_3OH (10 : 1 v/v) as mobile phase and mPEG-amine as reference, and visualized after coloration with iodine vapour.

The target was identified using the BRUKER NMR-300 with $CDCl_3$ as solvent, while its MW was confirmed with Voyager-DE STR matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Applied Biosystems, CA, USA); m/z data of samples were automatically acquired in the linear mode by using the linear method (accelerating voltage 20 kV, laser intensity 2400–2600, acquisition mass range 1000 ~ 25 000). Samples (0.5 μl, approximately 20 μg/ml) dissolved in a solvent containing 50% of acetonitrile/isopropanol in water with 0.1% of TFA were spotted on a 96 ∞ 2 well plate with 0.5 μl of a saturated solution of sinapinic acid in ACN. Samples were analysed after they were dried, and the final spectrum was the average of at least 200 scans.

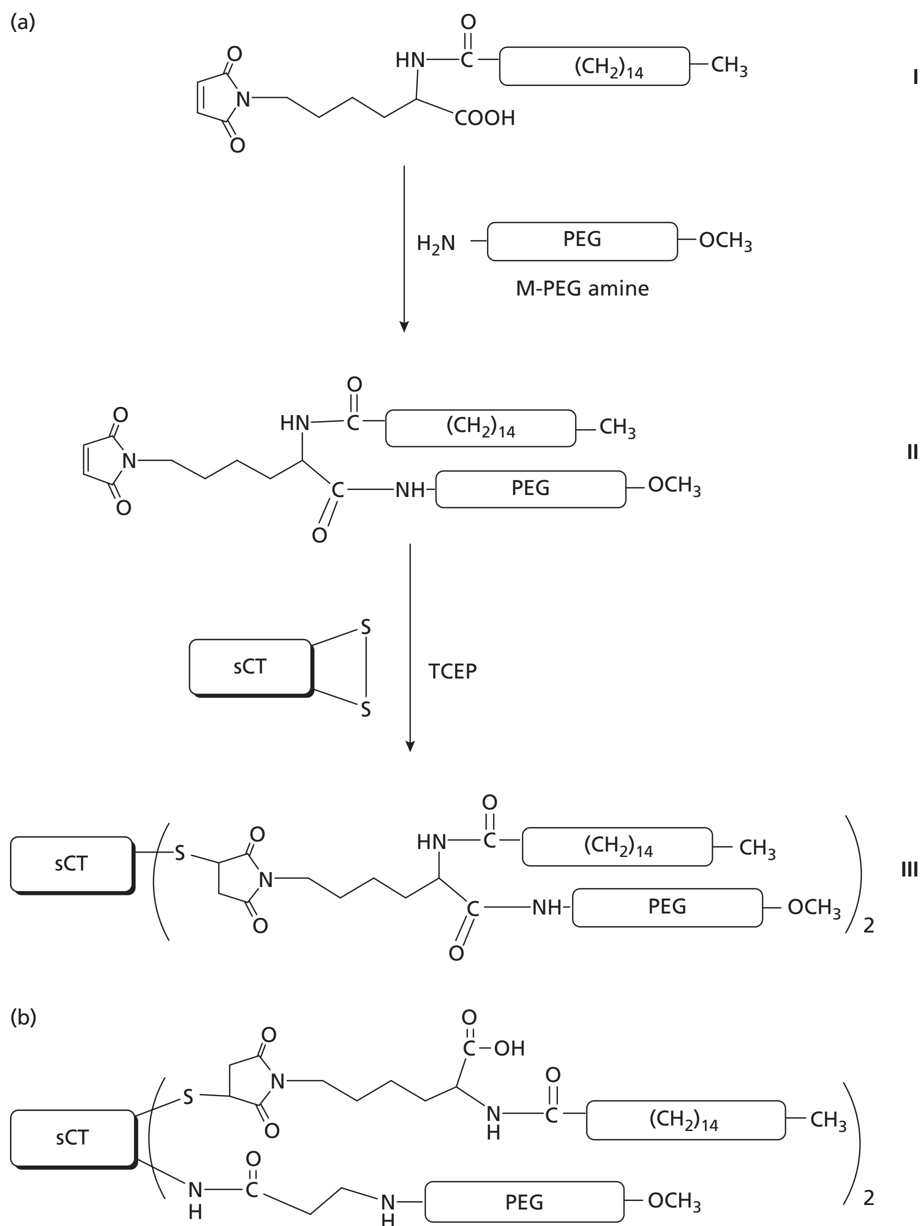

Figure 2 Synthesis pathway of Mal-PL-sCT (a) and structure of 2PEG-Mal-sCT (b)

