Synthesis, Characterization and *In Vivo* Activity of Salmon Calcitonin Coconjugated With Lipid and Polyethylene Glycol

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ABSTRACT: An irreversible lipidized salmon calcitonin (sCT) analog, Mal-sCT, was previously shown to have comparable hypocalcemic activity to sCT *in vivo*. This study reports on the synthesis, characterization and pharmacological activity of novel PEGylated Mal-sCT analogs. Mal-sCT, prepared by conjugating sCT via thio-ether bonds with aqueous-soluble palmitic acid derivative at Cys 1 and Cys 7, was reacted with mPEG-succinimide (mPEG-Suc, 5 kDa). The products were purified and then identified by MALDI-TOF MS and HPLC. Mal-sCT was conjugated with 1 (1PEG-Mal-sCT) or 2 (2PEG-Mal-sCT) PEG chains at Lys 11 and Lys 18, the former being the preferred site of conjugation at higher mPEG-Suc/Mal-sCT ratio. Circular dichroism analysis showed the PEGylated Mal-sCT analogs to possess a robust helical conformation, while size measurement by dynamic light scattering indicated a propensity of the peptides to self-aggregate in aqueous solutions. Both 1PEG-Mal-sCT and 2PEG-Mal-sCT were more stable in rodent intestinal fluids than sCT or Mal-sCT. However, 1PEG-Mal-sCT had comparable hypocalcemic activity to Mal-sCT when injected subcutaneously in the rat, while 2PEG-Mal-sCT was inactive. 1PEG-Mal-sCT was inactive when administered orally in the rat. This study suggested PEGylation of Mal-sCT increased the stability of the lipidized peptide to enzyme degradation, but did not enhance its hypocalcemic activity. J Pharm Sci 98:1438–1451, 2009

Keywords: salmon calcitonin; lipidization; PEGylation; hypocalcemic activity; stability; aggregation; conformation

INTRODUCTION

PEGylation has evolved to become an established method for enhancing the enzyme stability of peptide drugs,[1] including calcitonin.[2] The PEG chains (also called poly(ethylene oxide)) emanating from a peptide have been proposed to bind strongly with water molecules to form an effective shield against enzyme approach.[1] In addition, PEGylation is a method for prolonging the systemic circulation half-life of a peptide, as it can increase the molecular size of the peptide significantly enough to impede its clearance by renal filtration.[3] PEG conjugation may also facilitate the oral delivery of a peptide. A recent study has shown the poly(caprolactone)-*b*-poly-(ethylene oxide) (PCL-*b*-PEO) polymer to be

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efficiently transported across cell membrane into the Golgi apparatus and mitochondria of PC12 (rat pheochromocytoma cells) and NIH/3T3 (NIH Swiss mouse embryo cells) cells.[4] The translocation was believed to be aided by the aggregation of the polymer into spherical particles of 20–45 nm diameter in aqueous media. The mechanism of translocation was associated with the process of endocytosis.[5,6] Hence, by likening the hydrophobic PCL to a lipid chain, it is conceivable that PEGylated lipidized peptides might also be transported across cell membrane by the same mechanism.

We previously synthesized and evaluated a novel nonreversible lipid conjugate of salmon calcitonin (sCT), denoted as Mal-sCT.[7] Mal-sCT consisted of sCT conjugated at Cys 1 and Cys 7 via thio-ether bonds with a pair of water-soluble ε-maleimido lysine derivative of palmitic acid. This peptide self-aggregated in aqueous media and was capable of producing comparable hypocalcemic activity to sCT in the rat model. Mal-sCT was also more resistant than sCT to degradation in hepatic and intestinal milieu, but the level of enhanced stability was inadequate to allow Mal-sCT to be administered orally.[7] We hypothesized that PEGylation of Mal-sCT might not only sufficiently increase its stability to enzymatic degradation, but that the PEGylated lipidized sCT, through its aggregation into nanoparticles, might also have higher permeability across absorptive epithelia as has been demonstrated with the PCL-*b*-PEO nanoparticles.[4]

To test the hypothesis that PEGylation was advantageous for Mal-sCT, this article set out to evaluate the synthesis, characterization and pharmacological activity of sCT coconjugated with lipid and PEG. sCT consists of 32 amino acids, a proline amide at residue 32 and an N-terminal disulfide bridge between residues 1 and 7. The oral pharmacological bioavailability of sCT after intraduodenal administration was approximately 0.029–0.040%.[8] Previous experiments with Mal-sCT have indicated that sCT bioactivity was not affected by lipid conjugation via nonreversible thio-ether bonds at the Cys 1 and Cys 7 positions. Rather, the lipid conjugation had promoted peptide stability in liver extract and peptide association with cell membrane.[7] Studies conducted in other laboratories have shown PEG-modification of sCT at Lys 18 to enhance its oral[9] and nasal[10] bioactivity by approximately three- to sixfold. While lipid modification of sCT at Lys 11 and Lys 18 did not affect its *in vitro* activity.[11] On the basis of these collective data, we hypothesized that sCT might be coconjugated with PEG and lipid at the Lys and Cys moieties, respectively, without sacrificing its bioactivity.

MATERIALS AND METHODS

sCT was purchased from Unigene Laboratory (Boonton, NJ). mPEG-succinimidyl propionate (mPEG-Suc) (MW 5000) was from Nektar Therapeutics Corporation (Huntsville, AL). *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) (electrophoresis grade), trypsin (TPCK treated, from bovine pancreas), trifluoroethanol (TFE), triethylamine (TEA) and trifluoroacetic acid (TFA) were from Sigma-Aldrich Company (St. Louis, MO). Tris(hydroxymethyl)methylglycine (Tricine) was from Amersham Biosciences (Little Chalfont, Buckinghamshire, England), polyacrylamide gel (40%) stock solution with 38:1 w/w of acrylamide to *N*,*N*′-methylene bis(acrylamide) was from Bio-Rad (Hercules, CA) and the MicroBCA Protein Assay Kit was from Pierce Biotechnology (Rockford, IL). Acetonitrile and isopropanol of HPLC grade were supplied by Fisher Scientific (Irvine, CA). Milli-Q water was used throughout the study.

Synthesis

Mal-sCT

Mal-sCT was synthesized as described previously.[7] Briefly, a water-soluble ε-maleimido lysine derivative of palmitic acid was synthesized by reacting Pal-Suc with ε-maleimido lysine. The latter was generated from a reaction of α-Boc-lysine and methylpyrrolecarboxylate, with subsequent deprotection of the Boc group. The palmitic derivative was further conjugated with sCT via a thio-ether bond to produce Mal-sCT in aqueous solution under reductive condition maintained by TCEP. Mal-sCT was purified using the HPLC and identified by Electrospray Ionisation Mass Spectrometry (ESI-MS).

1PEG-Mal-sCT and 2PEG-Mal-sCT

Figure 1 shows the synthesis pathways for 1PEG-Mal-sCT and 2PEG-Mal-sCT. Mal-sCT and