European Journal of Pharmaceutics and Biopharmaceutics 96 (2015) 329–337

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research Paper

Acylation of salmon calcitonin modulates *in vitro* intestinal peptide flux through membrane permeability enhancement

Sofie Trier [a,b], Lars Linderoth [b], Simon Bjerregaard [b], Holger M. Strauss [b], Ulrik L. Rahbek [b], Thomas L. Andresen [a,*]

[a] Dept. of Micro- and Nanotechnology, Center for Nanomedicine and Theranostics, Technical University of Denmark, Building 423, Produktionstorvet, DK-2800 Kgs. Lyngby, Denmark
[b] Global Research, Novo Nordisk A/S, Novo Nordisk Park 1, DK-2760 Maaloev, Denmark

ARTICLE INFO

Article history:
Received 17 April 2015
Revised 24 August 2015
Accepted in revised form 2 September 2015
Available online 5 September 2015

Keywords:
Acylation
Peptide
Salmon calcitonin
Oral
Caco-2
Intestinal permeability
Lipid membranes

ABSTRACT

Acylation of peptide drugs with fatty acid chains has proven beneficial for prolonging systemic circulation, as well as increasing enzymatic stability and interactions with lipid cell membranes. Thus, acylation offers several potential benefits for oral delivery of therapeutic peptides, and we hypothesize that tailoring the acylation may be used to optimize intestinal translocation. This work aims to characterize acylated analogues of the therapeutic peptide salmon calcitonin (sCT), which lowers blood calcium, by systematically increasing acyl chain length at two positions, in order to elucidate its influence on intestinal cell translocation and membrane interaction.

We find that acylation drastically increases *in vitro* intestinal peptide flux and confers a transient permeability enhancing effect on the cell layer. The analogues permeabilize model lipid membranes, indicating that the effect is due to a solubilization of the cell membrane, similar to transcellular oral permeation enhancers. The effect is dependent on pH, with larger effect at lower pH, and is impacted by acylation chain length and position. Compared to the unacylated peptide backbone, N-terminal acylation with a short chain provides 6- or 9-fold increase in peptide translocation at pH 7.4 and 5.5, respectively. Prolonging the chain length appears to hamper translocation, possibly due to self-association or aggregation, although the long chain acylated analogues remain superior to the unacylated peptide. For K^{18}-acylation a short chain provides a moderate improvement, whereas medium and long chain analogues are highly efficient, with a 12-fold increase in permeability compared to the unacylated peptide backbone, on par with currently employed oral permeation enhancers. For K^{18}-acylation the medium chain acylation appears to be optimal, as elongating the chain causes greater binding to the cell membrane but similar permeability, and we speculate that increasing the chain length further may decrease the permeability.

In conclusion, acylated sCT acts as its own *in vitro* intestinal permeation enhancer, with reversible effects on Caco-2 cells, indicating that acylation of sCT may represent a promising tool to increase intestinal permeability without adding oral permeation enhancers.

1. Introduction

Acylation of therapeutic peptides with fatty acids is a widely used peptide alteration strategy in drug delivery, which has found particular use for prolonging peptide circulation in blood of otherwise rapidly cleared peptide drugs, e.g. by exploiting the affinity of serum albumin toward fatty acids [1] and for increasing enzymatic stability [2–5]. Acylation has been employed for a multitude of therapeutic peptides [1,3,6,7], including several marketed drugs (e.g. insulin and Glucagon-like peptide-1). An important drawback for alteration of therapeutic peptides is the risk of reducing potency; however, through a rational choice of acylation site and type and/or screening different acylation positions, it is often possible to limit the deleterious effects on biological activity [8,9].

The majority of reports regarding acylated peptides have focused on subcutaneous injection, whereas the potential for oral administration remains an emerging field. Acylation may benefit several of the challenges in oral delivery, e.g. the highly metabolic environment in the stomach and intestine [10], and the limited absorption of large, hydrophillic peptide drugs through the intestinal epithelial barrier [11]. A widely used method for predicting

* Corresponding author.
E-mail address: thomas.andresen@nanotech.dtu.dk (T.L. Andresen).

http://dx.doi.org/10.1016/j.ejpb.2015.09.001
0939-6411/

in vivo oral absorption is *in vitro* quantification of translocation across monolayers of the human colon cancer cell line (Caco-2), which has been shown to correlate well with oral bioavailability [12,13]. In a few cases, acylation has been shown to increase intestinal permeability of peptide drugs [6,8,14]; however, detailed investigations of systematic acyl variations are lacking. Such investigations will benefit rational design of new peptide drugs, as investigations of peptide interactions with model lipid membranes [15–17] can elucidate its influence on cellular membrane translocation potentially leading to higher oral bioavailability.

Calcitonin (CT) is a single-chain 32 amino acid peptide hormone, with an N-terminal disulphide bridge between positions 1 and 7, and a C-terminal amidated proline. It is produced in the thyroid gland and secreted in response to excess calcium in serum [18], and its primary biological function is the inhibition of osteoclast-mediated bone resorption. Calcitonin is employed in the treatment of bone-related disorders such as osteoporosis, osteoarthritis, Paget's disease, and hypercalcemia [19,20]. Whereas human calcitonin (hCT) is physically and chemically unstable [21], salmon calcitonin (sCT) is more stable, potent [22] and has a longer *in vivo* half-life [20,23]. Consequently, the therapeutic use is mainly based on sCT.