Synthesis of Mal-PL-sCT (**III**) was carried out under reductive conditions maintained by TCEP. Briefly, 24.34 mg (7.1 μmol) of sCT, 85.57 mg (15.4 μmol) of PEG-lipid and 12 mg (41.9 μmol) of TECP were stirred overnight in a 15-ml mixture of dimethylformamide (DMF) and water (2 : 1) under argon. The reaction mixture was analysed with Tricine-SDS-PAGE with Bio-Rad molecular markers (mixed with sCT) as reference samples.[27] Purification was carried out in a C-18 semi-preparative column (10 ∞ 250 mm, 10 μm) attached to an HPLC system, and the fractions containing the purified sample were pooled together and freeze-dried after evaporation of solvent with a rotary evaporator. The final product was a transparent gel-like semi-solid at room temperature. The yield was 80.29 mg (5.5 μmol) (yield 80.2%). Mal-PL-sCT was identified by MALDI-TOF MS, and its purity further characterized with Tricine-SDS-PAGE and HPLC analyses.

2PEG-Mal-sCT

2PEG-Mal-sCT (Figure 2b) was synthesized as previously reported.[23] Mal-sCT and mPEG-Suc (MW 5000) at molar ratios of 1 : 3.6 were dissolved in 200 μl of DMF, and 0.6 of TEA was added with stirring for 20 min. The reaction mix was added to 1 ml of saturated ammonium acetate buffer. 2PEG-Mal-sCT was freeze-dried after purification using a semi-preparative HPLC C-18 column and identified to have MW of 15 kDa.[23] Purified 2PEG-Mal-sCT was similar to Mal-PL-sCT in being a transparent gel-like semi-solid.

HPLC analysis was carried out using a Waters Symmetry300 C-18 column (4.6 ∞ 250 mm). The elution time profile was from 100% A (0.1% TFA in water) to 100% B (1:1 v/v mixture of isopropanol and acetonitrile with 0.1% TFA) in 40 min and maintaining at 100% B for another 5 min. Sample detection was carried out at λ = 214 nm.

Particle size

Particle size was determined using the Protein Solutions DynaPro dynamic light-scattering (DLS) instrument (Wyatt Technology Corporation, Santa Barbara, CA, USA) at a laser wavelength of 825.8 nm, a detector angle of 90º, and typical sample volume of 20 μl. sCT was dissolved at 1.0 mM in distilled water. Due to high photon counts, neither Mal-PL-sCT nor 2PEG-Mal-sCT could be detected at the same concentration as sCT. Instead, Mal-PL-sCT and 2PEG-Mal-sCT were detected at 11 and 2.5 μM, respectively. A lower concentration was used for 2PEG-Mal-sCT as it exceeded the higher limit of detection of the DLS instrument at 11 μM. Each light-scattering experiment consisted of 20 or more independent measurements, and the data were analysed using the DynaPro software, DYNAMICS (version 5.26.60). To minimize dust interference, all solutions were freshly prepared and centrifuged at 2000 rpm for 2 min (Eppendorf centrifuge 5417, Hamburg, Germany) immediately prior to analysis.

