A novel truncated LEAP2 palmitoylated at its N-terminal antagonizes effects of ghrelin

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Palmitoylated LEAP2 analog antagonizes effect of ghrelin

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Abbreviations
AgRP  agouti-related protein
ANOVA  analysis of variance
Dpr  diaminopropionic acid
ESI  electrospray ionization
GH  growth hormone
GHSR1a  growth hormone secretagogue receptor 1a
HPLC  high-performance liquid chromatography
IC50  maximal inhibitory concentration
Kd  dissociation constant of the radioligand
Ki  inhibition constant
LC  liquid chromatography
LEAP2  liver enriched antimicrobial peptide-2
MRM  multiple reaction monitoring
MS  mass spectrometer
NPY neuropeptide Y
s.c. subcutaneous

Key words:
LEAP2, ghrelin, GHSR1a, affinity to receptor, food intake regulation, growth hormone release

Recommended section
Drug discovery and translational medicine
Abstract

Ghrelin is secreted in stomach during fasting and targets the growth hormone secretagogue receptor (GHSR1a) in hypothalamus and brainstem to exert its orexigenic effect. Recently, liver enriched antimicrobial peptide-2 (LEAP2) was identified as an endogenous high affinity GHSR1a antagonist. LEAP2 is a 40 amino acid peptide with two disulfide bridges and GHSR1a affinity in the N-terminal hydrophobic part. In this study, we tested modified truncated N-terminal peptide LEAP2(1-14) along with its myristoylated, palmitoylated and stearoylated analogs to determine their affinity to and activation of GHSR1a and their anorexigenic effects after acute peripheral administration. The lipidized analogs bound GHSR1a with affinity similar to that of natural LEAP2, and lipidization significantly enhanced the affinity of LEAP2(1-14) to GHSR1a. According to the beta-lactamase reporter gene response, natural GHSR1a agonist ghrelin activated the receptor with nanomolar EC50. LEAP2(1-14) analogs behaved as inverse agonists of GHSR1a and suppressed internal activity of the receptor with EC50 values in the 10^-8 M range. LEAP2(1-14) analogs significantly lowered acute food intake in overnight fasted mice, and palmitoylated LEAP2(1-14) was the most potent. In free-fed mice, all LEAP2(1-14) analogs significantly decreased the orexigenic effect of the stable ghrelin analog [Dpr³]Ghrelin. Moreover, palmitoylated LEAP2(1-14) inhibited the growth hormone (GH) release induced by [Dpr³]Ghrelin and exhibited an increased stability in rat plasma compared with LEAP2(1-14). In conclusion, palmitoylated LEAP2(1-14) had the most pronounced affinity for GHSR1a, had an anorexigenic effect, exhibited stability in rat plasma, and attenuated [Dpr³]Ghrelin-induced GH release. Such properties render palmitoylated LEAP2(1-14) a promising substance for antiobesity treatment.

Significance statement

Agonist and antagonist of one receptor are rarely found in one organism. For ghrelin receptor (growth hormone secretagogue receptor, GHSR), endogenous agonist ghrelin and endogenous antagonist/inverse agonist liver enriched antimicrobial peptide-2 (LEAP2) co-exist and differently control GHSR signaling. As ghrelin has the unique role in food intake regulation, energy homeostasis, and cytoprotection, lipidized truncated LEAP2 analogs presented in this study could serve not only to reveal relationship between ghrelin and LEAP2 but also for development of potential anti-obesity agents.
Introduction

Liver-expressed antimicrobial peptide 2 (LEAP2) is a peptide of 40 amino acids originally isolated from human blood in 2003 (Krause et al., 2003). LEAP2 is expressed mainly in liver and jejunum (Ge et al., 2018). LEAP2 has 2 disulfide bonds and folds into a cationic globular structure (Henriques et al., 2010). In early studies (Krause et al., 2003), LEAP2 was reported to show in vitro antimicrobial activities at a micromolar concentration. However, LEAP2 physiological concentration in plasma is in the nanomolar range (Mani et al., 2019). Moreover, LEAP2 peptide sequence is highly conserved from fish to mammals (Li et al., 2021), implying that LEAP2 performs other important functions in addition to being an antimicrobial peptide. Ge et al. tested the activation of 168 known human G-protein coupled receptors by LEAP2 in agonist and antagonist modes (Ge et al., 2018). LEAP2 fully inhibited growth hormone secretagogue receptor 1a (GHSR1a), receptor of the orexigenic hormone ghrelin.

The ghrelin-GHSR1a system is involved in multiple biological functions, such as growth hormone (GH) secretion, food intake, reward-seeking behaviors, cardiovascular functions and glucose homeostasis (Zigman et al., 2006). These diversified functions suggest the complexity of GHSR1a intracellular signaling. Ghrelin is secreted during fasting in stomach and stimulates secretion of the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY) in AgRP/NPY neurons in the arcuate nucleus of hypothalamus (Cowley et al., 2003). Ghrelin consists of 28 amino acids and is unusual among peptide hormones because its Ser3 is octanoylated (Kojima et al., 1999). Ghrelin without the acyl group can neither bind GHSR1a nor induce GH release (Sato et al., 2014).

