International Journal of
Molecular Sciences

MDPI

Article

Palmitoylation of Prolactin-Releasing Peptide Increased Affinity for and Activation of the GPR10, NPFF-R2 and NPFF-R1 Receptors: In Vitro Study

Alena Karnošová [1,2], Veronika Strnadová [1], Lucie Holá [1,2], Blanka Železná [1], Jaroslav Kuneš [1,3] and Lenka Maletínská [1,*]

[1] Biochemistry and Molecular Biology, Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, 16610 Prague, Czech Republic; alena.karnosova@uochb.cas.cz (A.K.); veronika.strnadova@uochb.cas.cz (V.S.); lucie.cerna@uochb.cas.cz (L.H.); zelezna@uochb.cas.cz (B.Ž.); kunes@biomed.cas.cz (J.K.)
[2] First Faculty of Medicine, Charles University, 12108 Prague, Czech Republic
[3] Experimental Hypertension, Institute of Physiology of the Czech Academy of Sciences, 14200 Prague, Czech Republic
* Correspondence: maletin@uochb.cas.cz; Tel.: +420-220-183567

Abstract: The anorexigenic neuropeptide prolactin-releasing peptide (PrRP) is involved in the regulation of food intake and energy expenditure. Lipidization of PrRP stabilizes the peptide, facilitates central effect after peripheral administration and increases its affinity for its receptor, GPR10, and for the neuropeptide FF (NPFF) receptor NPFF-R2. The two most potent palmitoylated analogs with anorectic effects in mice, palm^{11}-PrRP31 and palm-PrRP31, were studied in vitro to determine their agonist/antagonist properties and mechanism of action on GPR10, NPFF-R2 and other potential off-target receptors related to energy homeostasis. Palmitoylation of both PrRP31 analogs increased the binding properties of PrRP31 to anorexigenic receptors GPR10 and NPFF-R2 and resulted in a high affinity for another NPFF receptor, NPFF-R1. Moreover, in CHO-K1 cells expressing GPR10, NPFF-R2 or NPFF-R1, palm^{11}-PrRP and palm-PrRP significantly increased the phosphorylation of extracellular signal-regulated kinase (ERK), protein kinase B (Akt) and cAMP-responsive element-binding protein (CREB). Palm^{11}-PrRP31, unlike palm-PrRP31, did not activate either c-Jun *N*-terminal kinase (JNK), p38, c-Jun, c-Fos or CREB pathways in cells expressing NPFF-1R. Palm-PrRP31 also has higher binding affinities for off-target receptors, namely, the ghrelin, opioid (KOR, MOR, DOR and OPR-L1) and neuropeptide Y (Y_1, Y_2 and Y_5) receptors. Palm^{11}-PrRP31 exhibited fewer off-target activities; therefore, it has a higher potential to be used as an anti-obesity drug with anorectic effects.

Keywords: prolactin-releasing peptide; GPR10; neuropeptide FF; NPFF-R2; NPFF-R1; binding properties; signaling pathways

Citation: Karnošová, A.; Strnadová, V.; Holá, L.; Železná, B.; Kuneš, J.; Maletínská, L. Palmitoylation of Prolactin-Releasing Peptide Increased Affinity for and Activation of the GPR10, NPFF-R2 and NPFF-R1 Receptors: In Vitro Study. *Int. J. Mol. Sci.* **2021**, *22*, 8904. https://doi.org/10.3390/ijms22168904

Academic Editor: Clara Balsano

Received: 1 July 2021
Accepted: 12 August 2021
Published: 18 August 2021

1. Introduction

Prolactin-releasing peptide (PrRP) was discovered as an endogenous ligand of the orphan G-protein coupled receptor GPR10 (also known as hGR3) in the hypothalamus and has been suggested to stimulate prolactin secretion [1,2]. However, soon after this finding, Lawrence et al. showed a reduction in food intake and body weight and an increase in energy expenditure after intracerebroventricular (ICV) PrRP injection in rats and questioned the role of PrRP in prolactin secretion [3,4]. The effects of PrRP, mostly mediated through the GPR10 receptor, which is widely expressed throughout the brain mainly in areas related to the regulation of food intake and energy homeostasis, confirm GPR10 knockout (KO) mouse studies showing an increase in body weight in KO mice [5–7].

PrRP occurs in two biologically active isoforms, PrRP31 and PrRP20. Our previous studies showed the induction of central c-Fos activation of regions related to food intake

after peripheral administration of PrRP31 or PrRP20 modified with either myristoyl or palmitoyl, but this central effect was not observed after peripheral administration of natural PrRP31 or PrRP20. Lipidized PrRP31 and PrRP20 analogs decrease food intake and body weight in mice, increase stability and prolong half-life compared to natural peptides [8–12]. PrRP20 and PrRP31 also strongly interact with the receptor of neuropeptide FF (NPFF), NPFF-R2 [13]. Lipidization of PrRP20 and PrRP31 increases in vitro binding affinities not only to GPR10 but also to NPFF-R2 [8,9]. However, lipidized PrRP20 showed lower solubility and bioavailability [8]; therefore, our further studies were focused on lipidized PrRP31 analogs.

PrRP, together with NPFF, belongs to the RF-amide peptide family, which contains a typical *C*-terminal amino acid sequence motif (RF-NH_2) essential for receptor activation. All RF-amide peptides have a high affinity for and activity on both NPFF receptors NPFF-R2 and NPFF-R1 and may also exert in vivo effects through these receptors [14]. Expression of both NPFF receptors has been found in hypothalamic areas that regulate feeding and energy homeostasis. Moreover, the ability of NPFF to regulate food intake was previously demonstrated, when ICV administration of NPFF was shown to result in decreased food intake in fasted rats [15,16].

Both NPFF receptors show the ability to regulate the cardiovascular system and modulate pain perceptions [17–19]. Despite the fact that antagonist of NPFF-R1 and NPFF-R2 RF9 prevents opioid-induced hyperalgesia and that NPFF induces an increase in arterial blood pressure in rats [20], our previous study did not prove the antagonistic activity of RF9 on NPFF-induced anorexigenic effects [21]. Conversely, RF9 exhibits an anorectic effect after ICV or subcutaneous administration in fasted mice [21].

Similar to NPFF, PrRP also appears to have antinociceptive properties [22,23]. Although PrRP has a high affinity for NPFF receptors, its ability to modulate pain perception through NPFF-1R and NPFF-2R has not been proven. Kalliomäki et al. studied the nociceptive properties of 1DMe, a stable NPFF analog, and PrRP in the central nervous system of rats and refuted the ability of PrRPs to regulate pain perception through NPFF receptors [22].

Many G-protein coupled receptors (GPCRs) share similar characteristic features. Receptors GPR10, NPFF-R1 and NPFF-R2 are members of the β-type rhodopsin GPCR family, which has important roles in the regulation of food intake and energy homeostasis [24]. GPR10 has a high percentage of amino sequence identity, especially in the transmembrane regions, with neuropeptide Y receptors, members of the β-type rhodopsin GPCR family, which are involved in food intake regulation [25]. Furthermore, Y receptors share a high percentage of amino sequence homology with NPFF-R1 and NPFF-R2 [26].

The mechanism of action of PrRP is not yet fully understood. PrRP31 and PrRP20 have been shown to mobilize Ca^{2+} from intracellular stores via GPR10 by activating the second messenger IP3 (inositol-1,4,5-trisphosphate), leading to an increase in cytoplasmic Ca^{2+} [27,28], which can subsequently activate the extracellular signal-regulated kinase (ERK) signaling cascade [29]. PrRPs displayed the ability to activate the phosphorylation of ERK, the c-Jun *N*-terminal kinase (JNK) pathway, the cAMP-responsive element binding protein (CREB) pathway and the protein kinase B (Akt/PKB) pathway, which plays a key role in the regulation of protein synthesis and maintenance of glucose homeostasis [30–32].

Maixnerová et al. previously showed that the first 20 amino acids of PrRP31 are important for the preservation of full in vivo activity [31]. This study compares the activity of two most potent PrRP31 analogs, palm^{11}-PrRP31 and palm-PrRP31, which contain palmitic acid attached to the N-terminus of the amino acid chain (palm-PrRP31) or to the position 11, where original Arg^{11} was replaced with Lys^{11} (palm^{11}-PrRP31) (Table 1). These analogs previously showed the ability to significantly decrease food intake and body weight after repeated peripheral administration [8,9], but the mechanism of action is still unclear. We aimed to identify the off-target activity of palm^{11}-PrRP31 and palm-PrRP31 to map the mechanism of action and to compare intracellular transduction pathways of anorexigenic receptors GPR10, NPFF-R2, and new strong target of PrRP31 analogs, NPFF-

R1. GPR10 is a highly selective receptor for PrRP31 and analogs related to PrRP31. To control the selectivity of PrRP31 for GPR10s, we used NPFF and its stable analog 1DMe in this study. To determine whether the possible analgesic effect of PrRPs is caused by off-target activity, opioid receptors were investigated.

Table 1. Structures of human prolactin-releasing peptide 31 (PrRP31), neuropeptide FF (NPFF) and its analogs.

Analog	Sequence
PrRP31	SRAHQHSMETRTPDINPAWYTGRGIRPVGRF-NH_2
$Palm^{11}$-PrRP31	SRTHRHSMEIK(γ-E (N-palm))TPDINPAWYASRGIRPVGRF-NH_2
Palm-PrRP31	(N-palm)SRTHRHSMEIRTPDINPAWYASRRGIRPVGRF-NH_2
NPFF	FLFQPQRF-NH_2
1DMe	yL(N-Me)FQPQRF-NH_2

2. Results

2.1. Binding Affinity for GPR10, NPFF-R2 and Potential Off-Target Receptors

2.1.1. Palmitoylated PrRP31 Analogs Have a High Binding Affinity for GPR10, NPFF-R2 and NPFF-R1

Based on previously published data, affinity for the GPR10 and NPFF-R2 of PrRP31 and its analogs was studied [8,9]. PrRP31 and its two palmitoylated analogs of PrRP31 (see Table 1 for structures) have a high binding affinity for the GPR10 and NPFF-R2 receptors, and their K_i values were in the nanomolar range (Table 2). Compared to natural PrRP31, palmitoylated analogs had a higher binding affinity for both of these receptors. $Palm^{11}$-PrRP31 showed a higher affinity for the receptor GPR10 than for the receptor NPFF-R2. NPFF and its stable analog 1DMe displayed negligible affinity for the GPR10 receptor. The affinities of NPFF and 1DMe to NPFF-R2 were detected to be in the nanomolar range (Table 2).

Table 2. Binding affinities of natural PrRP31, its analogs and other peptides to tested receptors.