Stability

The stability of the peptides in intestinal fluid was determined according to a method developed in our laboratory.[20] To obtain the intestinal fluid, three female Wistar rats (9 weeks old, about 220 g), which had been fasted for 24 h, were euthantized by intraperitoneal injection of ketamine (150 mg/kg) and xylazine (20 mg/kg). The protocol for euthanasia of rats was approved by the Animals Ethics Committee of the National University of Singapore (NUS). The intestine was exposed by an abdominal incision, and 30–40 cm of the small intestine, beginning from the duodenum, was excised. The luminal content was flushed out with 7.5–9.0 ml of phosphate buffer solution (PBS, pH 6.6) using a syringe (actual intestinal fluid was about 0.5–1.5 ml), and clear solution was obtained after centrifugation. The protein content in the intestinal fluid of each rat was separately determined using the MicroBCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). The pooled intestinal fluids were further diluted with PBS to yield a final protein content of 0.9 mg/ml. Degradation experiments were initiated by adding 30 μl of intestinal fluid to 566 μl of peptide solution (40 μM in PBS) to yield a final intestinal protein content of 45 μg/ml. After incubation for a predetermined time at 37ºC, 120 μl of the digested mix was withdrawn and 8 μl of acetic acid was added to quench the enzyme activity. Residual peptide in the intestinal fluid was quantified by HPLC analysis.

Peptide conformation

Peptide conformation was determined by circular dichroism (CD) spectroscopy. CD spectra were obtained at 20°C with a Jasco Circular Dichroism System 810 using a scanning range of 190–260 nm and an N_2 flow rate of 5 l/min. The sample cuvette (200 μl, 1.0 mm path lengh) was cleaned until blank samples (0, 20, and 50% of TFE in water) showed CD of less than 0.5 mDeg. The spectra of test samples, which comprised 0.10 mM of sCT or sCT conjugate dissolved in distilled water containing 0–50% of TFE, were recorded with the corresponding solvents serving as blanks.

In-vivo hypocalcaemic activity

The pharmacodynamic response of the conjugates was evaluated by analysis of plasma calcium concentration in the rat model.[28] The experimental protocols involving animals were approved by the Animals Ethics Committee of the NUS. Since sCT is commonly used to treat postmenopausal osteoporosis, only female rats were used in the study, but this does not preclude male rats from responding in the same way. Wistar female rats weighing 170–220 g (about 8 weeks old) were purchased from the NUS Centre for Animal Resources and housed at the NUS Animal Holding Unit. The rats were divided randomly into groups of six after 3 days of acclimatization.

Mal-PL-sCT and 2PEG-Mal-sCT samples were separately dissolved in normal saline (0.9% NaCl) to give 0.51 mg/ml (0.033 mM). sCT (0.033 mM) prepared in the same way and normal saline (0.9% NaCl) served as positive and negative controls, respectively. The subcutaneous dose for all the peptides was equivalent to 0.114 mg/kg of sCT. Control rats were administered with normal saline. Immediately before and at 1, 2, 4, 8, 12, 18 and 24 h after administration, blood samples (120–150 μl) were collected via saphenous vein puncture with a Microvette CB300LH (Sarstedt, Germany). Blood plasma was obtained by centrifuging the samples at 5000 rpm (Eppendorf Centrifuge 5145D) for 10 min at 15ºC.

The plasma calcium level was assayed by atomic absorption spectrometry (Perkin Elmer AAnalyst 100, MA, USA) as described previously by us.[20] A hollow calcium lamp at λ = 422.7 Å was used as the light source.[29] The slit width was 0.7 nm and a pure ethyne/compressed air flame was used.

Statistical analyses

Results are expressed as mean ± SD unless otherwise stated. Particle size and plasma calcium data were analysed by one-way ANOVA, with post-hoc Turkey's test applied for the comparison of group means at a P value of 0.05 (SPSS 12.0, SPSS Inc., Chicago, IL, USA). HPLC data at each time point for the degradation study were analysed by independent-sample t-test.

Results

Synthesis

Mal-PL-sCT

Analysis of the intermediate product, PEG-lipid, by silica-gel TLC indicated a highly purified product with an Rf value of 0.26 (Figure 3). Compared with the Rf value of 0.11 for the reactant, mPEG-amine, the intermediate product was more lipophilic, which was expected of mPEG-amine conjugated with Mal-Lys-Pal.