The N-terminal fragment of LEAP2 is enriched with hydrophobic amino acids, such as Met1, Pro3, Phe4 and Trp5, allowing it to bind the same ligand-binding pocket on GHSR1a as ghrelin (Wang et al., 2019). LEAP2 competes with ghrelin for binding to GHSR1a and decreases the constitutive activity of GHSR1a, acting as an inverse agonist by stabilizing an inactive conformation of the receptor (M’Kadmi et al., 2019). LEAP2 inhibits the major effects of ghrelin in vivo. LEAP2 subcutaneous (s.c.) pretreatment was reported to attenuate the orexigenic effect of s.c. administered ghrelin in mice. Moreover, LEAP2 inhibits ghrelin-induced GH release (Ge et al., 2018). However, subsequently, other researchers showed that specifically intracerebroventricular but not peripheral administration of LEAP2 to rats suppressed central ghrelin functions, including Fos expression in hypothalamic nuclei, the
promotion of food intake, blood glucose elevation, and body temperature reduction (Islam et al., 2020). Lean but also obese mice deficient in LEAP2 are more sensitive to the acute effects of administered ghrelin on food intake and GH secretion. LEAP2 deficiency lowers energy expenditure, reduces locomotor activity and increases food intake in females chronically fed a high-fat diet, resulting in increased body weight, body length and hepatic fat accumulation (Shankar et al., 2021).

The plasma levels of ghrelin and its inverse agonist LEAP2 have opposite trend in fasting and feeding/refeeding in mice and humans. Similarly, the LEAP2 levels are higher and ghrelin levels are lower in morbidly obese humans than in lean ones. The LEAP2 to ghrelin ratio is an obvious marker of obesity and decreases with body weight loss in obese people (Mani et al., 2019).

Research investigating the interrelation between ghrelin and LEAP2 faces the following two very basic problems: first, the instability of ghrelin caused by the labile ester bond between octanoic acid and the hydroxyl of Ser³, which is essential for ghrelin biological activity, and second, the extremely difficult synthesis of LEAP2 complicated with two S-S bonds. These problems could be overcome by first, using the ghrelin analog \([\text{Dpr}^3]\text{Ghrelin}\) with Ser³ replaced with diaminopropionic acid (Dpr) and octanoyl anchored to the peptide chain by stable amide bond (Bednarek et al., 2000; Maletinska et al., 2012) and second, by using shorter N-terminal peptides of LEAP2, whose syntheses are feasible and which have preserved biological activity (M'Kadmi et al., 2019).

The aim of this study was to design a series of novel truncated LEAP2 analogs to select the most stable and bioavailable GHSR1a inverse agonists. Biologically active LEAP2(1-14) was synthesized based on published data (M'Kadmi et al., 2019) and lipidized with different fatty acid residues (myristoyl, palmitoyl or stearoyl) at its C-terminus as the N-terminal part of LEAP2 is essential for receptor binding (M'Kadmi et al., 2019). LEAP2(1-14) and its lipidized analogs were characterized by evaluating their binding affinity to GHSR1a, their ability to affect GHSR1a internal activity, their opposing effect on ghrelin orexigenic action and GH release in mice, and their stability in rat plasma. Out of LEAP2 truncated lipidized analogs designed and tested in this study, palmitoylated LEAP2(1-14) showed the most potent inhibitory properties toward ghrelin induced GHSR1 activation.
Materials and methods

Peptide synthesis

**Ghrelin and [Dpr³]Ghrelin synthesis.** Ghrelin and [Dpr³]Ghrelin were assembled in a solid-phase ABI 433A synthesizer (Applied Biosystems, Foster City, CA) as previously described (Holubova et al., 2018). Ghrelin was used in the *in vitro* experiments, and its stable analog [Dpr³]Ghrelin was used in the *in vivo* experiments. Lipidization with the corresponding fatty acid was performed on a fully protected peptide on resin as the last step (Maletinska et al., 2012). Peptide purification and identification were carried out by analytical high-performance liquid chromatography (HPLC) and mass spectrometry (MS). The purity of the synthesized peptides was greater than 95%.

LEAP-2(38-77) (#075-40) was obtained from Pheonix Pharmaceuticals (Burlingame, CA USA).