Receptor	**GPR10**	**NPFF-R2**	**NPFF-R1**	**KOR**
	[^{125}I]-PrRP31	**[^{125}I]-1DMe**	**[^{125}I]-1DMe**	**[^{125}I]-Dynorphin**
		K_i [nM]		
PrRP31	4.58 ± 0.66	26.73 ± 9.01	40.39 ± 4.20	>10,000
$Palm^{11}$-PrRP31	3.44 ± 0.36	7.66 ± 1.33	13.52 ± 1.57	4278 ± 866
Palm-PrRP31	4.04 ± 0.01	0.77 ± 0.19	0.78 ± 0.11	106 ± 15
NPFF	>10,000	0.28 ± 0.06	1.08 ± 0.09	-
1DMe	>10,000	1.03 ± 0.23	0.79 ± 0.06	-
Dynorphin	-	-	-	0.36 ± 0.03
Receptor	**Y_1**	**Y_2**	**Y_5**	**GHSR**
	[^{125}I]-PYY	**[^{125}I]-PYY**	**[^{125}I]-PYY**	**[^{125}I]-Ghrelin**
		K_i [nM]		
PYY	2.92 ± 0.28	6.51 ± 0.71	3.06 ± 0.49	-
PrRP31	>10,000	>10,000	2863 ± 43	>10,000
$Palm^{11}$-PrRP31	>10,000	>10,000	362 ± 96	2800 ± 466
Palm-PrRP31	3147 ± 31	>10,000	32.62 ± 6.16	160 ± 16
Ghrelin	-	-	-	4.59 ± 0.41

- not determined; data presented as the means K_i values ± SEM and analyzed in Graph-Pad Software were performed in 2–5 independent experiments in duplicates. K_i was calculated using the Cheng-Prusoff equation [33].

To find another possible target of the two most potent palmitoylated analogs of PrRP31, binding to NPFF-R1 was tested. Membranes from CHO-K1 cells expressing the NPFF-R1 were isolated, and the K_d was determined to be 0.94 ± 0.06 nM by saturation experiments using the radioligand [^{125}I]-1DMe. Although natural PrRP31 bound to NPFF-R1 with a

lower affinity than to NPFF-R2, the binding affinity was still in the 10^{-8} M range (Table 2). Palmitoylation increased the binding affinities of both analogs to NPFF-R1. Palm-PrRP31 showed binding affinities in the nanomolar range to both NPFF receptors compared to palm^{11}-PrRP31 (Table 2).

2.1.2. Palm-PrRP31 Shows a Higher Affinity for Other Potential Off-Target Receptors than Palm^{11}-PrRP31

Several other potential off-target receptors of PrRP31 and its palmitoylated analogs were tested. The binding properties of PrRP31, palm^{11}-PrRP and palm-PrRP31 to receptors Y_1, Y_2, and Y_5, ghrelin receptor (also growth hormone secretagogue receptor—GHSR) and kappa-opioid receptor (KOR) were determined. The natural ligand PYY of Y receptors bound in the nanomolar range to the Y_1, Y_2, and Y_5 receptors (Table 2). From saturation binding experiments with [^{125}I]-PYY as a radioligand, the K_d for each receptor was determined. The K_d for Y_1 was 1.53 ± 0.08 nM, for Y_2 was 2.18 ± 0.85 nM and for Y_5 was 1.01 ± 0.27 nM. Natural PrRP31 had no affinity to the Y_1 and Y_2 receptors in the range of measured concentrations, but it showed a very low affinity to the Y_5 receptor. Compared to palm^{11}-PrRP31, palm-PrRP31 exhibited a relatively high affinity for the Y_5 receptor. Both palmitoylated analogs bound to Y_1 and Y_2 with a negligible low affinity (Table 2).

The K_d determined by a saturation binding experiment with [^{125}I]-dynorphin as a radioligand was 2.38 nM. The agonist dynorphin showed a very high affinity for the KOR receptor, but no binding was observed with natural PrRP31 (Table 2). Palmitoylation enhanced binding to the KOR receptor. Palm-PrRP31 bound to KOR with a higher affinity than palm^{11}-PrRP31, but both were in the 10^{-7}–10^{-6} M range.

Another tested potential off-target receptor was the ghrelin receptor GHSR. From saturation experiments using [^{125}I]-ghrelin as a radioligand, a K_d of 0.44 ± 0.12 nM was determined. Natural PrRP31 showed no binding to GHSR in competitive binding experiments in the measured range, but palmitoylated analogs showed a low binding affinity for this receptor (Table 2). Palm-PrRP31 had a higher affinity for GHSR than palm^{11}-PrRP31.

2.2. PrRP31 and Its Palmitoylated Analogs Stimulate Ca^{2+} Mobilization in CHO-K1 Cells Expressing GPR10 or NPFF-R2

Stimulation of Ca^{2+} in CHO-K1 cells expressing the GPR10 receptor was monitored using the calcium-sensitive dye Fura 2. No calcium mobilization was observed after stimulation with the NPFF-R2 agonists NPFF and 1DMe (Figure 1). On the other hand, natural PrRP31, palm^{11}-PrRP31 and palm-PrRP31 stimulated Ca^{2+} mobilization. Both of the lipidized analogs showed a similar Ca^{2+} release response, which was observed at lower concentrations compared to PrRP31.

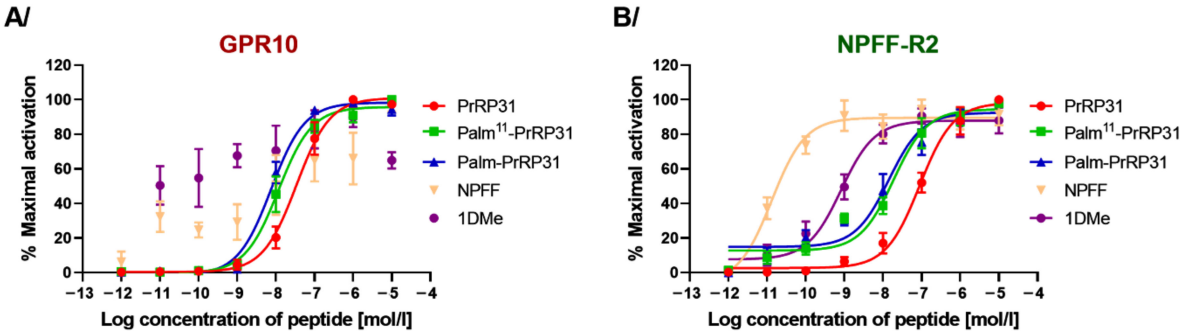


Figure 1. Intracellular Ca^{2+} mobilization in CHO-K1 cells expressing (**A**) GPR10 or (**B**) NPFF-R2. Data are presented as mean ± SEM, and the experiments were performed in duplicates and repeated two (GPR10) or three (NPFF-R2) times in duplicates.

The CHO-K1 cell line expressing NPFF-R2 with aequorin protein, which detects intracellular Ca^{2+} release, was used to study the agonist properties of PrRP31, its palmitoylated

analogs, NPFF and 1DMe. NPFF (EC_{50}, 0.24 ± 0.02 pM) and 1DMe (EC_{50}, 0.82 ± 0.15 nM) stimulated intracellular Ca^{2+} release at much lower concentrations than the GPR10 agonist PrRP31 (EC_{50}, 89.33 ± 0.84 nM) and its lipidized analogs. Palmitoylation of PrRP31 increased agonist activity at NPFF-R2, where the EC_{50} of palm^{11}-PrRP31 was 18.71 ± 1.31 nM and that of palm-PrRP31 was 14.16 ± 1.52 nM (Figure 1).

2.3. Palmitoylated PrRP31 Analogs Activate Different Intracellular Signaling Pathways in GPR10-, NPFF-R2- or NPFF-R1-Expressing Cells

To determine the intracellular mechanism of action of PrRP31 and its palmitoylated analogs, several signaling pathways were tested in cells expressing GPR10, NPFF-R2 or NPFF-R1 receptors using immunoblotting (Figures 2–5; Supplementary Figure S1). No changes in total protein levels were observed (Supplementary Figure S1B); therefore, only activated/phosphorylated proteins were quantified and compared. NPFF and 1DMe were used as negative controls to validate GPR10 selective properties.

To study PKB/Akt pathway activation, phosphorylation of Akt at Ser473 (Figure 2A) and Thr308 (Figure 2B) was tested. Both PrRP31 analogs, palm^{11}-PrRP31 and palm-PrRP31, showed significantly increased phosphorylation of Akt at Ser473 (Figure 2A) and Thr308 (Figure 2B) in cells with GPR10 and NPFF-R2 but also in cells expressing NPFF-R1. Natural PrRP31 did not significantly activate Akt (Figure 2) in cells with NPFF-R1. NPFF and 1DMe increased the phosphorylation of Akt at either Ser473 (Figure 2A) or Thr308 (Figure 2B) in cells containing NPFF-R2 and NPFF-R1, but they were less effective at GPR10.

The activation of the cAMP-dependent protein kinase (PKA) was also studied (Supplementary Figure S1A). No significant changes were observed after treatment with PrRP31, palmitoylated PrRP31 analogs, NPFF or 1DMe in cells expressing GPR10, NPFF-R2 and NPFF-R1.

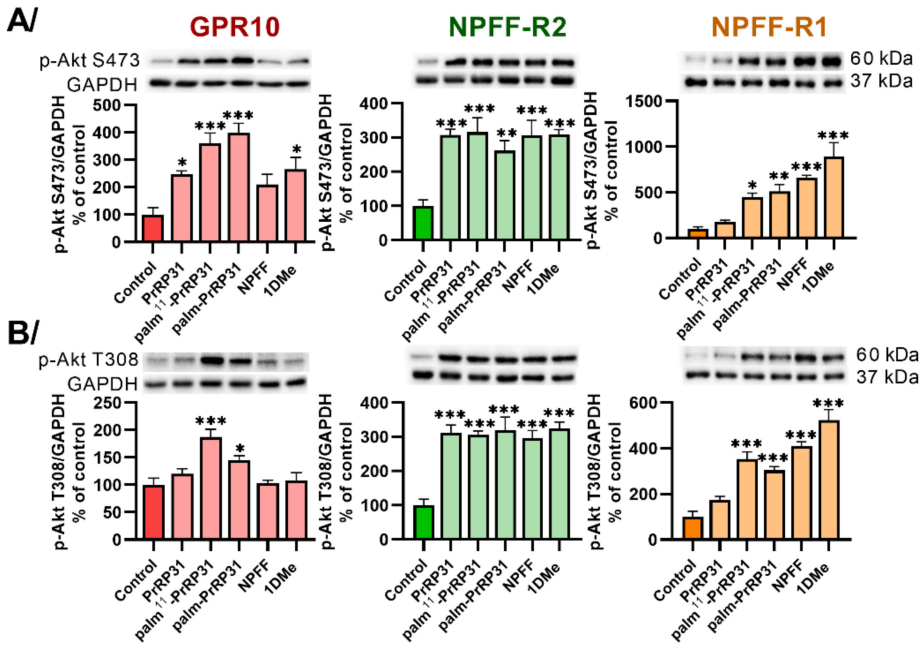


Figure 2. Induction of (**A**) Akt (S473) and (**B**) Akt (T308) phosphorylation after 5 min of incubation at 37 °C with peptides at final concentrations of 10^{-6} M in CHO-K1 cells expressing receptors GPR10, NPFF-R2 and NPFF-R1. Densitometric quantification was normalized to GAPDH, and the phosphorylation level in the untreated control was standardized as 100%. Data are presented as the mean ± SEM and analyzed by two-way ANOVA followed by Dunnett's post hoc test. Experiments were performed independently at least three times. Statistically significant differences from the control are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

