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Late-stage lipidation of fully elaborated tryptophan-containing peptides for improved pharmacokinetics

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ABSTRACT

The late-stage modification of native peptides to alter and/or enhance their properties and functions is attractive but formidably challenging. Peptide lipidation is one of the effective strategies to overcome short half-life and rapid clearance. Herein, we report a late-stage installation of a fatty acid lipid onto fully elaborated peptides, using glucagon as an example, through regio- and chemoselective functionalization of tryptophan with high potency and remarkable in vivo half-life extension.

Introduction

Peptide drugs enjoy great intrinsic advantages over other chemistry modalities due to their superior efficacy, high selectivity, and low toxicity.[1] However, most naturally occurring peptides are quickly degraded in serum and rapidly cleared from the body. The proteolytic instability and short circulating half-life significantly limit their use as effective therapeutics. Lipidation is one of the chemical modification methods which have been proved to be highly useful and practical for improving the physicochemical, pharmacological and even biological properties of peptides.[2–4]

The typical lipidation method is through acylation of peptide lysine side chain with long-chain, saturated fatty acid lipids.[2,5,6] It usually requires *de novo* synthesis of peptides and different amino protecting groups are implemented if the peptide has multiple lysine residues. Other methods such as cysteine *S*-alkylation,[7,8] serine *O*-esterification[9] were also reported. However, the ability to modify selected amino acid (AA) side chains for lipidation in fully elaborated peptides is still very limited.[2,10] The desired late-stage, site-specific lipidation approach would obviate the need for the protection/deprotection steps and the need for engineering peptides with limited synthetic handles or the use of unnatural amino acids. In order to achieve that, an orthogonal functional group can be introduced through late-stage functionalization of a peptide, which allows for further site-specific manipulation.

The glucagon-related peptides, including glucagon, GLP-1, GLP-2, GIP, oxyntomodulin (OXM), and their synthetic versions of dual[11] and/or triple[12] receptor agonist peptides, have been extensively studied for the use of diabetes treatment. Lipidation platform through acylation of lysine residue is currently the gold standard chemical approach for engineering long-acting peptide analogs.[5,6] This technology has an established track record for pharmacokinetic improvement in commercialized products, such as once-daily GLP-1R agonist Liraglutide,[13] insulin Levemir,[6] insulin Degludec,[14,15] as well as a phase 3 clinical candidate, once-weekly GLP-1RA Semaglutide.[16] Both Liraglutide and Semaglutide are lipidated in position 20 (the *N*-terminal His counted as position 1) of GLP-1 (7–36) amide. In order to achieve site-specific monoacylation, substitution of Lys[28] to Arg was applied in both cases. It should be noted that other positions were also reported for lipid attachment through Lys acylation or Cys alkylation in GLP-1RAs.[11,12,17] However, all of them require different levels of sequence alteration and/or functional group protection strategy.

Sequence alignment and analysis of glucagon-related peptides revealed that tryptophan (Trp) at position 25 is highly conserved across the panel and only one single Trp residue is incorporated in each peptide (Fig. 1a). We envisioned that it could be an ideal setting for late-stage, site-specific functionalization of such fully elaborated peptides. Although challenging, Trp-selective