**Synthesis of LEAP2(1-14) and its lipidized analogs.** Fmoc-¹Nle-²Thr(tBu)-³Pro-⁴Phe-⁵Trp(Boc)-⁶Arg(Pbf)-⁷Gly-⁸Val-⁹Ser(tBu)-¹⁰Leu-¹¹Arg(Pbf)-¹²Pro-¹³Ile-¹⁴Gly-¹⁵βAla-¹⁶Lys(Alloc)-NH₂ was assembled by solid-phase peptide synthesis starting from Agilent Amphisphere 40 RAM resin (0.36 mmol/g) 1100 mg resin, 0.4 mmol, using Fmoc chemistry, HATU/DIEA coupling conditions and piperidine/DMF for deprotection. All coupling steps (5 eq.) were performed twice for 10 min. After the completion of the synthesis, the Alloc group of Lys¹⁶ was deprotected twice in DCM using Pd(PPh₃)₄ 0.25 eq. and PhSiH₃ 25 eq. for 30 min. Then, the peptidyl resin was washed with DCM, dried and divided into 4 equal parts. Lipidization was performed on three parts by coupling each part with the three corresponding acids (10 eq.) using BOP as a coupling agent (10 eq.) and DIEA as the base for 30 min. The fourth part was directly deprotected to yield LEAP2(1-14). Final deprotection was performed with a TFA/TIS/H₂O (95/2.5/2.5) mixture for 3 h. Purification was performed on a Gilson PLC 2250 Preparative RP-HPLC system (Villiers le Bel, France) using a preparative column (Waters DeltaPak C18 Radial-Pak Cartridge, 100 Å, 40-100 mm, 15 µm particle size) in the gradient mode at a flow rate of 50.0 mL/min. Buffer A was 0.1% TFA in water, and buffer B was 0.1% TFA in acetonitrile.

**Peptide characterization.** The LC/MS system consisted of an HPLC-ZQ (Waters) equipped with an ESI source. The analyses were carried out using a Phenomenex Kinetex column (C18, 100 Å, 100x2.1 mm, 2.6 µm). A flow rate of 0.5 mL/min and a gradient of 0-100% B over 5
min were used as follows: eluent A, water/0.1% HCO₂H; eluent B, ACN/0.1% HCO₃H. Positive-ion electrospray (ESI+) mass spectra were acquired from 100 to 1500 m/z with a scan time of 0.2 s. Nitrogen was used for both the nebulizing and drying gas. All peptides were characterized with a purity of at least 95%.

The structures of all peptides are shown in Figure 1B.

Peptide iodination
Ghrelin was iodinated at His⁹ with Na[¹²⁵I] purchased from Izotop (Budapest, Hungary) using Iodo-Gen (Pierce, Rockford, IL, USA) according to (Maletinska et al., 2012). The identity of the peptides was verified by a MALDI-TOF Reflex IV mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The specific activity of ¹²⁵I-ghrelin was approximately 2000 Ci/mmol. Aliquots of purified radiolabeled ghrelin were dried by evaporation, kept at -20 °C and used in the binding studies within 1 month.

Cell culture
T-REx™ Tango™ GHSR-bla U2OS cells overexpressing GHSR1a and containing a β-lactamase reporter gene under the control of an upstream activation site response element were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The cells were maintained at 37 °C in a humidified incubator with 5% CO₂ in McCoy’s 5A medium supplemented with 10% dialyzed fetal bovine serum, 0.1 mM nonessential amino acids, 25 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 1% streptomycin/penicillin, 200 μg/mL zeocin, 50 μg/mL hygromycin, and 100 μg/mL geneticin according to Thermo Fisher’s protocol.

Competitive binding studies
The competitive binding studies were performed as previously described (Karnosova et al., 2021). In brief, T-REx™ Tango™ GHSR-bla U2OS cells were seeded on 24-well plates to reach a density of approximately 20,000 cells/well, which was found to be optimal for the binding experiment. The cells were allowed to grow for 3 days. 16 hours before experiment, doxycycline was added into final concentration 1.25 ng/mL. Ghrelin, LEAP2 and their analogs were used at final concentrations from 10⁻¹² to 10⁻⁵ M to compete with 0.1 nM [¹²⁵I]-ghrelin. The incubations were performed in a total volume of 250 μl of binding buffer (50 mM Tris-Cl pH 7.4, 118 mM NaCl, 5 mM MgCl₂, 4.7 mM KCl, 0.1% BSA, and 2 g/l glucose) for 60 minutes at 23 °C. The cells were rinsed with wash buffer (20 mM Tris pH 7.4, 118 mM NaCl, 4.7 mM KCl, and 5 mM MgCl₂) and subsequently lysed in 0.1 M NaOH. Radioactivity was determined by gamma counting (Wizard 1470 Automatic Gamma Counter;
PerkinElmer Life and Analytical Sciences, Waltham, MA). The experiments were carried out in duplicate at least three times.

Beta-lactamase-dependent fluorescence resonance energy transfer assay
T-REx™ Tango™ GHSR-bla U2OS cells were used to study the agonist/inverse agonist/antagonist properties of ghrelin, LEAP2 and their analogs in a complementation assay for GHSR1a activity involving arrestin recruitment and expression of β-lactamase. The cells were plated at 10,000 cells/well in a 384-well plate in assay medium, and the assay was performed according to Thermo Fisher’s protocol and according to our previous study (Holubova et al., 2018). Ghrelin, LEAP2 and their analogs were used at final concentrations ranging from $10^{-12}$ to $10^{-5}$ M. The fluorescent plate reader FlexStation 3 was operated at a 409 nm excitation wavelength and a 460 or 530 nm emission wavelength via bottom read. The experiments were carried out in duplicate at least three times.