Administration of sCT is currently limited to either parenteral or intranasal routes; however, several examples of oral delivery exist in the literature and, recently, several clinical trials have been completed [20,24]. Most of these reports deal with native sCT in combination with oral permeation enhancers or enzyme inhibitors [20,23,24], which is challenging due to the requirement for sufficiently high additive concentration to allow for intact peptide permeation. Other approaches include advanced delivery systems [25–28] and a few examples of modifying the peptide itself [6,29–31]. 2K PEGylation at Lys18 was shown to increase *in vivo* efficacy but not *in vitro* permeability, so the improvement was ascribed primarily to increased enzymatic stability [31]. Dual acylation with long chains at the sulphide bridge in a reversible or permanent manner was shown to increase oral bioavailability but the *in vitro* permeability was not investigated [6,29,30].

A previous study of acylated analogues of Glucagon-like peptide-2 (GLP-2), a negatively charged peptide hormone [32], revealed a non-trivial relationship between translocation ability and acyl chain length, which correlated with the level of peptide–membrane interactions.

In the present study we wish to investigate this effect in more detail, and have, similar to the GLP-2 analogues, synthesized and characterized a series of acylated sCT analogues where the systematically increasing acyl chains have been placed at two positions on the positively charged backbone. This allows an investigation into the effects of the acyl chain length, backbone charge and acylation placement on membrane interaction and *in vitro* intestinal permeability and, when compared to acylated GLP-2, enables elucidation of potential general trends of peptide acylations on membrane translocation properties.

2. Materials and methods

2.1. Materials

Resin and natural amino acids were purchased from Novabiochem (Germany). c8, c12 and c16 carboxylic acids, Fmoc-beta-Alanine and native sCT were provided by Novo Nordisk A/S. Palmitoyloleoylglycerophosphocholine (POPC) was purchased from Avanti Polar Lipids (USA). HEPES, Ovalbumin (OVA, from chicken egg white) and other standard chemicals were purchased from Sigma–Aldrich (Denmark).

Dulbecco's Modified Eagle Medium (DMEM), L-glutamine and penicillin/streptomycin were purchased from Lonza (Switzerland). Hank's Balanced Salt Solution (HBSS), foetal bovine serum (FBS), nonessential amino acid and other standard cell culture products were purchased from Gibco (Denmark). Radioactively labelled [^{3}H]mannitol, scintillation fluid (Microscint-40), luciferase substrate (SteadyLitePlus) and 96-well plates for luciferase assay (CulturPlate, black) were purchased from PerkinElmer (USA). 12 well Transwell plates for Caco-2 cell monolayers (polycarbonate, 12 mm, pore size 0.4 μM) were purchased from Corning Costar Corp. (USA). hCT-R BHK cells (Hollex-1, ZymoGenetics, US patent 5622839) were provided by Novo Nordisk and Caco-2 cells (HTB-37) were purchased from ATCC.

2.2. Peptide synthesis

The peptides were synthesized by automated Fmoc based SPPS, using a Rink Amide AM resin in a 0.25 mmol scale on a Prelude peptide synthesizer (Protein Technologies), using standard protocols (coupling with 6 eq. amino acid or fatty acid, diisopropylcarbodiimide (DIC) and ethyl 2-cyano-2-(hydroxyimino)acetate (Trade name Oxyma Pure) in N-Methyl-2-pyrrolidone (NMP) for 60 min at r.t.; deprotection with 20% piperidine in NMP for 2×10 min). Acetamidomethyl (Acm) was used as protecting groups for Cys. Formation of the disulphide bridge was performed by dissolving the peptides in H_2O (1 mg/1 ml), adjusting pH to 5.5 with acetic acid (AcOH) and oxidizing with a solution of 5% I_2/AcOH at room temperature for approximately 2 h. For N-ε-lysine acylated peptides the 4-Methyltrityl (Mtt) protecting group was used. Mtt was removed with neat hexafluoro-2-propanol (HFIP, 3×15 min) followed by washings with dichloromethane (DCM) and the acylation performed on a Prelude peptide synthesizer, using the same conditions as described above. The peptides were cleaved from the resin and deprotected using Trifluoroacetic acid (TFA)/H_2O/Thioanisole (TIS) 92.5:2.5:5 v/v for 3 h, followed by precipitation with diethylether. The peptide was dissolved in a suitable solvent (e.g. 95:5 H_2O:MeCN) and purified by preparative RP-HPLC on an XBridge c18 column (Waters) using MeCN/H_2O/TFA. The fractions were analyzed by UPLC, LCMS, and chemiluminescent nitrogen detection [33], and the appropriate fractions were pooled and portioned for lyophilization.

2.3. hCT receptor activation

The receptor potency of modified sCT analogues was assessed using a hCT-R transfected BHK cell line, similar to previously described for GLP-2 [32].

Briefly, hCT-R cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin, and the assay was performed in DMEM without phenol red, containing 10 mM HEPES, 1% glutamax, 0.1% OVA and 1% penicillin/streptomycin. Prior to experiments, cells were plated in 384-well plates (CulturPlate) at 1×10^5 cells/ml, centrifuged for 20 s at 1500 rpm, and incubated overnight (at 37 °C and 5% CO_2). After wash with assay medium, cells were incubated with peptide analogues for 3 h, prepared by 7-fold dilutions in the concentration range 0.1 pM–10 nM (and pure buffer as 10 fM point). Cells were then incubated with luciferase substrate for 30 min at room temperature while protected from light, and after a brief shake the luminescence was measured in a topcounter (Packard Topcount). Relative Luminescence Units (RLU) depend on peptide concentration as shown in Eq. (1):

$$\mathrm{RLU} = A + \frac{B - A}{1 + 10^{((\log \mathrm{EC}_{50} - x) \cdot C)}} \quad (1)$$