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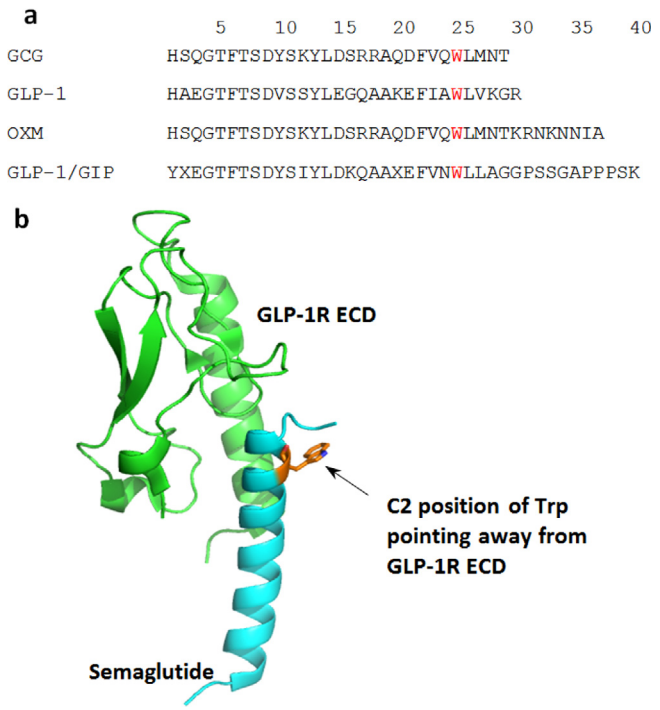
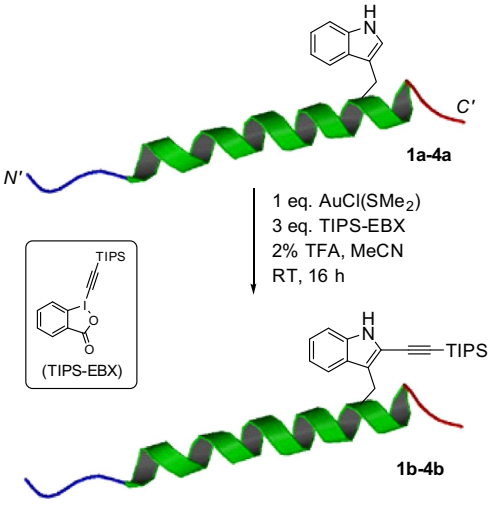


Fig. 1. (a) Sequences of glucagon (GCG), GLP-1, oxyntomodulin (OXM), and GLP-1/GIP dual agonist.[11] C-terminal amidation for GLP-1, and GLP-1/GIP dual agonist. X = 2-aminoisobutyric acid (or Aib). Trp is conserved across the panel and highlighted in red. (b) X-ray crystal structure of Semaglutide peptide in complex with GLP-1 receptor extracellular domain (PDB code: 4ZGM). The Trp[25] is highlighted with stick structure, whose C2 position pointing away from the GLP-1R ECD.



Scheme 1. A representative reaction scheme of Trp alkynylation in glucagon-related peptides.

Table 1
Trp alkynylation of glucagon-related peptides.

Entry	Peptide[a]	Yield, %[b]
1	GCG (**1a**)	44
2	GLP-1 (**2a**)	33
3	OXM (**3a**)	25
4	GLP-1/GIP coagonist (**4a**)	38

[a] See Fig. 1a for amino acid sequence.
[b] Reaction conditions: peptide (1 equiv), TIPS-EBX (3 equiv), $AuCl(SMe_2)$ (1 equiv), MeCN with <2% TFA/HOAc, RT, 16 h. The low isolated yields were attributed to the incomplete reaction conversion and purification loss on RP-HPLC C8 column.

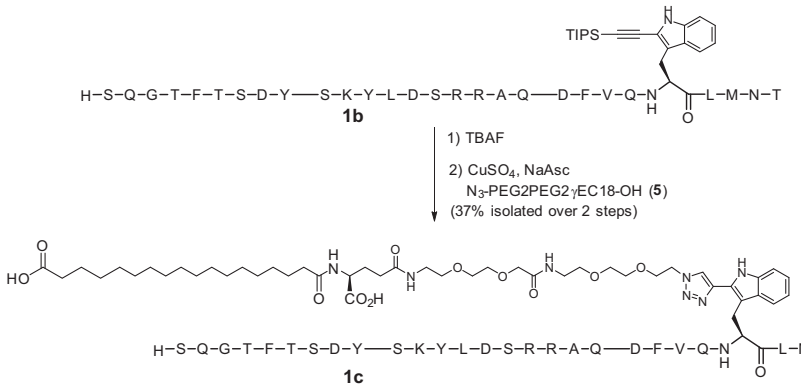
functionalization in peptides and proteins has been described. A rhodium carbenoid approach reported by Francis and coworkers, although giving a mixture of both N1 and C2 substituted products, is still useful for Trp-containing protein labeling.[18] More recently, a complete regio- and chemoselective Trp functionalization in peptides and proteins has also been reported.[19,20] This elegant method allows for direct installation of an orthogonal handle (i.e., an alkyne) at C2 position of Trp in the peptides. Since alkyne-azide cycloaddition "click" chemistry is the most widely used bioconjugation strategy for manipulating the properties of large molecules,[22] we thought this sequential Trp C2 alkynylation/click reaction offers a unique suite of late-stage lipidation of native peptides for pharmacokinetic (PK) improvement.