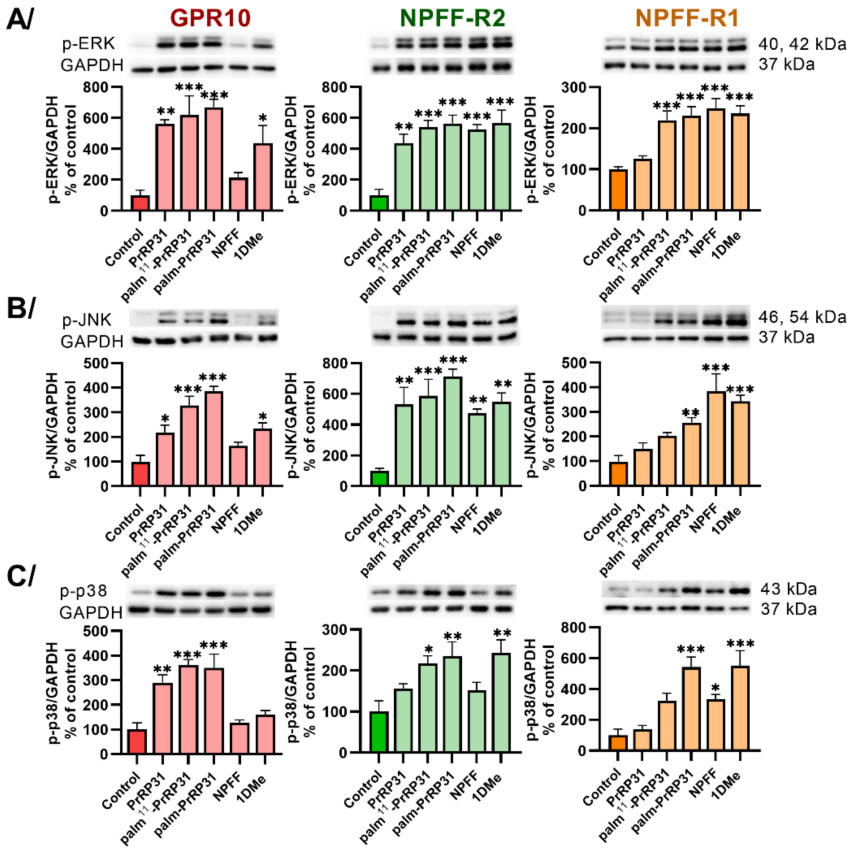


Figure 3. Induction of MAPK pathways: phosphorylation of (**A**) ERK and (**B**) JNK after 5 min and (**C**) p38 after 60 min of incubation at 37 °C with peptides at final concentrations of 10^{-6} M in CHO-K1 cells expressing receptors GPR10, NPFF-R2 and NPFF-R1. Densitometric quantification was normalized to GAPDH, and the phosphorylation level in the untreated control was standardized as 100%. Data are presented as the mean ± SEM and analyzed by two-way ANOVA followed by Dunnett's post hoc test. Experiments were performed independently at least three times. Statistically significant differences from the control are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

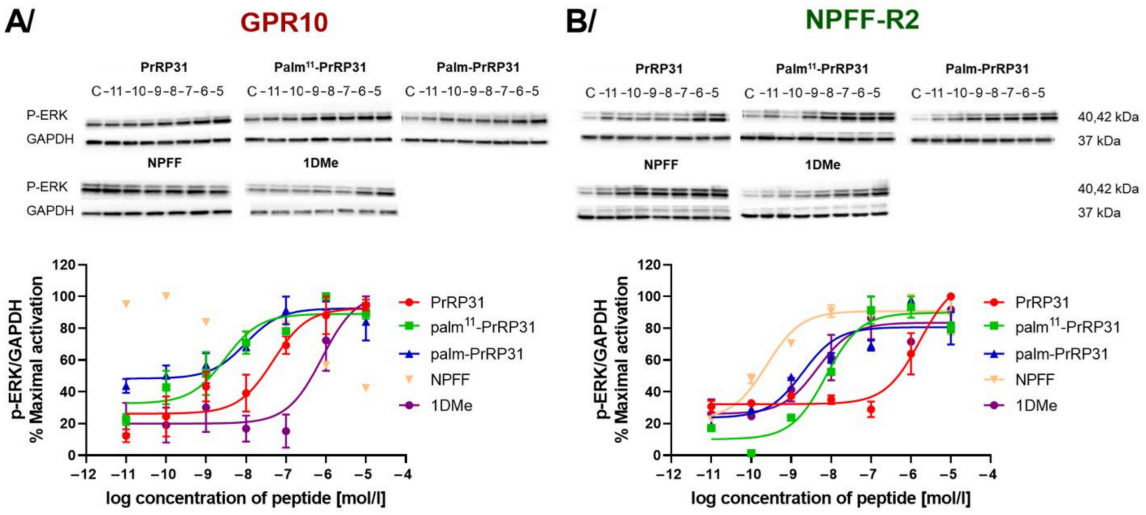


Figure 4. Dose-response phosphorylation of ERK in CHO-K1 cells expressing (**A**) GPR10 and (**B**) NPFF-R2 after 5 min of incubation at 37 °C with peptides at final concentrations from 10^{-11} to 10^{-5} M. Densitometric quantification was normalized to GAPDH. Data are presented as the mean ± SEM, and the experiments were performed independently at least two times and were analyzed using nonlinear regression.

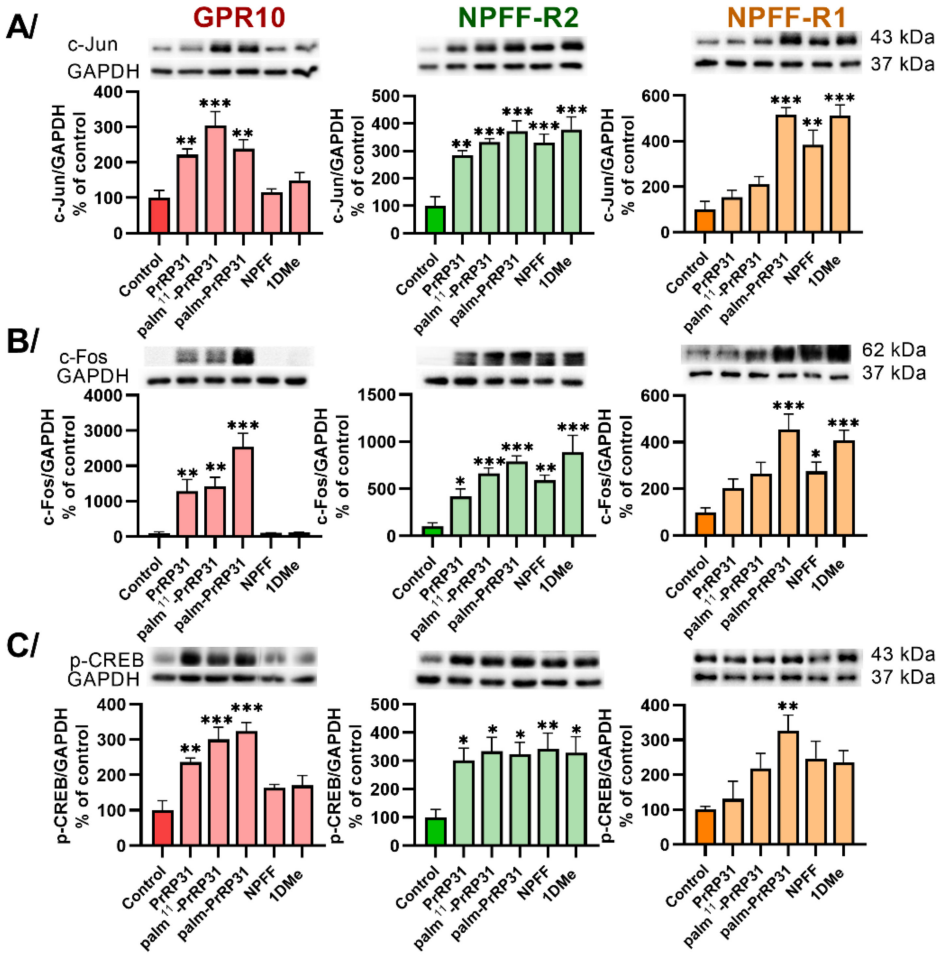


Figure 5. Activation of (**A**) c-Jun and (**B**) c-Fos after 60 min incubation and induction of (**C**) CREB phosphorylation after 5 min incubation at 37 °C with peptides in final concentrations 10^{-6} M in CHO-K1 cells expressing receptors GPR10, NPFF-R2 and NPFF-R1. Densitometric quantification was normalized to GAPDH and the phosphorylation level in the untreated control was standardized as 100%. Data are presented as mean ± SEM and analyzed by two-way ANOVA followed by Dunnett's post hoc test. Experiments were performed independently at least three times. Statistically significant differences from the control are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

One of the key signaling pathways of GPCR signaling, the mitogen-activated protein kinase (MAPK) pathway, was also studied. The phosphorylation of MAPKs, ERK, JNK and p38 was significantly increased in CHO-K1 cells expressing receptors GPR10, NPFF-R2 and NPFF-R1 after stimulation with palm-PrRP31 (Figure 3A–C). Palm^{11}-PrRP31 significantly increased the phosphorylation of ERK, JNK and p38 in CHO-K1 cells expressing GPR10 and NPFF-R2 (Figure 3A–C), but no significant increase in JNK and p38 was observed in cells with NPFF-R1 (Figure 3B,C). Natural PrRP31 was effective in cells expressing GPR10 and NPFF-R2 but did not activate ERK (Figure 3A), JNK (Figure 3B) or p38 (Figure 3C) in cells transfected with NPFF-R1.

To further characterize the signaling of receptors GPR10 and NPFF-R2, dose-response experiments were performed. The EC_{50} of ERK activation in cells expressing GPR10 was in the nanomolar range after stimulation with PrRP31, palm^{11}-PrRP and palm-PrRP31 (Figure 4A). Cells expressing NPFF-R2 showed a strong response with EC_{50} in nanomolar concentrations after stimulation with natural PrRP31, palmitoylated PrRP31 analogs, NPFF or 1DMe (Figure 4B).

Finally, three DNA-binding proteins, cyclic AMP-responsive element-binding (CREB), c-Jun and c-Fos protein, which activate transcription factors, were tested (Figure 5). Palm-

PrRP31 significantly increased the activation of c-Jun (Figure 5A) and c-Fos (Figure 5B) and the phosphorylation of CREB (Figure 5C) compared to the nontreated control in the CHO-K1 cells expressing GPR10, NPFF-R2 or NPFF-R1. Stimulation with palm^{11}-PrRP31 significantly increased the activation of all three DNA-binding proteins (Figure 5) in cells with GPR10 and NPFF-R2, but was ineffective in cells expressing the NPFF-R1 receptor. No activation in GPR10 after stimulation with NPFF and its stable analog 1DMe was observed, unlike in NPFF-R2 or NPFF-R1, where significantly increased activation was monitored.

The results showing signaling pathway activation determined using immunoblotting in CHO-K1 cells expressing GPR10, NPFF-R2 and NPFF-R1 incubated with peptides at final concentrations of 10^{-6} M are summarized in Table 3.

Table 3. Summary table of signaling pathways tested using immunoblot in cells expressing GPR10, NPFF-R2 and NPFF-R1.

