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High metabolic in vivo stability and bioavailability of a palmitoylated ghrelin receptor ligand assessed by mass spectrometry

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ABSTRACT

The constitutive activity of the ghrelin receptor is of high physiological and pathophysiological relevance. In-depth structure–activity relationship studies revealed a palmitoylated ghrelin receptor ligand that displays an in vitro binding affinity in the low nanomolar range. Activity studies revealed inverse agonistic as well as antagonistic properties and in vitro metabolic analysis indicated a high stability in blood serum and liver homogenate. For metabolic testing in vivo, a combined approach of stable isotopic labeling and mass spectrometry-based analysis was established. Therefore, a heavy isotopic version of the peptide containing a ^{13}C-labeled palmitic acid was synthesized and a 1:1 ratio of a ^{12}C/^{13}C-peptide mixture was injected into rats. Biological samples were analyzed by multiple reaction monitoring allowing simultaneous peptide detection and quantification. Measurements revealed a suitable bioavailability over 24 h in rat serum and subsequent high-resolution mass spectrometry investigations showed only negligible degradation and slow body clearance. Hence, this method combination allowed the identification and evaluation of a highly potent and metabolically stable ghrelin receptor ligand in vivo.

1. Introduction

The in vitro analysis of the metabolic stability of peptides can be easily carried out by incubating fluorescently labeled peptides in blood plasma or liver homogenate.[1,2] In contrast, the in vivo analysis of peptide biodistribution, biokinetics and metabolic stability is more challenging. Radiolabels suitable for imaging techniques such as positron emission tomography (PET) are most frequently used, but as it has been shown that they alter peptide properties and offer only a short radioactive half-life, non-radioactive stable isotopic labeling represents a powerful alternative.[3–5] The incorporation of heavy isotopes such as carbon-13 (^{13}C) does not change the chemical characteristics and therefore, biological features of heavy labeled peptides are close to identical to their unlabeled counterparts.[6,7] The detection of isotopically labeled peptide pairs can be performed by liquid chromatography tandem mass spectrometry (LC–MS/MS) technology[8] and quantitative proteomic analysis is possible by multiple reaction monitoring (MRM).[9] The use of these methods enables the quantification of peptides in in vivo samples such as serum, urine and feces[10] and the identification of peptide metabolites.[11]

One interesting target in the field of G protein-coupled receptor (GPCR) therapeutics is the ghrelin receptor. Since the receptor has been discovered in 1996 as an orphan receptor,[12] thorough studies revealed its involvement in several physiological and pathophysiological functions. First described as growth hormone secretagogue receptor (GHS-R1a), only its function in the process of growth hormone release was reported.[12,13] After the discovery of the endogenous ligand ghrelin in 1999 however, its importance in the regulation of appetite and food intake was revealed.[14,15] In addition, the receptor-ligand system is known to be implicated in learning and memory processes as well as in alcohol-related diseases. Furthermore, an overexpression of the receptor was

Abbreviations: b/D-Bth, β-(3-benzothienyl)-D-alanine; GPCR, G protein-coupled receptor; iv, intravenously; MRM, multiple reaction monitoring; MS, mass spectrometry; Pam, palmitic acid; PEG2, poly(ethylene glycol) 2 kDa; TAMRA, carboxytetramethylrhodamine.

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shown in several tumors.[16–20] This variety of functions makes the ghrelin receptor an interesting therapeutic target for drug design aiming to regulate its constitutive activity.

In the field of ghrelin receptor modulation, peptides such as [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P and derivatives are able to target the basal signaling of the receptor.[21] These analogs are of great interest, since peptides may be advantageous over other drug compounds that target GPCRs. They are less toxic than small molecules, possess a lower immunogenicity than antibody-based drugs and only little unspecific binding to molecules can be observed.[22,23] Nevertheless, the drawbacks of peptides are still their low metabolic stability and fast body clearance. To overcome these disadvantages, PEGylation, lipidation or the introduction of D-amino acids can be applied.[24,25] Lipidation of a compound leads to more hydrophobic analogs that can build micelles or bind to plasma proteins, for example, serum albumin.[26] Additionally, modification with lipids can improve pharmacokinetics and enhance membrane binding.[27,28] In consequence, peptides are protected from proteolytic enzymes and renal and hepatic excretion are reduced, which results in an extended serum half-life.[1,29] The coupling of poly(ethylene glycol) (PEG; $C_{2n}H_{4n+1}O_{n+1}$) enlarges the size of the peptide complex and thereby also reduces kidney filtration. Moreover, the introduction of PEG moieties improves water solubility and decreases the accessibility for proteolytic enzymes.[30,31] Another method to increase the half-life of peptides is the incorporation of non-natural and D-amino acids. Peptidases are very selective for the L-enantiomer of naturally occurring amino acids and the use of modified amino acids offers a powerful tool for prolonged peptide stability.[32,33]

Here, structure–activity relationship studies of the [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P-derived ghrelin receptor inverse agonist KbFwLL-NH_2[34] (b = benzothienyl-D-alanine, D-Bth) with either 2 kDa poly(ethylene glycol) (PEG2) or palmitic acid (Pam) were performed. In vitro metabolic stability was investigated in blood plasma and liver homogenate to identify the degradation process as well as possible degradation products. These studies led to the highly potent and efficient C-terminally palmitoylated ligand KbFwLK(Pam)-NH_2 featuring a remarkable metabolic stability. The combination of stable isotopic labeling and MS-analysis allowed further investigations of the bioavailability and the metabolic stability in vivo. A 1:1 mixture of the $^{12}C/^{13}C$-labeled peptide was administered iv to rats and subsequent investigations using MRM and high-resolution LC–MS/MS revealed a constant peptide level and no degradation in blood serum over 24 h. Thus, a ghrelin receptor inverse agonist with distinct in vivo bioavailability was identified emphasizing the advantages of this modern combined approach to study peptide ligands in vivo.