Experimental animals
Male C57Bl/6J mice (Charles River, Sulzfeld, Germany) were housed at a temperature of 23 °C and a daily cycle of 12 h light and dark (light from 6:00 AM). The mice were given ad libitum water and standard chow diet of ssniff R/M-H (cat. no. V1534; Spezialdiäten GmbH, Soest, Germany), which contained 58%, 9%, and 33% of calories from carbohydrate, fat, and protein, respectively. All experiments followed the ethical guidelines for animal experiments and the Act of the Czech Republic Nr. 246/1992 and were approved by the Committee for Experiments with Laboratory Animals of the Academy of Sciences of the Czech Republic (decision no. 38/2013 was issued on 8/4/2013).

Effect of LEAP2(1-14) and its lipidized analogs on acute food intake in mice
Twelve-week-old mice were placed in separate cages for 1 week with free access to water and food pellets. Anorexigenic activity was tested in mice fasted for 17 h. On the day of the food intake experiment, at 8:00 a.m., the mice were s.c. injected with 150 µl of saline or LEAP2(1-14) analogs (dissolved in saline) at a dose of 5 mg/kg of body weight (n = 5). Thirty minutes after the injection, the mice were given preweighed food pellets. The anorexigenic effect of the LEAP2(1-14) analogs on the orexigenic activity of [Dpr³]Ghrelin was tested in freely fed mice. On the day of the food intake experiment, at 8:00 a.m., the mice were s.c. injected with 150 µl of saline or LEAP2(1-14) analogs (dissolved in saline) at a dose of 5 mg/kg of body weight (n = 5). Fifteen minutes after the first injection, the mice were injected with saline or [Dpr³]Ghrelin at a dose of 1 mg/kg of body weight. Fifteen minutes after the second injection, the mice were given preweighed food pellets. Food intake was monitored every 30 min for at
least 7 hours. The animals had free access to water during the experiment. The results are expressed in grams of food consumed.

GH release after s.c. administration of [Dpr\textsuperscript{3}]Ghrelin and selected LEAP2(1-14) analogs to mice
The effects of LEAP2(1-14) and palm-LEAP2(1-14) on [Dpr\textsuperscript{3}]Ghrelin-induced GH release were determined in 8-week-old male C57BL/6J mice. At 8:00 a.m., freely fed mice were s.c. injected with 200 µl of saline or peptide (dissolved in saline) at a dose of 10 mg/kg of body weight (n = 5-8). Fifteen minutes after the first injection, the mice were injected with saline or [Dpr\textsuperscript{3}]Ghrelin at a dose of 1 mg/kg of body weight. Fifteen minutes after the second injection, the animals were sacrificed by decapitation, blood was collected, and the plasma was separated and stored at -80 °C until use. GH in the plasma samples was determined by a rat/mouse GH enzyme-linked immunosorbent assay kit (cat. no. EZRMGH-45K; Merck-Millipore, Burlington, Massachusetts, USA) according to the protocol recommended by the manufacturer.

Stability of selected LEAP2(1-14) analogs in rat plasma
The stability of LEAP2(1-14) and palm-LEAP2(1-14) in rat plasma was determined by liquid chromatography (LC) combined with mass spectrometry (MS). For LC, an UltiMate 3000 (Thermo, USA) consisting of a DGP-3600SD pump, a WPS-3000SL autosampler, and a TCC-3000SD column compartment was used. The MS detection of the eluates from the LC system was performed with a Q-TRAP 3200 mass spectrometer (AB Sciex, Canada).

The LC–MS method used a XBridge Premier BEH C18, particle size 2.5 µm, VanGuard Fit, and 2.1x50 mm column (Waters, USA). Mobile phase A consisted of 0.1% HCOOH in water, and mobile phase B was 0.1% HCOOH in acetonitrile. The gradient time profile was as follows: 0–5 min, from 100% A to 100% B; 5–7 min 100% B; 7–7.2 min from 100% B to 100% A; and 7.2–12 min 100% A. The flow rate was 0.200 mL/min. The column was maintained at 25 °C, and the autosampler temperature was adjusted to 15 °C. The injection volume was 2 µL. For the analysis, the data acquisition and management software Analyst version 1.6 was employed (AB Sciex). Specific MS methods utilizing multiple reaction monitoring (MRM) were developed for the studied peptides. The general MS setup was as follows: a turbo-V ion source equipped with an electrospray ionization (ESI) probe in the positive mode, ion spray voltage 5500 V, curtain gas 15 psig, source temperature 450 °C, ion source gas (1) 50 psig, and ion source gas (2) 60 psig.
Stock solutions of LEAP2(1-14) and palm-LEAP2(1-14) were prepared by dissolving the compounds in 0.1% HCOOH/H2O to a final concentration of 1 mg/mL. Blank rat plasma (90 µL) placed in 1.5 mL Protein LoBind Tubes (Eppendorf, USA) and thermostated at 37 °C (Incubator NB-T205, N-Biotek, Korea) was spiked with 10 µL of the appropriate peptide solution. All stability experiments were carried out in triplicate. At specified time intervals, i.e., 0, 1, 2, 4, 8, and 24 h, the plasma samples were taken up from the incubator, and the endogenous proteins were precipitated with 400 µL of 0.1% HCOOH in acetonitrile/H2O, 8/2 (v/v) solution. Then, the samples were centrifuged for 5 min at 14 000 × g at 4 °C (Micro Star R17, VWR, Germany) before the supernatant was transferred to 1.8 mL glass vials for the subsequent LC–MS analysis.