	PrRP31			**Palm^{11}-PrRP31**			**Palm-PrRP31**			**NPFF**			**1DMe**		
Receptor	**GPR10**	**NPFF-R2**	**NPFF-R1**	**GPR10**	**NPFF-R2**	**NPFF-R1**	**GPR10**	**NPFF-R2**	**NPFF-R1**	**GPR10**	**NPFF-R2**	**NPFF-R1**	**GPR10**	**NPFF-R2**	**NPFF-R1**
p-ERK	↑ **	↑ **	-	↑ ***	↑ ***	↑ ***	↑ ***	↑ ***	↑ ***	-	↑ ***	↑ ***	↑ *	↑ ***	↑ ***
p-JNK	↑ *	↑ **	-	↑ ***	↑ ***	-	↑ ***	↑ ***	↑ **	-	↑ **	↑ ***	↑ *	↑ **	↑ ***
p-p38	↑ **	-	-	↑ ***	↑ *	-	↑ ***	↑ **	↑ ***	-	-	↑ *	-	↑ **	↑ ***
p-Akt S473	↑ *	↑ ***	-	↑ ***	↑ ***	↑ *	↑ ***	↑ **	↑ **	-	↑ ***	↑ ***	↑ *	↑ ***	↑ ***
p-Akt T308	-	↑ ***	-	↑ ***	↑ ***	↑ ***	↑ *	↑ ***	↑ ***	-	↑ ***	↑ ***	-	↑ ***	↑ ***
p-PKA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c-Jun	↑ **	↑ **	-	↑ ***	↑ ***	-	↑ **	↑ ***	↑ ***	-	↑ ***	↑ **	-	↑ ***	↑ ***
c-Fos	↑ **	↑ *	-	↑ **	↑ ***	-	↑ ***	↑ ***	↑ ***	-	↑ **	↑ *	-	↑ ***	↑ ***
p-CREB	↑ **	↑ *	-	↑ ***	↑ *	-	↑ ***	↑ *	↑ **	-	↑ **	-	-	↑ *	-

↑ significant activation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), - no significant changes.

2.4. Agonist and Antagonist Properties of PrRP31 and Its Palmitoylated Analogs at Other Potential Off-Target Receptors

Using the beta-lactamase reporter gene assay with a FRET substrate, receptor activation was studied to establish agonist and antagonist properties of natural PrRP31 and palmitoylated PrRP31 analogs.

Both tested palmitoylated PrRP analogs were strong agonists of the GPR10 receptor, and their EC_{50} values were in the picomolar range (Table 4). Palm^{11}-PrRP31 had stronger agonist activity on GPR10 than the analog palm-PrRP31.

Table 4. Agonist properties on GPR10 and other potential off-target receptors determined using β-lactamase assay.

Receptor	**GPR10**	**Y_5**	**GHSR**	**KOR**	**DOR**	**MOR**	**ORL-1**
	EC_{50} [pM]			**EC_{50} [nM]**			
PrRP31	530.3 ± 70.5	N	N	N	N	N	N
PYY		19.4 ± 2.5					
Ghrelin			2.8 ± 2.5				
U-50488				1.4 ± 1.0			
Deltorphin II					5.6 ± 9.9		
DAMGO						14.7 ± 1.9	
Nociceptin							3.8 ± 0.6
Palm^{11}-PrRP31	39.1 ± 5.1	583.3 ± 121.1	1068.1 ± 272.2	>10,000	N	N	N
Palm-PrRP31	71.8 ± 6.4	56.5 ± 18.4	1273.5 ± 167.9	>10,000	N	N	N

Data presented as the means EC_{50} values ± SEM and analyzed in Graph-Pad Software and performed in 2–3 independent experiments in duplicates; N-no agonist properties.

Natural PrRP31 was not effective at any tested possible off-target receptor. Both palm^{11}-PrRP31 and palm-PrRP31 did not show any agonist activity on the DOR, MOR and

ORL-1 opioid receptors, but they did have very weak agonist activity on the KOR (Table 4). In addition, lipidized analogs exerted weak agonist effects on GHSR.

Compared to palm^{11}-PrRP31, palm-PrRP31 showed much stronger agonist activity on GHSR and the Y_5 receptor (Table 4); therefore, antagonist activity on receptors Y_5, GHSR and opioid receptors was tested only with PrRP31 and palm^{11}-PrRP31. No antagonist properties of PrRP31 and palm^{11}-PrRP31 were observed with receptors Y_5 (Figure 6), GHSR or opioid receptors (KOR, DOR, MOR, ORL-1) (Supplementary Figure S2A,B). Palm^{11}-PrRP31 was shown to be a positive allosteric modulator for the Y_5 receptor, enhancing PYY activity (Figure 6B).

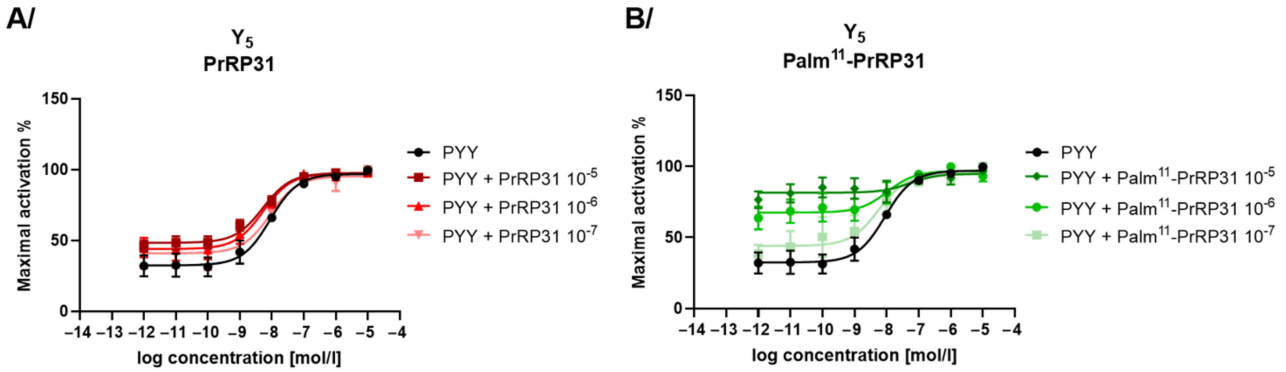


Figure 6. Antagonist mode assay showing effect of (**A**) PrRP31 and (**B**) palm^{11}-PrRP31 at Y_5 receptor together with PYY agonist. Data are presented as mean ± SEM, and the experiments were performed in duplicates and repeated at least two times and analyzed using nonlinear regression.

3. Discussion

Palmitoylated analogs of neuropeptide PrRP31 previously showed anorexigenic effects and central c-Fos activation after peripheral administration, as well as increased central insulin and leptin signaling, suggesting great potential for the treatment of not only obesity but also neurodegenerative disorders [9,34,35]. PrRP31 has a high affinity for its receptor GPR10, but it also binds to NPFF-R2 [13]. Based on the results of our previous studies the mechanism of action of the two most potent palmitoylated PrRP31 analogs, palm^{11}-PrRP31 and palm-PrRP31 on the anorexigenic receptors GPR10 and NPFF-R2 was mapped.

Palmitoylation increased the binding properties of PrRP31 to both of these receptors. Palm^{11}-PrRP31 had a higher affinity for the GPR10 receptor than palm-PrRP31, and both analogs displayed an affinity for the NPFF-R2 in the nanomolar range. In this study, several possible off-target receptors of PrR31 were tested. Both of the PrRP31 analogs showed a stronger affinity for the NPFF-R1 than natural PrRP31. Therefore, NPFF-R1 is now considered another relevant target of lipidized PrRP31 analogs.

The activation of intracellular Ca^{2+} mobilization in the CHO-K1 AequoScreen cell line expressing NPFF-R2 showed the agonist properties of PrRP31 and its palmitoylated analogs. Palmitoylation increased the agonist properties of PrRP31 on the receptor NPFF-R2. However, NPFF and its stable analog 1DMe have much stronger agonist activity on its NPFF-R2 receptor than palm^{11}-PrRP31 and palm-PrRP31. The activation of GPR10 was studied using the β-lactamase assay with a FRET substrate and a FLIPR calcium assay measuring intracellular Ca^{2+} mobilization. The EC_{50} values of palm^{11}-PrRP31 and palm-PrRP31 were in the picomolar range, and the activation was increased three times after palmitoylation. Previous studies suggested that GPR10 is coupled with Gi/o proteins [30,32]. Other studies have shown the ability of PrRP to stimulate cAMP in rat PC12 cells [36] and CHO-K1 cells expressing GPR10 [37], which pointed to Gs protein coupling. However, Langmead et al. revealed the PrRP-induced mobilization of intracellular Ca^{2+} after GPR10 activation, and the PrRP's inability to suppress cAMP levels after forskolin stimulation in HEK293 cells transfected with GPR10. These results suggested that GPR10 is coupled with the Gq

protein [27]. We observed intracellular Ca^{2+} mobilization after stimulation with PrRP31 and its palmitoylated analogs, which may suggest that GPR10 is coupled with either Gi or Gq. This also supported our finding that PKA was not activated after stimulation with PrRP31 and its palmitoylated analogs; thus, GPR10 was not coupled with Gs proteins.

In this study, the intracellular signaling pathways of PrRP31 and its palmitoylated analogs in CHO-K1 cells transfected with GPR10, NPFF-R2, or NPFF-R1 were explored using immunoblotting, and the possible signal transduction of GPR10 was suggested (Figure 7). Haykawa et al. previously showed the activation of Akt in rat pituitary GH3 cells after 5 min of stimulation with PrRP [32]. Our study found significant induction of Akt phosphorylation at T308 and S473 in CHO-K1 cells expressing GPR10, NPFF-R2 and NPFF-R1 after 5 min of stimulation with palm^{11}-PrRP31 and palm-PrRP31, but no significantly increased phosphorylation was observed after stimulation with natural PrPR31 in cells with NPFF-R1. Palmitoylation helped stabilize PrRP31 and increased the induction activity of Akt through the receptors GPR10, NPFF-R2 and NPFF-R1. Previous studies demonstrated that PrRP activated the MAP kinases ERK and JNK in rat GH3 cells [30] and PC12 cells [38]. Our results showed significant activation of JNK, ERK and p38 MAPKs after PrRP31 incubation in CHO-K1 cells expressing GPR10, and of JNK and ERK in cells with NPFF-R2. Both palmitoylated PrRP31 analogs also significantly increased the phosphorylation of all three tested MAPKs in GPR10 and NPFF-R2-expressing cells. Dose-response experiments showed the ability of PrRP31, palm^{11}-PrRP31 and palm-PrRP31 to activate ERK phosphorylation in cells with GPR10 and NPFF-R2 in the nanomolar range. JNK and ERK activation play important roles in cell proliferation, differentiation and apoptosis by promoting the formation of AP1 complexes, important transcription factors controlling the cell cycle, through the activation of c-Fos and c-Jun [39]. Similar to ERK and JNK, p38 is also connected with cell cycle regulation, regulation of stress responses, immune responses and cell differentiation [40]. Palm^{11}-PrRP31 and palm-PrRP31 were found to significantly increase p38 phosphorylation in cells expressing GPR10 and NPFF-R2, and stimulation with palm-PrRP31 induced p38 phosphorylation in cells expressing NPFF-R1. Both inducible transcription factors, c-Fos and c-Jun, were significantly activated after stimulation with PrRP31 and its palmitoylated analogs in cells with GPR10 and NPFF-R2. Conversely, NPFF-R1 significantly activated c-Fos and c-Jun only after stimulation with palm-PrRP31. The transcription factor CREB is also important for the regulation of cell proliferation, cell survival and differentiation, for maintaining glucose homeostasis, and has an important role in activating immune responses [40,41]. Likewise, c-Fos and c-Jun activation and phosphorylation of the transcription factor CREB were significantly increased in cells expressing GPR10 and NPFF-R2 after stimulation with PrRP31 and its analogs. Compared to palm^{11}-PrRP31, palm-PrRP31 showed a higher activity in cells transfected with NPFF-R1 in all tested signaling pathways. The results show that PrRP31, palm^{11}-PrRP31 and palm-PrRP31 may play important roles in the regulation of cell proliferation and affect immune responses. These findings suggest that dysregulation of glucose homeostasis and inflammatory responses linked with obesity could be treated with PrRP31 analogs.