2. Material and methods

2.1. Materials

N^{α}-Fmoc-protected amino acids were purchased from Novabiochem (Schwalbach, Germany) and Iris Biotech GmbH (Marktredwitz, Germany). *tert*-Butyloxycarbonyl (Boc) was used as side chain protecting group for Lys and Trp, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) for Lys as orthogonal protecting group. The 4-(2′,4′-dimethoxyphenyl-9-fluorenylmethoxycarbonyl-aminomethyl)-phenoxy (Rink amide) resin, 5(6)-carboxytetramethylrhodamine (TAMRA) and 1-hydroxy-benzotriazole (HOBt) were purchased from Novabiochem (Schwalbach, Germany). Acetonitrile (ACN), 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HATU), *N′N′*-diisopropylcarbodiimide (DIC), hydrazine and sodium-dodecylsulfate (SDS) were from Sigma–Aldrich (Taufkirchen, Germany). Trifluoroacetic acid (TFA), 4-dimethylaminopyridine (DMAP), *t*-butanol, methanol, thioanisole, 1,2-ethanedithiol were obtained from Fluka (Taufkirchen, Germany). Diethyl ether, dichloromethane, methanol and dimethylformamide (DMF) were from Biosolve (Valkenswaard, The Netherlands). Ethanol (EtOH) was purchased from AppliChem (Darmstadt, Deutschland).

For cell culture the following media and supplements were used: DMEM with higher glucose (4.5 g/l) and with L-glutamine, phosphate buffered saline (PBS), bovine serum albumin (BSA), penicillin and streptomycin were purchased from PAA Laboratories (Pasching, Austria). Hygromycin B was obtained from InvivoGen Europe (Toulouse, France). The disodium salt of ethylenediaminetetraacetic acid (EDTA) was purchased from AppliChem (Darmstadt, Germany). Trypsin-EDTA and fetal calf serum (FCS) were obtained from Gibco Life Technologies (Basel, Switzerland). Sodium hydroxide (NaOH) and formic acid (HCOOH) were purchased from Grüssing GmbH (Filsum, Germany). Sodium formate (HCOONa) and disodium tetraborate decahydrate ($Na_2B_4O_7$) were obtained from MERCK (Darmstadt, Germany). Lithium chloride (LiCl) was obtained from Sigma (Taufkirchen, Germany). Ammonium formate was obtained from Paul Lohmann GmbH (Emmerthal, Germany) and Pefabloc SC from Fluka (Taufkirchen, Germany). [2-^{3}H(N)]-myo-inositol, ^{125}I-His-Ghrelin and scintillation cocktail Optiphase 'Hisafe' 3 were from PerkinElmer (Rodgau, Germany). Anion-exchange resin AG 1-X8 was obtained from BioRad (München, Germany). Throughout the experiments, Eppendorf® Protein LoBind tubes (low-bind tube, Hamburg, Germany) were used to ensure no loss of peptide owing to unspecific binding.

2.2. Peptide synthesis

The automated multiple peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) was used for synthesis of compounds on the Rink amide resin (13.5 μmol) with Fmoc/*t*-Bu strategy as described recently.[35] Coupling of distinct amino acids and palmitic acid was performed manually with 5 equiv Fmoc amino acid, 5 equiv DIC and 5 equiv HOBt in DMF. PEG2 was coupled with 3 equiv MeO-PEG2-NHS, 6 equiv DIC and DMAP in DCM. Coupling of 2,4,6,8,10,12,14,16-^{13}C-sodium palmitate was carried out at the Lys[6] side chain with 2 equiv palmitate, 5 equiv HOBt and 5 equiv DIC in DMF/MeOH. Peptides were labeled with 1.5 equiv 5(6)-TAMRA, 1.5 equiv HATU and 3 equiv DIPEA in DMF/DCM. The Dde protection group was used to modify peptides side-specifically and cleaved selectively with 2% hydrazine in DMF. The elongated peptides were cleaved from the resin in one step with 90% TFA and 10% scavenger (1,2-ethanedithiol/thioanisole 3:7). Purification was achieved by preparative HPLC on a reversed-phase C18 column (Phenomenex Jupiter 10u Proteo 90 Å: 250 × 21.2 mm; 7.8 μm; 90 Å). Peptides were analyzed by MALDI-TOF MS (UltraflexII, Bruker, Bremen, Germany), ESI-ion trap MS (HCT, Bruker, Bremen, Germany) and by analytical reversed-phase HPLC on columns VariTide RPC (Varian: 250 × 4.6 mm; 6 μm; 200 Å) or Vydac RP-18 (Grace Vydac: 250 × 4.6 mm; 5 μm; 300 Å) and Phenomenex Jupiter 4u Proteo 90 Å (Phenomenex: 250 × 4.6 mm; 4 μm; 90 Å). Peptides for in vivo studies were resalted twice with 20 mM HCl. The observed masses were in full agreement with the calculated masses and peptide purity ⩾95% could be obtained according to the analytical HPLC using at least two different systems (Table S1).

2.3. Cell culture

COS7 cells stably transfected with the human ghrelin receptor were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose and 10% (v/v) FCS, 0.4 mg/ml