Statistical analysis
The data are presented as the means ± SEM. The competitive binding experiments were analyzed by GraphPad Software (San Diego, CA, USA) according to Motulsky (Motulsky and Neubig, 2002). The competitive binding curves were plotted compared to the best fit of single-binding site models. The half maximal inhibitory concentration (IC50) values were obtained from a nonlinear regression analysis, and the inhibition constants (Ki values) were calculated from the IC50 values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The dissociation constant of the radioligand (Kd) value obtained from the saturation binding experiments was 0.38 nM. The beta-lactamase assay results were analyzed by a nonlinear regression as log agonist versus response using GraphPad software. The EC50 values were determined as the concentration of the peptide that yielded 50% of the maximal response. The data are representative of at least three experiments, each performed in duplicate.

The data from the food intake and GH release experiments were calculated using GraphPad Prism software. A one-way and two-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test, was used when appropriate as described in the tables and figure legends; P < 0.1 was considered statistically significant.
Results

LEAP2, LEAP2(1-14) and its lipidized analogs compete with ghrelin for binding to GHSR1a

Based on previously published data, the affinity for GHSR1a of ghrelin, its stable analog [Dpr^3]Ghrelin (Holubova et al., 2018), LEAP2, LEAP2(1-14), and its lipidized analogs was studied. All peptides competed with ^125*I-ghrelin for binding to GHSR1a overexpressed in U2OS cells; ghrelin and [Dpr^3]Ghrelin had a K_i in a nanomolar range as shown in Figure 1. LEAP2 and its N-terminal fragment LEAP2(1-14) competed with ^125*I-ghrelin for binding to GHSR1a with K_i in the 10^-8 M range. Palmitoylation and myristoylation enhanced, but stearoylation lowered, the affinity of LEAP2(1-14) for GHSR1a.

LEAP2, LEAP2(1-14) and its lipidized analogs show inverse agonist and antagonist properties in a GHSR1a activation assay

GHSR1a activation was detected by a T-REx™ Tango™ GHSR-bla U2OS Cell-based Assay using a fluorometric microplate reader (FlexStation). Ghrelin and [Dpr^3]Ghrelin acted as strong GHSR1a agonists activating the receptor with a nanomolar EC_{50} (Figure 1 and 2A). Natural LEAP2 and lipidized LEAP2(1-14) analogs acted as inverse agonists of GHSR1a and suppressed GHSR1a internal activity with EC_{50} values in the 10^-8 M range. LEAP2(1-14) was a less effective inverse agonist, with an EC_{50} value that was three times higher.

Dose–response curves of ghrelin in the absence or presence of increasing concentrations of LEAP2 analogs served to determine the antagonist properties of all LEAP2 compounds. As shown in Figure 2B, increasing the concentration of natural LEAP2 up to 1 μM increased the EC_{50} of ghrelin by more than ten times. In contrast, nonlipidized LEAP2(1-14) had lower antagonist activity (Figure 2C). On the other hand, increasing the concentration of all lipidized LEAP2(1-14) analogs (Figure 2D-F) up to 1 μM increased the EC_{50} of ghrelin by more than one thousand times.

LEAP2(1-14) and its lipidized analogs decrease acute food intake in mice

The effects of s.c. administered LEAP2(1-14) and its lipidized analogs on acute food intake were tested in fasted mice (Figure 3), and the cumulative food intake was recorded for 420 minutes after the administration of the peptides. LEAP2(1-14) and the myristoylated and stearoylated LEAP2(1-14) analogs showed a weak anorexigenic effect, but the palmitoylated analog strongly reduced the cumulative food intake in fasted mice.
The ability of LEAP2(1-14) and its lipidized analogs to modulate the orexigenic effects of [Dpr³]Ghrelin in mice was tested in free-fed mice (Figure 4A-D). All peptides significantly reduced [Dpr³]Ghrelin-induced food intake. LEAP2(1-14) and myristoylated LEAP2(1-14) did not, but palmitoylated and stearoylated LEAP2(1-14) fully inhibited [Dpr³]Ghrelin orexigenic action.