In this study, we tested potential off-target receptors of PrRP31, which are related to food intake and energy metabolism. Because PrRP and NPFF were found to have antinociceptive properties [16,17,22,23], their agonist and antagonist activities on opioid receptors were studied using a β-lactamase assay. In our study, palmitoylation increased the binding properties of natural PrRP31. Palm^{11}-PrRP31 was found to have a lower affinity for KOR than palm-PrRP31, but they both had negligible ability to activate the KOR receptor in either agonist mode or antagonist mode. We did not observe any agonist or antagonist activity of either PrRP31 palmitoylated analog on the other opioid receptors MOR, DOR and ORL-1. The possible pain modulation properties of PrRP do not seem to be linked to opioid receptors, which supports the idea that GPR10 is involved in pain processing regulation. A study by Laurent et al. using GPR10 KO suggested that the central anti-opioid activity of NPFF in mice is regulated by GPR10. Moreover, they suggested that the dual coupling of GPR10 with Gq and Gi may be the reason for PrRP's involvement in

different neuronal networks [23]. GPR10 could be involved either in pain modulation or food intake regulation, depending on the type of G protein coupled with GPR10.

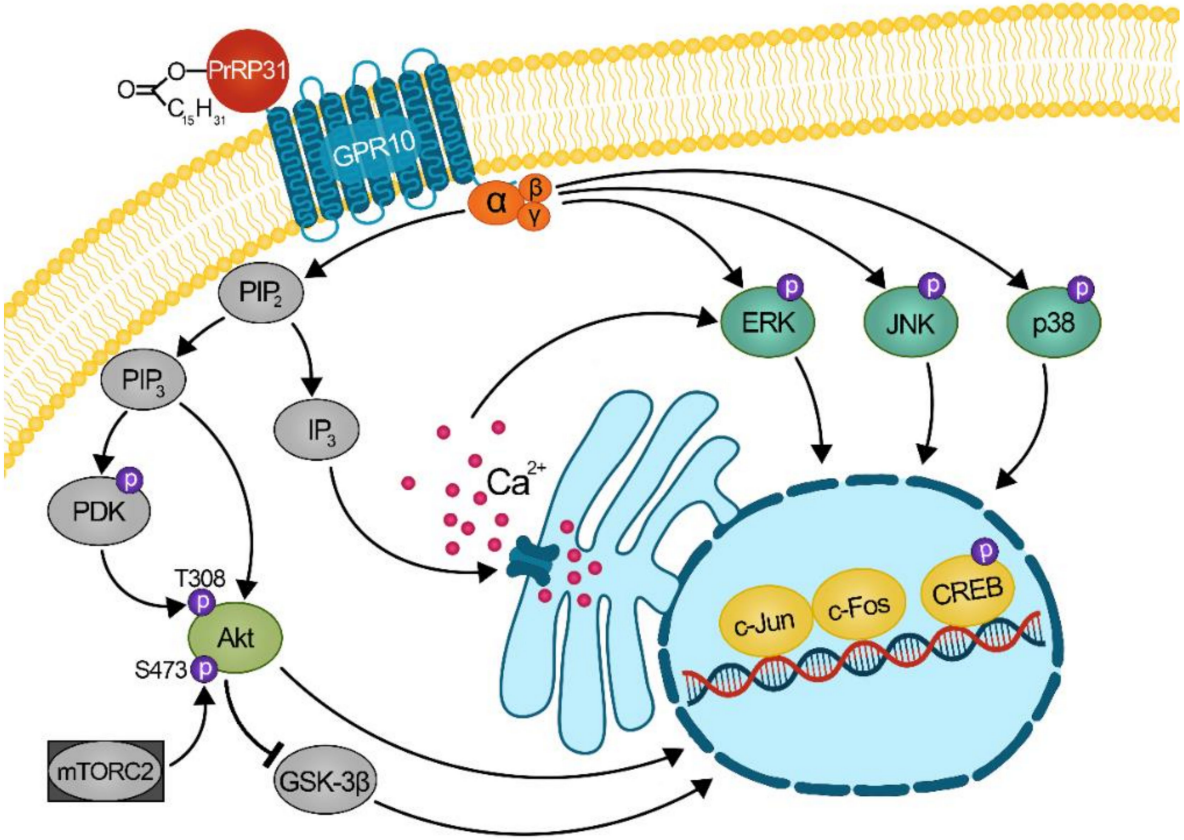


Figure 7. Scheme of mechanism of action of palmitoylated PrRP31 analogs at GPR10: ERK, extracellular signal-regulated kinase; JNK, c-Jun *N*-terminal kinase; CREB, cAMP-responsive element binding protein; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PDK, phosphoinositide-dependent kinase 1; Akt, protein kinase B; mTORC2, mammalian target of rapamycin complex 2; GSK-3β, glycogen synthase kinase-3β.

NPY, together with PYY and pancreatic polypeptide (PP), controls energy homeostasis though NPY receptors. NPY receptors are expressed throughout the central nervous system but can also be found in the peripheral nervous system [42]. The affinity of PrRP31 and its palmitoylated analogs for the receptors Y_1, Y_2 and Y_5 was tested. No binding affinity of PrRP31 and palmitoylated analogs for the Y_2 receptor was observed and a negligible affinity of palm-PrRP31 for Y_1 was detected. However, natural PrRP31, palm^{11}-PrRP31 and palm-PrRP31 bound and activated the Y_5 receptor with a K_i and an EC_{50} in the micromolar range. PrRP31 affinity and agonist activity were increased with the attached palmitoyl group. Y_1 and Y_5 receptors are expressed in the same neurons, and they both have important regulatory functions in food intake and energy balance [42]. Although NPY is an orexigenic peptide, Y_1 and Y_5 receptor deletion leads to obesity and decreases food intake [43]. This study showed that palm^{11}-PrRP31 and palm-PrRP31 had agonist activity ranging from 10^{-7} to 10^{-8} M on the Y_5 receptor, and palm^{11}-PrRP31 was also shown to be a positive allosteric modulator, which suggests that PrRP31 analogs could mediate the in vivo ability to reduce food intake through Y_5 receptors.

Finally, the off-target properties of the palmitoylated PrRP31 analogs on the receptor of the orexigenic peptide ghrelin were studied. Palm-PrRP31 had a higher affinity for the GHSR receptor than palm^{11}-PrRP31, but both analogs had negligible activity on GHSR.

Palm^{11}-PrRP31 and palm-PrRP31 displayed higher affinity for GPR10 and NPFF-R2 receptors than natural PrRP31, and stimulation with PrRP31 analogs activated transcription factors c-Fos, c-Jun and CREB and also activated PKB/Akt, MAPK pathways in cells expressing these receptors. A new strong target of palmitoylated analogs was found to be NPFF-R1. Palm-PrRP31 induced activation of tested signaling pathways in cells expressing NPFF-R1. Both analogs revealed negligible affinity and ability to activate receptors Y, opioid receptors and GHSR, but palm-PrRP31 showed higher off-target binding affinity for these possible off-target receptors. Palm^{11}-PrRP31 was a more selective agonist of anorexigenic receptors GPR10 and NPFF-R2, with less off-target activity; therefore, it has higher potential for the treatment of obesity and neurodegenerative diseases.

4. Materials and Methods

4.1. Material

Human PrRP31, palm^{11}-PrRP31, palm-PrRP31, neuropeptide FF (NPFF), its stable analog 1DMe (see Table 1 for structures), and ghrelin (ghr) were synthetized and purified as described previously [9,31]. PrRP31 palmitoylation was performed on fully protected peptide on resin as a last step [44]. Peptide purification and identification were determined by analytical high-performance liquid chromatography and by using a Q-TOF micro MS technique (Waters, Milford, MA, USA). Purity of the synthesized peptides was greater than 95%.

Human peptide YY (PYY) (#SC319) was obtained from the PolyPeptide Group (Strasbourg, France). The selective KOR agonists (±)-trans-U-50488 methanesulfonate salt (#D8040) were purchased from Sigma-Aldrich (St. Louis, MS, USA). [D-Pro10]-dynorphin A (#021-17), used as an agonist of KOR in binding experiments, was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). Selective agonists of mu-opioid receptor (MOR) DAMGO (#1171), opioid receptor-like 1 (ORL-1) agonist nociceptin (#0910) and agonist of delta-opioid receptor (DOR) [D-Ala2]-deltorphin II (#1180) were obtained from Tocris (Bristol, UK).

4.2. Peptide Iodination

Human PrRP31 and 1DMe were iodinated at Tyr20 and D-Tyr1, respectively, with Na[^{125}I] purchased from Izotop (Budapest, Hungary) using IODO-GEN (Pierce, Rockford, IL, USA), as described previously [44]. Radioligands [^{125}I]-PYY, [^{125}I]-dynorphin A and [^{125}I]-ghrelin were iodinated at Tyr20 (Tyr27), Tyr1 and His9, respectively. The identity of peptides was determined by a MALDI-TOF Reflex IV mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The specific activity of all ^{125}I-labeled peptides was approximately 2100 Ci/mmol. The radiolabeled peptides were kept in aliquots at −20 °C and used in experiments within 1 month.

4.3. Cell Cultures

All used cells were maintained at 37 °C in a humified incubator with 5% CO_2. Growth and assay media were prepared according to manufacturer protocols, and cells were cultured as required. Chinese hamster ovary cells (CHO-K1) stably expressing receptors GPR10 (#K1732) or kappa-opioid receptor (KOR) (#K1533) and human bone osteosarcoma epithelial cells (U2OS) stably expressing receptors of NPY (Y_1 (#K1803), Y_2 (#K149), Y_5 (#K1782)), mu-opioid receptor (MOR) (#K1523), delta-opioid receptor (DOR) (#K1778), opioid receptor-like 1 (ORL-1) (#K1786) and ghrelin receptor (GHSR) (#K1819) were all obtained from Thermo Fisher Scientific Inc. Brand (Waltham, MA, USA). CHO-K1 cell lines containing NPFF-R2 (#ES-490-A) and NPFF-R1 (#ES-491-C) were obtained from Perkin Elmer (Waltham, MA, USA).