Figure 3 TLC analysis of the PEG-lipid (A), the mixture (B) of PEG-lipid and mPEG-amine, and mPEG-amine (C)

MALDI-TOF MS showed a polydispersed peak (normal distribution ranging from 4587.9 to 6486.7) with average MW of 5557.9, which would correspond to PEG-amine (average MW 5197) conjugated with Mal-Lys-Pal (MW 464). The ^{1}H NMR spectrum (in $CDCl_3$) for PEG-lipid showed peaks corresponding to 6.685 ppm (2H, maleimido-H), 4.390 ppm (1H, lysine-α-H), 2.245 ppm (2H, $–COCH_2(CH_2)_{13}CH_3$), 2.137 ppm (H_2O), 1.2470 ppm (26H, $–COCH_2$ $(CH_2)_{13}CH_3$), 0.8742 ppm ($–COCH_2(CH_2)_{13}CH_3$) and 3.3–4.0 ppm (476 H, corresponding to 119 $–CH_2CH_2O–$, calculated MW of 5236). These results indicate that the target PEG-lipid was successfully synthesized.

Reaction between sCT and the PEG-lipid was very clean. Tricine-SDS-PAGE analysis suggested the generation of a dominant product having MW of between 25 and 37 kDa (not shown). Further purification resulted in a highly purified compound with similar MW to 2PEG-Mal-sCT (Figure 4). The higher apparent MW of the conjugates in Tricine-SDS-PAGE was due to the association of water molecules with the PEG chains during gel electrophoresis,[1] a common phenomenon associated with PEGylated proteins.[30] Compound purity was also confirmed by HPLC analysis (results not shown). Elution times for sCT, Mal-PL-sCT and 2PEG-Mal-sCT were 21.5, 31.6 and 33.0 min, respectively, suggesting a lipophilicity ranking order of sCT < Mal-PL-sCT < 2PEG-Mal-sCT. Retention of the positively charged lysine 11 and lysine 18 moieties in Mal-PL-sCT was likely to make it more hydrophilic than 2PEG-Mal-sCT, whose lysine groups were conjugated to PEG via amide bonds. MALDI-TOF MS analysis (Figure 5) showed a dominant polydispersed peak clustered at MW 15.5 kDa, which corresponded to a molecule of sCT (3.4 kDa) conjugated with two molecules of PEG-lipid (average MW 5.6 kDa), and minor polydispersed peaks at 5 and 9 kDa, which might be attributed to free PEG-lipid (5.6 kDa) and a conjugate of sCT with only one PEG-lipid, respectively. Given the tendency of lower MW compounds to show higher MS response, and taking into consideration the Tricine-SDS-PAGE and HPLC data, these impurities might be regarded as being present at negligible levels in the Mal-PL-sCT sample.

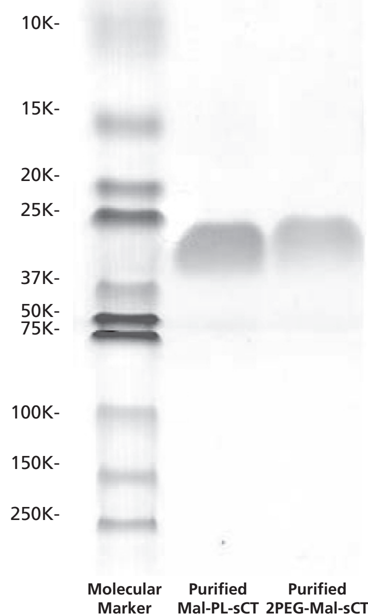

Figure 4 Tricine-SDS-PAGE analysis of molecular markers (lane 1), purified Mal-PL-sCT (lane 2) and purified 2PEG-Mal-sCT (lane 3)