Palm-LEAP2(1-14) analog reduces [Dpr³]Ghrelin-induced GH release

LEAP2(1-14) and its palmitoylated analog alone did not affect the plasma GH levels after an s.c. injection in 2-month-old mice. As the level of GH is naturally low in plasma, the ability of the peptides to inhibit [Dpr³]Ghrelin-induced GH release was tested (Figure 5). Palmitoylated LEAP2(1-14) significantly decreased the [Dpr³]Ghrelin-induced release of GH. However, this effect on GH release was not observed after the administration of a nonlipidized LEAP2(1-14).

Stability of LEAP2(1-14) and palm-LEAP2(1-14) in rat plasma.

As clearly shown in Figure 6, palm-LEAP2(1-14) is significantly more stable in blood plasma than its nonlipidized form. Thus, the lipidization of the original peptide improves its stability, which is highly desirable. Consequently, intact palm-LEAP2(1-14) lasts in living organisms for a rather long time, and its action is protracted.
Discussion

N-terminal fragments of LEAP2 were previously shown to sustain inverse agonist properties toward GHSR1a and decrease ghrelin-induced food intake (M’Kadmi et al., 2019). As the lipidization of peptides is described as a potential tool to increase their stability and overcome their inability to cross the blood–brain barrier (Zhang and Bulaj, 2012), we lipidized the LEAP2(1-14) peptide. Palmitoic acid not only increased the stability of the peptide, but also its affinity and its inverse agonist action on the GHSR1a receptor. Palm-LEAP2(1-14) abrogated [Dpr³]Ghrelin-induced effects on both food intake and GH release in mice.

LEAP2(1-14), which was used in this study, differs from the truncated N-terminal peptide in M’Kadmi’s study (M’Kadmi et al., 2019) by a β-Ala-Lys-NH₂ linker employed for the subsequent lipidization (Figure 1). In the in vitro studies, we confirmed that the lipidization of LEAP2(1-14) did not deteriorate its ability to bind to GHSR1a-transfected cells. LEAP2(1-14) and its lipidized analogs presented $K_i$ values comparable to that of natural LEAP2 in the $10^{-9}$-$10^{-8}$ M range. Palmitoylation and myristoylation even increased the affinity of LEAP2(1-14) for GHSR1a such that palm-LEAP2(1-14) had a higher affinity for the GHSR1a receptor than myr-LEAP2(1-14). Stearoylation slightly decreased the affinity of LEAP2(1-14) for GHSR1a but did not seem to reduce the in vivo effects of stear-LEAP2(1-14), suggesting that the better stability of the compound likely compensated for its decreased binding ability.

Based on the observation that LEAP2 did not affect GHSR1a-mediated β-arrestin recruitment, Ge et al. asserted that LEAP2 had no inverse agonist activity (Ge et al., 2018). However, this statement was disproved by M’Kadmi et al., who demonstrated the impact of LEAP2 on the basal level of inositol phosphate 1 (IP1) and the discovery that natural LEAP2 and its N-terminal fragments acted as inverse agonists (M’Kadmi et al., 2019). Moreover, M’Kadmi previously explained that β-arrestin recruitment at GHSR1a was low under basal conditions, which, thus, could lead to misleading results (M’Kadmi et al., 2015). All tested LEAP2-derived compounds decreased GHSR1a constitutive activity with an EC$_{50}$ of approximately $10^{-8}$-$10^{-7}$ M (Figure 1 and 2). Lipidized LEAP2(1-14) analogs had similar EC$_{50}$ compared to that of natural LEAP2.

Furthermore, Ge et al. claimed that LEAP2 was a noncompetitive antagonist of GHSR1a (Ge et al., 2018). However, two other studies (M’Kadmi et al., 2019; Wang et al., 2019) showed that LEAP2 acted as a competitive antagonist of GHSR1a and that LEAP2 and ghrelin shared
a common ligand-binding pocket on GHSR1a. In the present study, we verified that LEAP2 acts as a competitive antagonist (Figure 2). Ghrelin’s EC50 in the GHSR1a antagonist activity assay increased as the LEAP2 concentration increased, and LEAP2 did not change the maximal effect elicited by ghrelin (M’Kadmi et al., 2019). Similar to the inverse agonist assay, LEAP2(1-14) showed reduced antagonist activity compared with natural LEAP2. However, myr-LEAP2(1-14), palm-LEAP2(1-14) and stear-LEAP2(1-14) had even higher antagonist activity than natural LEAP2. Wang et al. explained that the differences across published studies (Ge et al., 2018; M’Kadmi et al., 2019), could be caused by the slow dissociation of LEAP2 from GHSR1a (Wang et al., 2019). LEAP2 preincubated with GHSR1a remains bound to the receptor, thus featuring noncompetitive antagonism in certain assays (Wang et al., 2019). In our study, even though LEAP2 was preincubated with GHSR1a, it acted as a competitive antagonist. Non-competitive antagonism and also lack of constitutive activity reported by Ge et al could be explained by the fact that in our study, incubation of LEAP2(1-14) analogs together with ghrelin was much longer than in Ge et al.’s study, and therefore there was enough time to establish equilibrium between ghrelin and LEAP2(1-14) analogs.