Based on the X-ray crystal structure of Semaglutide in complex with GLP-1R extracellular domain (Fig. 1b),[16] the C2 position of Trp is solvent exposed and pointing away from the receptor protein. Therefore, grafting a fatty acid lipid on C2-position of Trp in glucagon-related peptides would potentially have minimal negative impact on its biological activity, yet very likely with improved PK profiles.

Results and discussion

With this in mind, we chose native glucagon (GCG, **1a**) peptide as our model peptide because of its short half-life, around 4–7 min in human plasma.[23] Under slightly modified conditions,[20,21] the initial trial reaction of **1a** and Waser reagent (TIPS-EBX, 3 equiv) in the presence of $AuCl(SMe_2)$ (1 equiv) catalyst at room temperature gave a full conversion of starting material to the desired product **1b** based on LCMS analysis (see Scheme 1). It was isolated in 44% yield with reverse-phase HPLC C8 column (Table 1, entry 1). Remarkably, reactive amino acid residues (such as His, Ser, Phe, Try, Lys, Thr and Met) were all tolerated. Interestingly, although Waser reagent is a hypervalent iodine oxidant, the Met residue in GCG peptide was intact, with no trace amount of overoxidized product observed during the reaction. With this result, we were encouraged to test the alkynylation reactions of the aforementioned glucagon-related peptides in the Fig. 1a panel, which all suffer from the high clearance and short half-lives with regular formulation.[24] Therefore, GLP-1 peptide **2a**, bearing a 48% homology to GCG peptide, transformed to Trp-alkynylated product **2b** in 33% yield (entry 2). Oxyntomodulin (OXM, **3a**), a naturally occurring dual agonist binding both the GLP-1 receptor and the glucagon receptor, contains the full amino acid sequence of GCG followed by extra an 8 AAs extension at C-terminus. The alkynylation of OXM only resulted in 25% isolated yield of product **3b** (entry 3). Another reported dual agonist **4a**[11] with activation on both the GLP-1 receptor and the GIP receptor went on alkynylation reaction to give 38% yield of desired product **4b** (entry 4). This material was analyzed by MS/MS, confirming that the TIPS ethynyl group ends up on Trp residue in the peptide.

In order to enhance the PK properties of those peptides, we further reacted the alkynylated model peptide GCG **1b** with the fatty acid lipid **5**, containing PEG2PEG2γEC18-OH unit, an established long-chain, saturated lipid for once-weekly administration of GLP-1R agonists.[16] Initial desilylation of the TIPS ethynyl GCG peptide **1b** with polymer-supported fluoride (10X) led to no reaction. Nevertheless, the treatment of **1b** with TBAF immediately gave the desilylated product ethynyl GCG, which further reacted with azido-containing fatty acid lipid **5** under CuAAC condition to give the Trp[25]-lipidated GCG peptide **1c** in 37% yield over 2 steps (Scheme 2).



Scheme 2. Late-stage lipidation of GCG peptide, involving the steps of desilylation with TBAF and conjugation by CuAAC. N_3-PEG2PEG2γEC18-OH (5): (*S*)-1-azido-22-carboxy-10,19,24-trioxo-3,6,12,15-tetraoxa-9,18,23-triazahentetracontan-41-oic acid.

The activity of the lipidated GCG **1c** remains potent against the glucagon receptor with EC_{50} = 0.24 ± 0.04 nM (n = 2). Although less potent compared with the native GCG (EC_{50} = 0.012 nM), the lipidated GCG **1c** could potentially be a very attractive option for the treatment of Type 2 diabetes patients with obesity when co-dosing with long-acting GLP-1RAs in a reasonable combination as well as for the treatment of hypoglycemia.

Next, we examined whether the lipidation strategy translates into better pharmacokinetic profiles. The PK properties of the lipidated GCG **1c** were evaluated in male Wistar Han naïve rats (n = 3 per group) following i.v. or s.c. administration (Fig. 2). Following i.v. administration, **1c** exhibited low systemic plasma clearance of 0.27 mL/min/kg with elimination half-life of 6.8 h. The volume of distribution was 0.14 L/kg. The subcutaneous bioavailability of **1c** was 55% (Table 2). This remarkable extension of half-life (compared to native GCG) was presumably attributed to the strong albumin binding and reduced clearance, even without sequence modification to protect toward DPP-IV degradation.