Particle size

sCT had mean radii of 1.1 ± 0.1 nm (mean ± polydispersity) at 1.0 mM, which translated to a calculated MW of 4.69 kDa, comparable with the MW of sCT (3.43 kDa). Dilution resulted in very low photon counts, reaching levels similar to pure water, indicating that sCT exists mainly as monomers. The particle size of Mal-PL-sCT and 2PEG-Mal-sCT could not be determined at this concentration as the photon count exceeded the limit of detection. The mean particle radii for Mal-PL-sCT at 11 μM was 28.9 ± 9.4 nm, some 10-fold larger than the particle size based on its molecular weight (14.6 kDa). Photon counts of 2PEG-Mal-sCT at 11 μM also exceeded the detection limit of the DLS instrument, suggesting that 2PEG-Mal-sCT had a much larger particle size than Mal-PL-sCT at the same concentration. This was confirmed by the mean radii measured for 2PEG-Mal-sCT at 2.5 μM, which at 67.7 ± 21.0 nm was significantly larger than that of Mal-PL-sCT ($P < 0.05$) and was 30-fold larger than the particle radius of 2.0 nm expected based on its molecular weight. The larger sizes of Mal-PL-sCT and 2PEG-Mal-sCT were earlier alluded to by the Tricine-SDS-PAGE data (Figure 4), and might be attributed to the overall effect of molecular aggregation and the association of water molecules to the PEG chains.[1,30,31] Viscous drag, in particular, could significantly reduce particle mobility, leading to high readings on the DLS instrument. The larger size of 2PEG-Mal-sCT, relative to Mal-PL-sCT, was possibly a reflection of the PEG chains at lysine 11 and lysine 18 (Figure 2b).

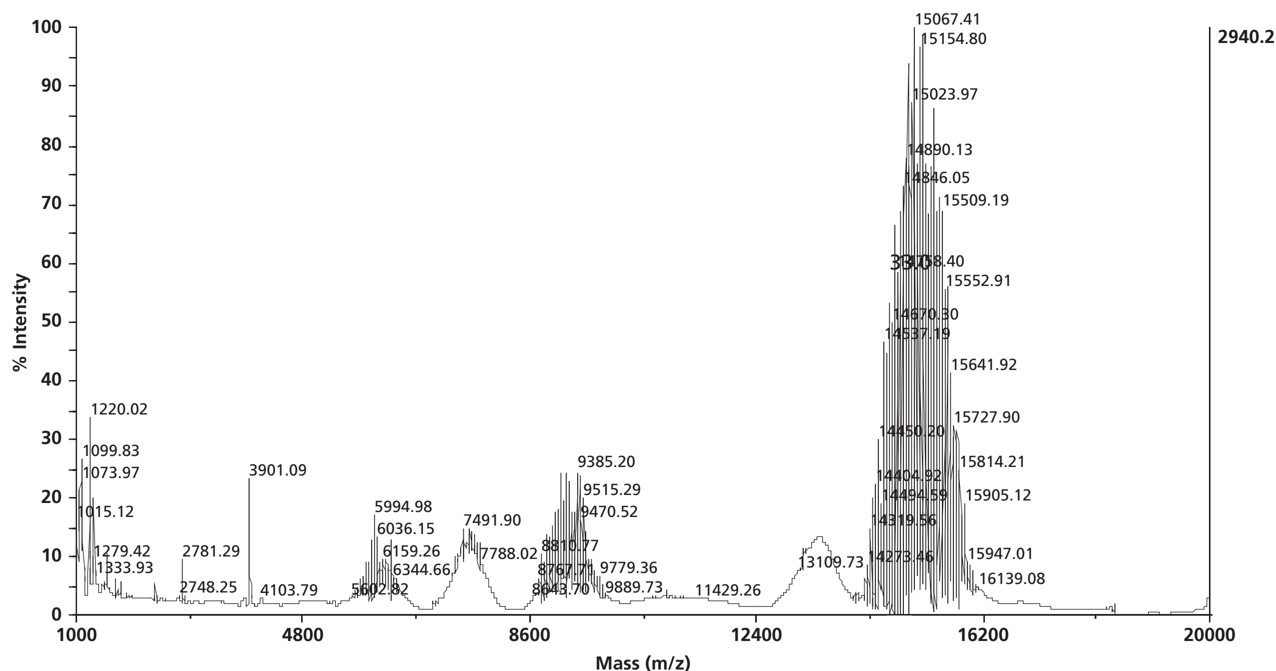

Figure 5 MALDI-TOF MS spectrum for Mal-PL-sCT

Peptide conformation

TFE, an organic solvent with low dielectric constant, is commonly used to simulate the in-vivo membrane environment.[32] Both CD spectra of Mal-PL-sCT and 2PEG-Mal-sCT showed a positive peak at 192 nm and two negative peaks with intensity of 208 and 222 nm, with peak intensity increasing slightly at higher TFE concentration (not shown). CD spectra for Mal-PL-sCT and 2PEG-Mal-sCT were comparable, indicating both conjugates assumed the helical structure in media with different dielectric constants. In contrast, sCT exhibited similar CD spectra only at higher TFE concentrations, with comparable results obtained with other organic solvents (such as methanol, ethanol, isopropanol and acetonitrile (results not shown)) at the same concentrations. The helical structure is contributed mainly by the residues between Val 8 and Tyr 22.[33] In previous work,[20,21] we have shown that sCT did not show the characteristic peaks in the absence of TFE, suggesting it lacked an apparent helical structure in aqueous solutions.