To investigate the antagonist properties of LEAP2 analogs in vivo, we focused on two well-established actions of ghrelin, namely, food intake (i) and GH release (ii).

(i) By activating GHSR1a in hypothalamic neurons, an orexigenic neural pathway is stimulated, resulting in increased food intake (Nakazato et al., 2001). Fasting is associated with increased levels of ghrelin; thus, we first tested the ability of LEAP2(1-14) and its lipidized analogs to affect fasting-induced food intake in mice (Figure 3). We already know that N-terminal LEAP2 analogs decrease food intake in fasted mice similarly to natural LEAP2 (Fernandez et al., 2022; M’Kadmi et al., 2019). Particularly, palm-LEAP2(1-14) had the highest anorexigenic effect. We tested the ability of LEAP2 analogs to suppress the orexigenic effect of exogenously administered stable ghrelin analog [Dpr3]Ghrelin used in our previous studies in free-fed mice (Maletinska et al., 2012). LEAP2(1-14) attenuated [Dpr3]Ghrelin-induced food intake to the same extent as described by M’Kadmi (M’Kadmi et al., 2019). More interestingly, palm-LEAP2(1-14) and stear-LEAP2(1-14) fully inhibited [Dpr3]Ghrelin-induced food intake (Figure 4), while myr-LEAP2(1-14) was less potent in inhibition. Overall, these data indicate that palmitoylated LEAP2(1-14) suppresses orexigenic ghrelin function similarly to natural LEAP2 in previous studies (Ge et al., 2018) and is the best of all lipidized LEAP2(1-14) analogs. Following these results, we focused on palm-
LEAP2(1-14) and nonlipidized LEAP2(1-14) to study GH release and conducted plasmatic stability experiments.

(ii) The activation of GHSR1a by ghrelin in pituitary cells led to robust GH release (Kojima et al., 1999) and natural LEAP2 suppressed ghrelin-induced GH secretion in mice (Ge et al., 2018; Islam et al., 2020). In this study (Figure 5), we compared the ability of LEAP2(1-14) and its palmitoylated analog to inhibit [Dpr3]Ghrelin-induced GH secretion. LEAP2(1-14) did not affect [Dpr3]Ghrelin-induced GH release, while palm-LEAP2(1-14) inhibited such GH release. The inability of LEAP2(1-14) to inhibit GH release might be caused by its lower stability or bioavailability in organisms compared to that of palm-LEAP2(1-14).

Finally, we tested the stability of LEAP2(1-14) and its palmitoylated analog in rat plasma (Figure 6). The stability of palm-LEAP2(1-14) was significantly higher than that of LEAP2(1-14). The prolongation of the peptide half-life by lipidization is generally achieved by its increased binding to serum albumin, which carries free fatty acids and multiple other endogenous ligands and drugs in the blood (Kurtzhals et al., 1995). If the peptide is bound to albumin and does not circulate freely in the blood, its circulation time is prolonged (Made et al., 2014).

Taken together, the *in vitro* and *in vivo* properties of the N-terminal LEAP2 peptide LEAP2(1-14) and its analogs lipidized with myristic, palmitic or stearic acid were tested and compared. All peptides exhibited a high binding affinity for GHSR1a-transfected cells and a high ability to inhibit GHSR1a constitutive activity, comparable to natural LEAP2. Palm-LEAP2(1-14) showed high *in vivo* stability and potent anorexigenic effects, and a single s.c. injection fully inhibited [Dpr3]Ghrelin-induced food intake and GH release. In conclusion, the novel LEAP2 analog palm-LEAP2(1-14) has great potential for the treatment of obesity. Our future studies will focus on further examining the interplay between LEAP2 and ghrelin in an organism and the potential actions of LEAP2 analogs in the brain.

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Footnotes
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Figures

Figure 1: Overview of lipid chains and sequences of ghrelin and LEAP2 analogs. (A) Fatty acid residues [C8, C14, C16, C18] applied in the current study. (B) Overview of sequences of ghrelin and LEAP2 analogs. Binding affinities of ghrelin and LEAP2 analogs to GHSR1a based on competition with $^{125}\text{I}$-ghrelin as the means of $K_i$ values ± SEM. $K_i$ values were calculated using the Cheng-Prusoff equation. Agonist and inverse agonist effect of peptides on GHSR1a activation presented as the means EC$_{50}$ values ± SEM. Data analyzed in Graph-Pad Software were performed in 3–5 independent experiments in duplicates.

Figure 2: Inverse agonist and antagonist GHSR1a activation potency (A) Inverse agonist mode assay showing effect of LEAP2 compounds on internal GHSR1a activation. (B-F) Antagonist mode assay showing effect of (B) LEAP2, (C) LEAP2(1-14), (D) myr-LEAP2(1-14), (E) palm-LEAP2(1-14) and (F) stear-LEAP2(1-14) on GHSR1a activation. The maximal ghrelin effect on U2OS cells expressing GHSR1a was standardized as 100%. Data are presented as the mean ± SEM. The experiments were performed in duplicates and repeated at least three times and analyzed using nonlinear regression.