Finally, parathyroid hormone (PTH) belongs to the same B-family G protein-coupled receptors (GPCRs) as glucagon-related peptides. The bioactive portion of this hormone is PTH (1–34) with a product Teriparatide already in the market for the treatment of osteoporosis. However, its half-life is only 4 min. The only reported long-acting version of this hormone is Fc fusion protein, which showed to treat hypoparathyroidism more effectively and for a longer period of time than PTH in rats and monkeys.[25] By examining the amino acid sequence of PTH (1–34), only one single Trp is incorporated in the sequence and the molecular model of hPTH (1–34) binding to its receptor indicating Trp C2 position has no direct interaction with receptor and is solvent exposed.[26] Hence, hPTH (1–34) is a perfect setting for chemical late-stage lipidation to improve pharmacokinetics. Thus, hPTH (1–34) (sequence: SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF) was treated with TIPS-EBX and gold catalyst to afford TIPS ethynyl PTH (28% yield), which underwent desilylation with TBAF (52% yield) and followed by click reaction with azido-PEG2PEG2γEC18-OH (**5**) to furnish the desired lipidated PTH (see supporting information).

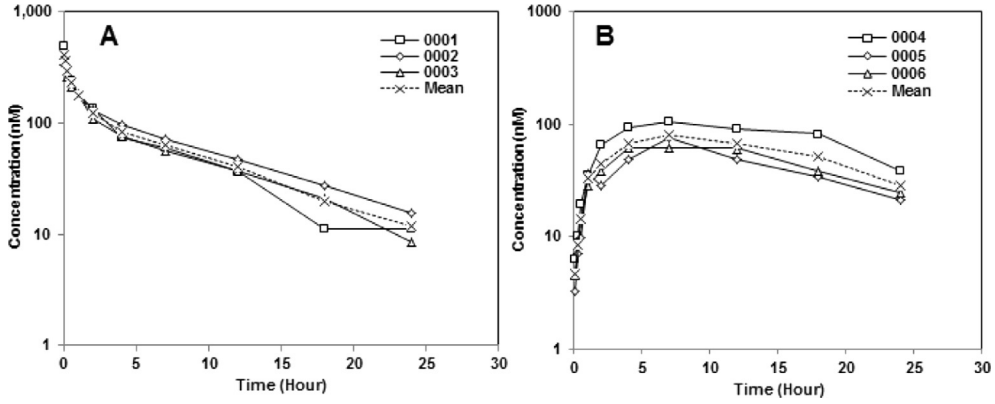


Fig. 2. Plasma concentration vs. time profiles of lipidated GCG **1c** in rats following IV (A) and SC (B) administration. GCG **1c** was formulated in mannitol buffer (5% mannitol, 6 mM sodium phosphate, pH 8) and dosed IV or SC at 1 mL/kg, n = 3 rats/group. Concentrations of GCG **1c** were determined by LCMS assay.

Table 2
Pharmacokinetics of GCG **1c** in male Wistar Han naïve rats following IV and SC dosing.[a]

Route	Dose (mg/kg)	$AUC_{0-\infty}$ (μM·h)	CL_p (mL/min/kg)	Vd_{ss} (L/kg)	$T_{1/2}$ (h)
IV	0.1	1.5 ± 0.2	0.27 ± 0.03	0.14 ± 0.00	6.8 ± 0.7
Route	Dose (mg/kg)	$AUC_{0-\infty}$ (μM h)	C_{max} (nM)	T_{max} (h)	F (%)
SC	0.2	1.6 ± 0.5	80 ± 22	7	55

[a] GCG **1c** was formulated as a solution and administered in rats as described above. Plasma concentrations of GCG **1c** were determined by LCMS assay. Values are means ± SD.

Conclusion

In summary, we have developed a novel lipidation strategy for fully elaborated peptides such as glucagon-related peptides and PTH at the very late stage without the need of *de novo* peptide synthesis. The first step regio- and chemoselective Trp C2 alkynylation were examined across the panel of listed peptides and the subsequent lipidation chemistry was showcased with GCG and PTH model peptides. The model lipidated GCG peptide demonstrated remarkable improvement of PK profiles compared to the native peptide. Naturally, this late-stage sequential modification, Trp C2 alkynylation coupled with click reaction, could be extended to the potential applications of protein labeling and cell penetrating peptides.

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A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2017.02.031.

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