Stability

Statistical analysis by paired two-tailed *t*-test of data representing the percentage of peptide load remaining following incubation with diluted rodent intestinal fluid indicated that Mal-PL-sCT was significantly more stable than sCT ($P < 0.05$) at 2–5 min (Figure 6). Previous data has shown that lipid conjugation alone (i.e. Mal-sCT) did not improve the intestinal enzymatic stability of sCT ($P > 0.05$),[20] suggesting an added protection conferred by PEGylation of the conjugated lipid. 2PEG-Mal-sCT was even more stable, retaining 39.4 ± 14.3% ($n = 3$) of the initial load at 20 min, about 13-fold higher than that for Mal-PL-sCT, despite sharing the same molecular weight and functional groups as Mal-PL-sCT.

In-vivo hypocalcaemic activity

The in-vivo bioactivity of Mal-PL-sCT was evaluated by measuring its ability to lower plasma calcium levels after subcutaneous injection in the rat model. Rats dosed with the vehicle (0.9% NaCl) served as negative controls, while those administered with the equivalent sCT dose were positive controls. Baseline plasma calcium levels were not significantly different between the rats in the control and treatment groups prior to treatment initiation. Rats administered with the vehicle also did not show significant differences in plasma calcium levels before and after dosing.

Mal-PL-sCT after subcutaneous injection produced significantly lower plasma calcium levels at 2–8 h post-injection compared to the vehicle (Figure 7), but the calcium levels were significantly higher than corresponding levels observed in the sCT control group. After 8 h, Mal-PL-sCT showed a trend of sustained activity, but there was no significant difference in plasma calcium levels between the Mal-PL-sCT-treated and vehicle groups. By comparison, 2PEG-Mal-sCT produced insignificant hypocalcaemic activity at all time points.

Discussion

This paper set out to evaluate a novel conjugation strategy for improving the deliverability of peptide and protein drugs. The strategy involved forming a chemically active PEG-lipid with a tri-block linker to introduce accessible PEG and lipid moieties simultaneously to a protein/peptide drug. Using sCT as the model drug, this paper explored the feasibility of synthesis, characterization and activity of the resulting

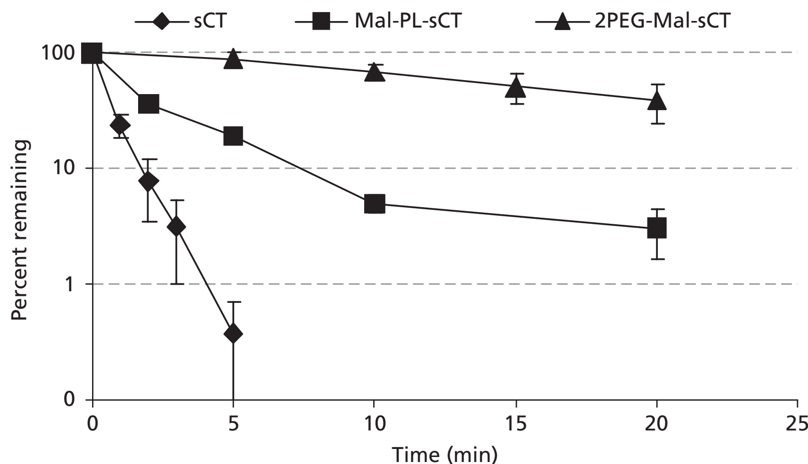

Figure 6 Degradation of sCT, Mal-PL-sCT and 2PEG-Mal-sCT by diluted intestinal solution. 40 μM of sCT and its conjugate were incubated with diluted intestinal solution (45 μg protein/ml) at 37ºC (mean ± SD, n = 3)