Figure 3: Effect of LEAP2(1-14) analogs on cumulative food intake after s.c. administration to fasted mice. All LEAP2(1-14) analogs were administered s.c. at a dose of 5 mg/kg. The food intake was monitored every 30 min for at least 7 hours (n = 5). The data are presented as means ± SEM and were analyzed by 2-way ANOVA followed by Bonferroni post hoc test. Significance is *P < 0.1; **P < 0.01; ***P < 0.001, vs. saline-treated group (n = 5).

Figure 4: Effect of LEAP2(1-14) analogs on [Dpr$^3$]Ghrelin-induced cumulative food intake after s.c. administration to free fed mice. All LEAP2(1-14) analogs were administered s.c. at a dose of 5 mg/kg, 15 minutes after injection, the mice were injected with saline or [Dpr$^3$]Ghrelin at dose of 1 mg/kg of body weight. The food intake was monitored every 30 min for at least 7 hours. Effect of (A) LEAP2(1-14), (B) myr-LEAP2(1-14), (C) palm-LEAP2(1-14), (D) stear-LEAP2(1-14). The data are presented as means ± SEM and were analyzed by 1-way ANOVA followed by Bonferroni post hoc test. Significance is *P < 0.1; **P < 0.01; ***P < 0.001, vs. saline + [Dpr$^3$]Ghrelin treated group (n = 5).

Figure 5: Effect of LEAP2(1-14) and palm-LEAP2(1-14) on [Dpr$^3$]Ghrelin-induced GH release in 2-months-old mice. Mice were s.c. injected with 200 µl of saline or LEAP2(1-14) analogs at dose of 10 mg/kg of body weight (n = 5-8), and after 15 minutes with saline or [Dpr$^3$]Ghrelin at dose of 1 mg/kg of body weight. Blood was collected after 15 minutes and GH was measured in blood plasma using a commercially available ELISA assay kit. Data are presented as means ± SEM and were evaluated by 1-way ANOVA followed by Bonferroni post hoc test. Significance is *P < 0.1; **P < 0.01; ***P < 0.001, vs. [Dpr$^3$]Ghrelin-treated group.
Figure 6: Stability of LEAP2(1-14) and palm-LEAP2(1-14) in rat plasma monitored by LC-MS. Experiments in all time points were accomplished in triplicate. The data are presented as means ± SEM.
Fig. 1

A

octanoyl- (C8:0)
myristoyl- (C14:0)
palmitoyl- (C16:0)
stearyl- (C18:0)

B

G S S F L S P E H Q R V Q R K E S K K P P A K L Q P R
octanoyl
Ghrelin

G S D O F L S P E H Q R V Q R K E S K K P P A K L Q P R
[Dpr]Ghrelin

M T P F W R G V S L R P I G A S C R D D S E C I T
LEAP2
E Q A V S L S C R R K R C L R

<table>
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<tr>
<th>Peptide</th>
<th>Competitive binding assay $K_i$ [nM]</th>
<th>GHSR agonist potency $EC_{50}$ [nM]</th>
<th>GHSR inverse agonist potency $EC_{50}$ [nM]</th>
</tr>
</thead>
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<tr>
<td>Nle TPFWRGVSRLPIG BA K NH$_2$ LEAP2(1-14)</td>
<td>11.01 ± 0.96</td>
<td>-</td>
<td>152.54 ± 19.24</td>
</tr>
<tr>
<td>Nle TPFWRGVSRLPIG BA K myristoyl myr-LEAP2(1-14)</td>
<td>3.64 ± 0.39</td>
<td>-</td>
<td>45.38 ± 7.41</td>
</tr>
<tr>
<td>Nle TPFWRGVSRLPIG BA K NH$_2$ palm-LEAP2(1-14)</td>
<td>1.91 ± 0.09</td>
<td>-</td>
<td>52.43 ± 7.95</td>
</tr>
<tr>
<td>Nle TPFWRGVSRLPIG BA K NH$_2$ stearoyl stear-LEAP2(1-14)</td>
<td>53.62 ± 7.88</td>
<td>-</td>
<td>51.11 ± 7.88</td>
</tr>
</tbody>
</table>
Fig. 2
Fig. 3

- saline
- LEAP2(1-14)
- myr-LEAP2(1-14)
- palm-LEAP2(1-14)
- stear-LEAP2(1-14)

food intake [g]

0.0 | 0.5 | 1.0

0 | 100 | 200 | 300 | 400

time [min]

* * ** *** ****

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Fig. 4
Fig. 5

GH in plasma [ng/ml]

- Saline
- [Dpr²]Ghrelin
- LEAP2(1-14)
- [Dpr²]Ghrelin + LEAP2(1-14)
- [Dpr²]Ghrelin + palm-LEAP2(1-14)

Significance levels: ***
Fig. 6