4.4. Cell Membrane Isolation

Pellets of CHO-K1 cells containing NPFF-R2, NPFF-R1 and KOR receptors were homogenized in ice-cold homogenizing buffer (20 mM HEPES pH 7.1, 5 mM $MgCl_2$,

0.7 mM bacitracin) with a DIAX 100 Homogenizer (Heidolph Instruments, Schwabach, Germany) and centrifuged in an ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) at 26,000× *g* for 15 min at 4 °C. The pellets were homogenized in ice-cold homogenization buffer, and the previous steps were repeated 2 more times. After the third centrifugation, pellets were resuspended in ice-cold storage buffer (50 mM Tris-Cl pH 7.4, 0.5 mM EDTA, 10 mM $MgCl_2$, 10% sucrose), and aliquots were stored at −80 °C. The concentration of isolated membrane proteins was determined by a PierceTM BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

4.5. Competitive Binding Experiments

Competition binding experiments were performed according to [45]. [^{125}I]-PrRP31 was used to compete with human PrRP31, palmitoylated PrRP31 analogs, NPFF, and 1DMe in CHO-K1 cells expressing human GPR10 as described previously [31]. Binding experiments using U2OS cells were optimized and performed in assay buffer (50 mM Tris-Cl pH 7.4, 118 mM NaCl, 5 mM $MgCl_2$, 4.7 mM KCl, 0.1% BSA) for cells stably expressing GHSR and (25 mM HEPES, 120 mM NaCl, 5 mM $MgCl_2$, 4.7 mM KCl, 1 mM $CaCl_2$, 0.5% BSA, 2 g/L glucose) for cells containing receptors Y_1, Y_2 and Y_5. PrRP31 or lipidized analogs of PrRP31,ghr, or PYY were used at final concentrations from 10^{-12} to 10^{-5} M to compete with 0.1 nM [^{125}I]-ghr, or [^{125}I]-PYY radioligands. Cells were incubated for 60 min at room temperature (RT).

Plasma membranes isolated from CHO-K1 cells containing receptors NPFF-R2, NPFF-R1 and KOR were used at a concentration of 5 µg of protein/tube, and binding experiments were performed in assay buffer (50 mM Tris-HCl pH 7.4 + 60 mM NaCl + 1 mM MgCl2 + 0.5% BSA). [^{125}I]-1DMe was used to compete with human PrRP31, palmitoylated PrRP31 analogs, NPFF or 1DMe in isolated membranes with NPFF-R2 or NPFF-R1, and [^{125}I]-dynorphin A was used to compete with human PrRP31 and palmitoylated PrRP31 analogs in isolated membranes with KOR. The studied peptides and radioligands were incubated with plasma membranes for 60 min at RT and subsequently filtered in a Brandel cell harvester (Biochemical and Development Laboratories, Gaithersburg, MD, USA) using Whatman GF/B filters preincubated in 0.3% polyethylenimine. Filters were rinsed three times with 2 mL of wash buffer (50 mM Tris pH 7.4 + 60 mM NaCl).

Radioactivity was determined by a γ-counter Wizard 1470 Automatic Gamma Counter (Perkin Elmer). Experiments were carried out in duplicate at least three times, and K_i was calculated using the Cheng-Prusoff equation.

4.6. Cell Signaling Detection by Immunoblotting

Activation of signaling pathways was studied in the CHO-K1 cell lines containing GPR10, NPFF-R2 and NPFF-R1. Cells were seeded in 24-well plates at 30,000 cells/well in assay medium (growth medium without selective antibiotics) and were grown for 2 days. The day before the experiment, the medium was changed to serum-free medium. On the day of the experiment, cells were incubated with PrRP31, lipidized PrRP31 analogs, NPFF or 1DMe at final concentrations from 10^{-11} to 10^{-5} M for 5 min or 60 min at 37 °C and then washed three times with ice-cold phosphate-buffered saline (PBS) pH 7.4. Cells were lysed with Laemmli sample buffer (62.5 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol, 50 mM NaF, and 1 mM Na_3VO_4). Samples were stored at −20 °C. Electrophoresis and immunoblotting were performed as described previously [45]. For detection of signaling pathways, primary monoclonal antibodies (see Table 5 for the antibodies used) purchased from Cell Signaling Technology (Danvers, MA, USA) were used.

Table 5. Primary antibodies used for immunoblotting and their dilutions.

Antibody Against	Source	Dilution
Phospho-Akt (Thr308) (#2965)	Rabbit	1:1000, 5% BSA, TBS/T-20
Phospho-Akt (Ser473) (#4060)	Rabbit	1:1000, 5% BSA, TBS/T-20
Akt (#4691S)	Rabbit	1:1000, 5% BSA, TBS/T-20
Phospho-CREB (Ser133) (#9196)	Mouse	1:1000, 5% milk, TBS/T-20
CREB (#9104S)	Mouse	1:1000, 5% milk, TBS/T-20
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#4370S)	Rabbit	1:2000, 5% BSA, TBS/T-20
p44/42 MAPK (Erk1/2) (#9107S)	Mouse	1:2000, 5% milk, TBS/T-20
Phospho-SAPK/JNK (Thr183/Tyr185) (#4668)	Rabbit	1:1000, 5% BSA, TBS/T-20
SAPK/JNK (#9252)	Rabbit	1:1000, 5% BSA, TBS/T-20
Phospho-p38 MAPK (Thr180/Tyr182) (#4511)	Rabbit	1:1000, 5% BSA, TBS/T-20
p38 MAPK (#9212)	Rabbit	1:1000, 5% BSA, TBS/T-20
Phospho-PKA C (Thr197) (#5661)	Rabbit	1:1000, 5% BSA, TBS/T-20
c-Fos (#2250)	Rabbit	1:1000, 5% BSA, TBS/T-20
c-Jun (#9165)	Rabbit	1:1000, 5% BSA, TBS/T-20
GAPDH (#97166)	Mouse	1:1000, 5% milk, TBS/T-20

4.7. Calcium Mobilization Assays

Measuring the intracellular Ca^{2+} level in CHO-K1 cells containing GRP10 was performed using the calcium-sensitive dye Fura-2 according to the manufacturer's protocol (Molecular Devices, Sunnyvale, CA, USA). The day before the experiment, cells were seeded at 40,000 cells/well in 96-well plates in growth media and kept at 37 °C in an incubator with 5% CO_2 overnight. Peptides were tested at concentrations from 10^{-12} to 10^{-5} M. Fura-2 fluorescent dye was detected using a FlexStation 3 fluorometric plate reader (Molecular Devices), and excitation was measured at 340 nm and 380 nm and emission at 510 nm.

The intracellular Ca^{2+} level was measured using the AequoScreen stable CHO-K1 cell line containing NPFF-R2 purchased from Perkin Elmer according to the manufacturer's protocol. Cells at 80–90% confluence cultured in media without selective antibiotics were detached (PBS pH 7.4 + 0.5 mM EDTA) and centrifuged. Cells resuspended in phenol red-free DMEM with 0.1% protease-free BSA and 5 µM coelenterazine h (Thermo Fisher Scientific Inc. Brand) were seeded at 50,000 cells/well in 96-well plates and incubated in the dark at RT with gentle agitation for 4 h. Peptides were tested at concentrations from 10^{-12} to 10^{-5} M. Luminescent light emission was recorded using a FlexStation 3 plate reader.

4.8. Cell Signaling Determined Using Beta-Lactamase Reporter System

Cell lines containing beta-lactamase reporter genes with different receptors, GPR10, Y_5, GHSR and opioid receptors, were used to study the agonist/antagonist properties of PrRP31 and lipidized PrRP31 analogs. Cells were seeded 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 [10]. Receptor agonists were tested at concentrations from 10^{-12} to 10^{-5} M. The concentration of the agonist in antagonist assay mode ranged from 10^{-12} to 10^{-5} M, and the potential antagonists PrRP31 and palm^{11}-PrRP31 were tested at concentrations from 10^{-7} or 10^{-6} to 10^{-5} M. Fluorescence was detected at 409 nm excitation and 460 and 530 nm emissions using the FlexStation 3 fluorometric plate reader.

4.9. Statistical Analysis

Data were analyzed by GraphPad Software (San Diego, CA, USA) and are presented as the means ± SEM. The saturation and competitive binding experiments were analyzed according to [46] using the Cheng-Prusoff equation [33]. The competitive binding curves were plotted compared to the best fit for single-binding site models, and half maximal inhibitory concentration (IC_{50}) values were obtained from nonlinear regression analysis. From saturation binding experiments, the dissociation constant (K_d) and number of binding

sites/cell (Bmax) were calculated. Inhibition constants (K_i) were calculated from IC_{50} values, K_d and the concentration of radioligands.

Experiments using immunoblotting were analyzed using one-way ANOVA followed by Dunnett's post hoc test; $p < 0.05$ was considered statistically significant. Dose-response curves were obtained from nonlinear regression.

The beta-lactamase assay results were analyzed by nonlinear regression as log agonist versus response, and EC_{50} values were determined in agonist mode using GraphPad software. Data are representative of at least two experiments, each performed in duplicate.

Ca^{2+} release assay data are shown as the percentage of maximal response, and the results were analyzed by nonlinear regression as log agonist versus response using GraphPad software. Data are representative of at least three experiments, each performed in duplicate.

5. Conclusions

Lipidized PrRP31 analogs have great potential for the treatment of obesity and neurodegenerative diseases. The in vitro properties of the two most potent palmitoylated analogs, palm-PrRP31 and palm^{11}-PrRP31, were tested and compared. Palmitoylation of PrRP31 increased not only the activity and binding affinity to GPR10 and NPFF-R2, which are both connected with food intake regulation, but also the binding properties and activity to NPFF-R1. Therefore, NPFF-R1 is a new target of lipidized PrRP31 analogs. Both analogs activated the cellular signaling of the PKB/Akt and MAPK pathways and activated the transcription factors c-Fos, c-Jun and CREB in cells expressing GPR10 and NPFFR-2. Activation of all previously mentioned cellular pathways in cells expressing NPFF-R1 was observed only after incubation with palm-PrRP31. Palm-PrRP31 also showed higher off-target activity on GHSR receptors and Y receptors than palm^{11}-PrRP31; therefore, the more selective palm^{11}-PrRP31 has a better potential for obesity treatment. Our future studies will focus on further development of palmitoylated PrRP analogs with minimized off-target activity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22168904/s1, Supplementary Figure S1: Induction of (A) PKA phosphorylation after 5 min incubation at 37 °C with peptides in final concen-18 trations 10-6 M in CHO-K1 cells expressing receptors GPR10, NPFF-R2 and NPFF-R1, Supplementary Figure S2: Antagonist mode of FRET assay showing effect of PrRP31 and palm11-PrRP31 at (A) opioid receptors and 23 (B) GHSR.

Author Contributions: A.K. measured the data, wrote the manuscript and prepared the figures; V.S. measured the data and contributed to the writing of the manuscript, L.H. assisted in data measurements and preparation of the pictures; L.M., B.Ž. and J.K. designed the experiments, contributed to the writing and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Grant Agency of the Czech Republic (GACR 21-03691S, RVO:67985823 and RVO:61388963) of the Academy of Sciences of the Czech Republic.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We gratefully acknowledge the generous help of Miroslava Blechová., for peptide synthesis and Aleš Marek, for radioiodination.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, the collection, analyses, or interpretation of data, the writing of the manuscript or in the decision to publish the results.