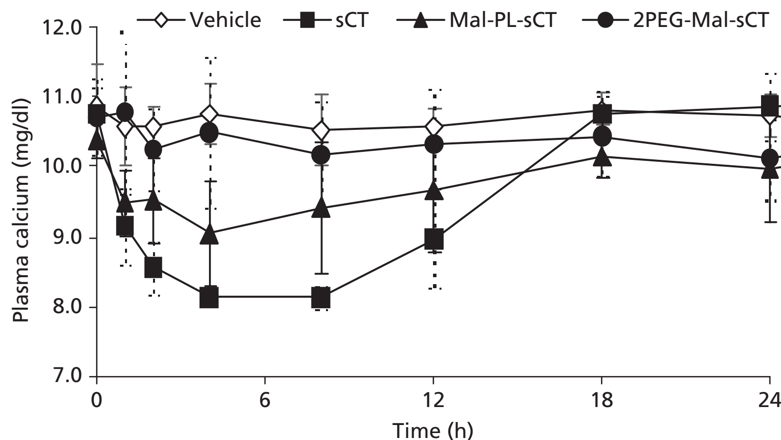

Figure 7 Plasma calcium level of rats administered with vehicle (normal saline), sCT, Mal-PL-sCT and 2PEG-Mal-sCT by subcutaneous injection. sCT, Mal-PL-sCT and 2PEG-Mal-sCT, dissolved in normal saline at 0.11, 0.51 and 0.51 mg/ml (equivalent to 0.11 mg/ml of sCT), respectively, were dosed at 200 μl/rat (200 g). Data represent mean ± SD, n = 6

conjugate, Mal-PL-sCT, and compared it to 2PEG-Mal-sCT, in which the same PEG and lipid moieties were separately conjugated to sCT at four different positions.

A simple scheme of the conjugation method is shown in Figure 1. A novel, chemically reactive PEG-lipid was successfully synthesized via condensation of Pal-Lys-Mal (**I**) and mPEG-amine. TLC analysis indicated that the PEGylated Pal-Lys-Mal was more lipophilic than the mPEG-amine (Figure 3), and positive identification was carried out with MALDI-TOF MS and NMR data. Further conjugation of the PEG-lipid with sCT at the cysteine 1 and cysteine 7 positions via maleimido lysine linkage yielded the target conjugate, Mal-PL-sCT. The conjugation process was highly efficient, giving a yield of 80.2% after purification.

Analyses by Tricine-SDS-PAGE and CD suggest that Mal-PL-sCT and 2PEG-Mal-sCT share similar molecular weight and robust helical structure. The conformations of these two peptides were similar to that of another conjugate, 1PEG-Mal-sCT,[23] which contained only one conjugated PEG group, at either lysine 11 or lysine 18. However, Mal-PL-sCT and 2PEG-Mal-sCT are different in several aspects. Firstly, Mal-PL-sCT has a smaller dynamic radius than 2PEG-Mal-sCT, possibly because linkage with the PEG-lipid results in a more compact structure than when the PEG and lipid moieties are separately conjugated at multiple positions. Secondly, while both Mal-PL-sCT and 2PEG-Mal-sCT exhibited significantly enhanced stability against intestinal enzymatic degradation, 2PEG-Mal-sCT was the more stable peptide. The enhanced stability of 2PEG-Mal-sCT might be attributed to the loss of free amine groups at lysine 11 and lysine 18 following PEG conjugation, as lysine 11 and lysine 18 are known substrates of trypsin, a potent protease in intestinal fluid.[34] This assumption is supported by the observed lower stability of Mal-PL-sCT, compared to 1PEG-Mal-sCT,[23] against intestinal enzymes, even though Mal-PL-sCT and 1PEG-Mal-sCT have comparable hydrodynamic sizes.