References

1. Hinuma, S.; Habata, Y.; Fujii, R.; Kawamata, Y.; Hosoya, M.; Fukusumi, S.; Kitada, C.; Masuo, Y.; Asano, T.; Matsumoto, H.; et al. A prolactin-releasing peptide in the brain. *Nature* **1998**, *393*, 272–276. [CrossRef]

2. Matsumoto, H.; Noguchi, J.; Horikoshi, Y.; Kawamata, Y.; Kitada, C.; Hinuma, S.; Onda, H.; Nishimura, O.; Fujino, M. Stimulation of Prolactin Release by Prolactin-Releasing. *Biochem. Biophys. Res. Commun.* **1999**, *259*, 321–324. [CrossRef]
3. Lawrence, C.B.; Celsi, F.; Brennand, J.; Luckman, S.M. Alternative role for prolactin-releasing peptide in the regulation of food intake. *Nat. Neurosc.* **2000**, *3*, 645–646. [CrossRef] [PubMed]
4. Lawrence, C.B.; Ellacott, K.L.J.; Luckman, S.M. PRL-releasing peptide reduces food intake and may mediate satiety signaling. *Endocrinology* **2002**, *143*, 360–367. [CrossRef] [PubMed]
5. Bjursell, M.; Lenneras, M.; Goransson, M.; Elmgren, A.; Bohlooly, Y.M. GPR10 deficiency in mice results in altered energy expenditure and obesity. *Biochem. Biophys. Res. Commun.* **2007**, *363*, 633–638. [CrossRef] [PubMed]
6. Gu, W.; Geddes, B.J.; Zhang, C.; Foley, K.P.; Stricker-Krongrad, A. The prolactin-releasing peptide receptor (GPR10) regulates body weight homeostasis in mice. *J. Mol. Neurosc.* **2004**, *22*, 93–103. [CrossRef]
7. Prazienkova, V.; Funda, J.; Pirnik, Z.; Karnosova, A.; Hruba, L.; Korinkova, L.; Neprasova, B.; Janovska, P.; Benzce, M.; Kadlecova, M.; et al. GPR10 gene deletion in mice increases basal neuronal activity, disturbs insulin sensitivity and alters lipid homeostasis. *Gene* **2021**, *774*, 145427. [CrossRef]
8. Maletinska, L.; Nagelova, V.; Ticha, A.; Zemenova, J.; Pirnik, Z.; Holubova, M.; Spolcova, A.; Mikulaskova, B.; Blechova, M.; Sykora, D.; et al. Novel lipidized analogs of prolactin-releasing peptide have prolonged half-lives and exert anti-obesity effects after peripheral administration. *Int. J. Obesity* **2015**, *39*, 986–993. [CrossRef]
9. Prazienkova, V.; Holubova, M.; Pelantova, H.; Buganova, M.; Pirnik, Z.; Mikulaskova, B.; Popelova, A.; Blechova, M.; Haluzik, M.; Zelezna, B.; et al. Impact of novel palmitoylated prolactin-releasing peptide analogs on metabolic changes in mice with diet-induced obesity. *PLoS ONE* **2017**, *12*, e0183449. [CrossRef]
10. Prazienkova, V.; Ticha, A.; Blechova, M.; Spolcova, A.; Zelezna, B.; Maletinska, L. Pharmacological characterization of lipidized analogs of prolactin-releasing peptide with a modified C-terminalaromatic ring. *J. Physiol. Pharmacol.* **2016**, *67*, 121–128. [PubMed]
11. Pirnik, Z.; Kolesarova, M.; Zelezna, B.; Maletinska, L. Repeated peripheral administration of lipidized prolactin-releasing peptide analog induces c-fos and FosB expression in neurons of dorsomedial hypothalamic nucleus in male C57 mice. *Neurochem. Int.* **2018**, *116*, 77–84. [CrossRef]
12. Pirnik, Z.; Zelezna, B.; Kiss, A.; Maletinska, L. Peripheral administration of palmitoylated prolactin-releasing peptide induces Fos expression in hypothalamic neurons involved in energy homeostasis in NMRI male mice. *Brain Res.* **2015**, *1625*, 151–158. [CrossRef]
13. Engstrom, M.; Brandt, A.; Wurster, S.; Savola, J.M.; Panula, P. Prolactin releasing peptide has high affinity and efficacy at neuropeptide FF2 receptors. *J. Pharmacol. Exp. Therapeut.* **2003**, *305*, 825–832. [CrossRef]
14. Elhabazi, K.; Humbert, J.P.; Bertin, I.; Schmitt, M.; Bihel, F.; Bourguignon, J.J.; Bucher, B.; Becker, J.A.; Sorg, T.; Meziane, H.; et al. Endogenous mammalian RF-amide peptides, including PrRP, kisspeptin and 26RFa, modulate nociception and morphine analgesia via NPFF receptors. *Neuropharmacology* **2013**, *75*, 164–171. [CrossRef]
15. Murase, T.; Arima, H.; Kondo, K.; Oiso, Y. Neuropeptide FF reduces food intake in rats. *Peptides* **1996**, *17*, 353–354. [CrossRef]
16. Sunter, D.; Hewson, A.K.; Lynam, S.; Dickson, S.L. Intracerebroventricular injection of neuropeptide FF, an opioid modulating neuropeptide, acutely reduces food intake and stimulates water intake in the rat. *Neurosci. Lett.* **2001**, *313*, 145–148. [CrossRef]
17. Mouledous, L.; Mollereau, C.; Zajac, J.M. Opioid-modulating properties of the neuropeptide FF system. *BioFactors* **2010**, *36*, 423–429. [CrossRef] [PubMed]
18. Nicklous, D.M.; Simansky, K.J. Neuropeptide FF exerts pro- and anti-opioid actions in the parabrachial nucleus to modulate food intake. *Am. J. Physiol. Regulat. Integr. Compar. Physiol.* **2003**, *285*, 1046–1054. [CrossRef] [PubMed]
19. Panula, P.; Aarnisalo, A.A.; Wasowicz, K. Neuropeptide FF, a mammalian neuropeptide with multiple functions. *Progress Neurobiol.* **1996**, *48*, 461–479. [CrossRef]
20. Simonin, F.; Schmitt, M.; Laulin, J.-P.; Laboureyras, E.; Jhamandas, J.H.; MacTavish, D.; Matifas, A.; Mollereau, C.; Laurent, P. RF9, a potent and selective neuropeptide FF receptor antagonist, prevents opioid-induced tolerance associated with hyperalgesia. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 466–471. [CrossRef]
21. Maletinska, L.; Ticha, A.; Nagelova, V.; Spolcova, A.; Blechova, M.; Elbert, T.; Zelezna, B. Neuropeptide FF analog RF9 is not an antagonist of NPFF receptor and decreases food intake in mice after its central and peripheral administration. *Brain Res.* **2013**, *1498*, 33–40. [CrossRef] [PubMed]
22. Kalliomäki, M.L.; Pertovaara, A.; Brandt, A.; Wei, H.; Pietilä, P.; Kalmari, J.; Xu, M.; Kalso, E.; Panula, P. Prolactin-releasing peptide affects pain, allodynia and autonomicreflexes through medullary mechanisms. *Neuropharmacology* **2004**, *46*, 412–424. [CrossRef] [PubMed]
23. Laurent, P.; Becker, J.A.; Valverde, O.; Ledent, C.; de Kerchove d'Exaerde, A.; Schiffmann, S.N.; Maldonado, R.; Vassart, G.; Parmentier, M. The prolactin-releasing peptide antagonizes the opioid system through its receptor GPR10. *Nat. Neurosci.* **2005**, *8*, 1735–1741. [CrossRef]
24. Cardoso, J.C.; Felix, R.C.; Fonseca, V.G.; Power, D.M. Feeding and the rhodopsin family g-protein coupled receptors in nematodes and arthropods. *Front. Endocrinol.* **2012**, *3*, 157. [CrossRef] [PubMed]
25. Marchese, A.; Heiber, M.; Nguyen, T.; Heng, H.H.Q.; Saldivia, V.R.; Cheng, R.; Murphy, P.M.; Tsui, L.C.; Shi, X.; Gregor, P.; et al. Cloning and Chromosomal Mapping of Three Novel Genes, GPR9, GPR10, and GPR14, Encoding Receptors Related to Interleukin 8, Neuropeptide Y, and Somatostatin Receptors. *Genomics* **1995**, *29*, 335–344. [CrossRef]

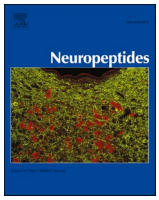
26. Bonini, J.A.; Jones, K.A.; Adham, N.; Forray, C.; Artymyshyn, R.; Durkin, M.M.; Smith, K.E.; Tamm, J.A.; Boteju, L.W.; Lakhlani, P.P.; et al. Identification and characterization of two G protein-coupled receptors for neuropeptide FF. *J. Biol. Chem.* **2000**, *275*, 39324–39331. [CrossRef] [PubMed]
27. Langmead, C.J.; Szekeres, P.G.; Chambers, J.K.; Ratcliffe, S.J.; Jones, D.N.; Hirst, W.D.; Price, G.W.; Herdon, H.J. Characterization of the binding of [(125)I]-human prolactin releasing peptide (PrRP) to GPR10, a novel G protein coupled receptor. *British J. Pharmacol.* **2000**, *131*, 683–688. [CrossRef]
28. Roland, B.L.; Sutton, S.W.; Wilson, S.J.; Luo, L.; Pyati, J.; Huvar, R.; Erlander, M.G.; Lovenberg, T.W. Anatomical distribution of prolactin-releasing peptide and its receptor suggests additional functions in the central nervous system and periphery. *Endocrinology* **1999**, *140*, 5736–5745. [CrossRef]
29. Chuderland, D.; Seger, R. Calcium regulates ERK signaling by modulating its protein-protein interactions. *Commun. Integr. Biol.* **2008**, *1*, 4–5. [CrossRef] [PubMed]
30. Kimura, A.; Ohmichi, M.; Tasaka, K.; Kanda, Y.; Ikegami, H.; Hayakawa, J.; Hisamoto, K.; Morishige, K.; Hinuma, S.; Kurachi, H.; et al. Prolactin-releasing peptide activation of the prolactin promoter is differentially mediated by extracellular signal-regulated protein kinase and c-Jun N-terminal protein kinase. *J. Biol. Chem.* **2000**, *275*, 3667–3674. [CrossRef] [PubMed]
31. Maixnerova, J.; Spolcova, A.; Pychova, M.; Blechova, M.; Elbert, T.; Rezacova, M.; Zelezna, B.; Maletinska, L. Characterization of prolactin-releasing peptide: Binding, signaling and hormone secretion in rodent pituitary cell lines endogenously expressing its receptor. *Peptides* **2011**, *32*, 811–817. [CrossRef]
32. Hayakawa, J.; Ohmichi, M.; Tasaka, K.; Kanda, Y.; Adachi, K.; Nishio, Y.; Hisamoto, K.; Mabuchi, S.; Hinuma, S.; Murata, Y. Regulation of the PRL promoter by Akt through cAMP response element binding protein. *Endocrinology* **2002**, *143*, 13–22. [CrossRef] [PubMed]
33. Cheng, Y.; Prusoff, W.H. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108. [CrossRef] [PubMed]
34. Holubova, M.; Zemenova, J.; Mikulaskova, B.; Panajotova, V.; Stohr, J.; Haluzik, M.; Kunes, J.; Zelezna, B.; Maletinska, L. Palmitoylated PrRP analog decreases body weight in DIO rats but not in ZDF rats. *J. Endocrinol.* **2016**, *229*, 85–96. [CrossRef]
35. Maletinska, L.; Popelova, A.; Zelezna, B.; Bencze, M.; Kunes, J. The impact of anorexigenic peptides in experimental models of Alzheimer's disease pathology. *J. Endocr.* **2019**, *240*, R47–R72. [CrossRef]
36. Nanmoku, T.; Takekoshi, K.; Isobe, K.; Kawakami, Y.; Nakai, T.; Okuda, Y. Prolactin-releasing peptide stimulates catecholamine release but not proliferation in rat pheochromocytoma PC12 cells. *Neurosci. Lett.* **2003**, *350*, 33–36. [CrossRef]
37. Wang, Y.; Wang, C.Y.; Wu, Y.; Huang, G.; Li, J.; Leung, F.C. Identification of the receptors for prolactin-releasing peptide (PrRP) and Carassius RFamide peptide (C-RFa) in chickens. *Endocrinology* **2012**, *153*, 1861–1874. [CrossRef]
38. Nanmoku, T.; Takekoshi, K.; Fukuda, T.; Ishii, K.; Isobe, K.; Kawakami, Y. Stimulation of catecholamine biosynthesis via the PKC pathway by prolactin-releasing peptide in PC12 rat pheochromocytoma cells. *J. Endocrinol.* **2005**, *186*, 233–239. [CrossRef]
39. Cargnello, M.; Roux, P.P. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* **2011**, *75*, 50–83. [CrossRef]
40. Canovas, B.; Nebreda, A.R. Diversity and versatility of p38 kinase signalling in health and disease. *Nat. Rev. Mol. Cell Biol.* **2021**. [CrossRef]
41. Wen, A.Y.; Sakamoto, K.M.; Miller, L.S. The role of the transcription factor CREB in immune function. *J. Immunol.* **2010**, *185*, 6413–6419. [CrossRef] [PubMed]
42. Loh, K.; Herzog, H.; Shi, Y.C. Regulation of energy homeostasis by the NPY system. *Trends Endocrinol. Metab.* **2015**, *26*, 125–135. [CrossRef] [PubMed]
43. Nguyen, A.D.; Mitchell, N.F.; Lin, S.; Macia, L.; Yulyaningsih, E.; Baldock, P.A.; Enriquez, R.F.; Zhang, L.; Shi, Y.C.; Zolotukhin, S.; et al. Y1 and Y5 receptors are both required for the regulation of food intake and energy homeostasis in mice. *PLoS ONE* **2012**, *7*, e40191. [CrossRef] [PubMed]
44. Maletinska, L.; Pychova, M.; Holubova, M.; Blechova, M.; Demianova, Z.; Elbert, T.; Zelezna, B. Characterization of new stable ghrelin analogs with prolonged orexigenic potency. *J. Pharmacol. Exp. Ther.* **2012**, *340*, 781–786. [CrossRef] [PubMed]
45. Spolcova, A.; Mikulaskova, B.; Holubova, M.; Nagelova, V.; Pirnik, Z.; Zemenova, J.; Haluzik, M.; Zelezna, B.; Galas, M.C.; Maletinska, L. Anorexigenic lipopeptides ameliorate central insulin signaling and attenuate tau phosphorylation in hippocampi of mice with monosodium glutamate-induced obesity. *J. Alzheimers Dis.* **2015**, *45*, 823–835. [CrossRef] [PubMed]
46. Motulsky, H.; Neubig, R. Analyzing radioligand binding data. *Curr. Protoc. Neurosci.* **2002**, *7*. [CrossRef] [PubMed]