More importantly, unlike 2PEG-Mal-sCT, which showed no hypocalcaemic activity, Mal-PL-sCT retained some

hypocalcaemic activity of sCT. The structure–activity relationship of sCT is complicated and not well understood because of its inherent structural flexibility in different environments. The *N*-terminal loop formed by the disulfide between cysteine 1 and cysteine 7 is not required for the full hypocalcaemic activity of sCT,[35–37] although the propensity of residues between Val 8 and Tyr 22 to form helices has been linked to the enhanced potency of sCT in comparison with other native calcitonins.[38] In a previous study we have shown that Mal-sCT, a non-reversible lipid-modified sCT derivative, assumes a robust helical structure independent of the polarity of its aqueous environments.[24] Pharmacokinetic and pharmacodynamic correlation indicates that Mal-sCT was less potent than sCT in hypocalcaemic activity, suggesting that, while the helical structure is an active conformation, other structural factors may have contributed to the hypocalcaemic activity of sCT.[21] These factors include structural flexibility and the presence of certain residues, e.g. Val 8 and Arg 24.[39] Such divergent structural requirements for the activity of sCT[40] are in line with the complex structure–activity relationships associated with other therapeutic proteins and peptides, such as insulin.[41] The implication of our study is that the conjugation of multiple functional groups to a peptide via a tri-block linker may significantly reduce the risk of loss of pharmacological activity. This is particularly important for those peptides and proteins whose structure and activity relationships remain unknown, and where functional group modification does not result in apparent secondary or tertiary structural changes,[42] which is the case for 2PEG-Mal-sCT and Mal-PL-sCT.

The tri-block conjugation design provides new opportunities to further improve and optimize currently available delivery systems for peptides. It can be used to develop novel PEGylated lipids as stealth lipids, and further as targeting delivery platforms when the PEG chains are conjugated with specific receptor ligands (e.g. folic acid) or antibodies.[14,43] In addition, modification with a PEG-lipid may potentially be useful for optimizing the biodistribution of peptide drugs. The design has a structural similarity to the diblock polymer poly(caprolactone)-b-poly(ethylene oxide) (PCL-b-PEO), as PCL may be likened to a lipid-like polymer.[44] The modification of PCL by PEO resulted in efficient transport of the polymer as nanocontainers across the cell membrane into the Golgi apparatus and mitochondria of PC12 (rat pheochromocytoma cells) and NIH/3T3 (NIH Swiss mouse embryo cells) cells, and the same may be true of lipidized peptide conjugates. The tri-block design can be further modified to yield reversible lipidized analogues of sCT,[21,22] e.g. by using cysteine rather than lysine in the tri-block linker to conjugate the PEG-lipid with sCT, and/or by using other reversible bonds between either of the two functional groups to adjust the properties of the resulting conjugates to different in-vivo environments.

To our knowledge, this paper is the first study of the biophysical properties and bioactivity of a therapeutic peptide co-conjugated with a PEG-lipid with a tri-block linker. Being a proof of concept evaluation, this study has not focused on improving the bioactivity of Mal-PL-sCT, although it is likely that the hypocalcaemic activity of Mal-PL-sCT could be improved by modifying the size of the conjugated PEG chain. In one published study,[25] sCT conjugated with a 2-kDa PEG chain has been shown to retain its bioactivity, whereas lower bioactivity was observed when the MW of the PEG was increased to 5 or 12 kDa.[25] Despite its failure to demonstrate a superior bioactivity for Mal-PL-sCT relative to sCT, this study offers a novel synthetic pathway where multiple functional groups may be conjugated to a peptide via a tri-block linker without a risk of obliterating the intrinsic bioactivity of the peptide. It is conceivable that the design of the novel PEG-lipid developed in this study may prove useful for optimization of the biophysical properties, and the stability and/or bioactivity, of other peptide or protein drugs.

Conclusions

In summary, a novel chemically active PEG-lipid was designed, synthesized and identified, and its conjugate with sCT, Mal-PL-sCT, was studied in comparison with 2PEG-Mal-sCT. Mal-PL-sCT, like 2PEG-Mal-sCT, has a propensity to aggregate, as shown by DLS study. Mal-PL-sCT has enhanced intestinal enzyme stability, although it is inferior to 2PEG-Mal-sCT in this aspect. However, in contrast to 2PEG-Mal-sCT, which showed no hypocalcaemic activity, Mal-PL-sCT retained some in-vivo hypocalcaemic activity.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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