Neuropeptides 98 (2023) 102319

Contents lists available at ScienceDirect

Neuropeptides

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



Search for lipidized PrRP analogs with strong anorexigenic effect: *In vitro* and *in vivo* studies

Veronika Strnadová [a], Alena Karnošová [a,d], Miroslava Blechová [a], Barbora Neprašová [a,b], Lucie Holá [a,d], Anna Němcová [a,c], Aneta Myšková [a,c], David Sýkora [c], Blanka Železná [a], Jaroslav Kuneš [a,b], Lenka Maletínská [a,*]

[a] *Institute of Organic Chemistry and Biochemistry, CAS, Prague 166 10, Czech Republic*
[b] *Institute of Physiology, CAS, Prague 142 00, Czech Republic*
[c] *University of Chemistry and Technology, Prague 166 28, Czech Republic*
[d] *First Faculty of Medicine, Charles University, Prague 121 08, Czech Republic*

ARTICLE INFO

Keywords:
Prolactin-releasing peptide
Lipidization
GPR10
NPFF-R2
NPFF-R1
ERK
Akt activation
Stability
Food intake

ABSTRACT

Prolactin-releasing peptide (PrRP) is an anorexigenic neuropeptide that attenuates food intake and increases energy expenditure. We designed three series of new lipidized PrRP31 analogs of different lengths of fatty acids attached at amino acids 1 or 11 directly or *via* linkers, part of them acetylated at the N-terminus and/or modified with dichlorophenylalanine ($PheCl_2$) at the C-terminus. We tested their affinity for and activation of signaling pathways relevant to receptors GPR10, NPFF-R2, and NPFF-R1, effect on food intake in fasted or freely fed mice and rats, and stability in rat plasma. We aimed to select a strong dual GPR10/NPFF-R2 agonist whose affinity for NPFF-1 was not enhanced. The selected potent analog was then tested for body weight-lowering potency after chronic administration in mice with diet-induced obesity. PrRP31 analogs lipidized by monocarboxylic fatty acids showed strong dual affinity for both GPR10 and NPFF-R2 and activated MAPK/ERK1/2, Akt and CREB in cells overexpressing GPR10 and NPFF-R2. The selected analog stabilized at N- and C-termini and palmitoylated through the TTDS linker to Lys^{11} is a powerful dual agonist GPR10/NPFF-R2 at not enhanced affinity for NPFF-R1. It showed strong anti-obesity properties in mice with diet-induced obesity and became a potential compound for further studies.

1. Introduction

Anorexigenic prolactin-releasing peptide (PrRP) is an endogenous ligand of the G-protein-coupled receptor GPR10 belonging to the RF-amide peptide family, reviewed in (Lin, 2008; Prazienkova et al., 2019a). PrRP interacts with its receptor GPR10, but it also has high binding affinity for the neuropeptide FF receptor type 2 (NPFF-R2), another receptor from the RF-amide peptide family (Engstrom et al., 2003; Maletinska et al., 2015).

PrRP and GPR10 are expressed mainly in the brainstem and hypothalamus, areas connected with appetite regulation and energy expenditure, as reviewed in (Quillet et al., 2016). Intracerebroventricular (ICV) administration of PrRP resulted in reduced food intake and increased energy expenditure in rodents (Lawrence et al., 2002; Lawrence et al., 2000).

PrRP is naturally found in two isoforms, PrRP20 and PrRP31, with

Abbreviations: Akt, Protein kinase B; ANOVA, Analysis of variance; BW, Body weight; CREB, cAMP response element-binding protein; DIO, Diet-induced obese; EC_{50}, Effective concentrations; FI, Food intake; FRET, Fluorescence-resonance energy transfer; GABA, Gamma-aminobutyric acid; gWAT, Gonadal white adipose tissue; HFD, High-fat diet; ICV, Intracerebroventricular; iWAT, Inguinal white adipose tissue; K_d, Dissociation constant; K_i, Inhibition constants; KO, Knockout; LC–MS, Liquid chromatography–mass spectrometry; MAPK/ERK, Mitogen-activated protein kinase/Extracellular signal-regulated kinase; Myr, Myristoyl; N-Ac, *N*-acetyl; NPFF, Neuropeptide FF; NPFF-R1, Neuropeptide FF receptor type 1; NPFF-R2, Neuropeptide FF receptor type 2; $PheCl_2$, Dichlorophenylalanine; PrRP, Prolactin-releasing peptide; pWAT, Perirenal white adipose tissue; SAR, Structure-activity relationship; SC, Subcutaneously; TTDS, 1,13-diamino-4,7,10-trioxatridecan-succinamic acid; γE, Gamma glutamic acid.

* Corresponding author at: Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Flemingovo náměstí 2, Prague 6, 166 10, Czech Republic.

E-mail address: maletin@uochb.cas.cz (L. Maletínská).

https://doi.org/10.1016/j.npep.2022.102319
Received 8 October 2022; Received in revised form 18 November 2022; Accepted 28 December 2022
Available online 13 January 2023

identical C-termini and similar biological activities (Hinuma et al., 1998). Several structure-activity relationship (SAR) studies have been published (Boyle et al., 2005; Deluca et al., 2013; Roland et al., 1999) showing that the deletion of the N-terminal part decreased PrRP20 affinity to GPR10 (Roland et al., 1999) and that for preservation of *in vitro* signaling, the C-terminal 13 peptide PrRP(19–31) was necessary (Boyle et al., 2005). In shorter PrRP20 analogs, PrRP(4–20), PrRP(8–20) and PrRP(14–20), the stability of the α-helical structure, which facilitates ligand recognition by the receptor, was decreased, and the biological activity was reduced (Deluca et al., 2013). C-terminal 20-peptide (PrRP20) preserves full biological activity *in vivo* after ICV administration in fasted mice (Maixnerova et al., 2011). PrRP with phenylalanine 31 modified by a bulky side chain or halogenated aromatic ring retained binding affinity to GPR10 and a strong anorexigenic effect after central ICV administration to fasted mice (Maletinska et al., 2011). Structure-activity relationship studies have been reviewed in (Prazienkova et al., 2019a).

In our previous study (Maletinska et al., 2015), lipidization was introduced to achieve the central anorexigenic effect of PrRP after its peripheral administration and to improve PrRP stability. When fatty acids with different lengths were attached at the N-terminus of PrRP20 or PrRP31, the binding affinity of the lipidized PrRP analogs was preserved for GPR10 and increased for NPFF-R2, and the stability in plasma and half-life in blood were prolonged compared to those of natural PrRP (Maletinska et al., 2015). Moreover, the food-intake-lowering effects of myristoylated-, palmitoylated- or stearoylated-PrRP31 after peripheral administration were reported for the first time. Several of our studies proved that the simultaneous high binding potency of lipidized PrRP analogs to both GPR10 and NPFF-R2 yields in a prolonged anorexigenic effect in fasted mice and an attenuating effect on body weight in rodent models of diet-induced obesity after their repeated peripheral administration (Holubova et al., 2016; Mikulaskova et al., 2018; Prazienkova et al., 2016; Prazienkova et al., 2017).

We have shown recently that palmitoylation of PrRP31 analogs increased the binding affinity of PrRP31 not only for anorexigenic receptors GPR10 and NPFF-R2 but also for another NPFF receptor, NPFF-R1 (Karnosova et al., 2021). Both NPFF receptors have been found in the hypothalamic area that regulates feeding and energy homeostasis and the cardiovascular system and modulate pain perception (Mouledous et al., 2010; Nicklous and Simansky, 2003; Panula et al., 1996) (Fang et al., 2007). In rodents, *Npffr1* and *Npffr2* mRNA are predominantly expressed in the CNS, with relatively low expression in peripheral tissues. NPFF-R1 was also found to be involved in the regulation of stress and reproduction. Inhibition of NPFF-R1 was proposed to connected with secretion of gonadotropins (reviewed in (Anjum et al., 2021; Hu et al., 2019))(Koller et al., 2021).

Even though a lipidized PrRP analog with a selective affinity to GPR10 was reported as an advantageous solution for PrRP *in vivo* (Pflimlin, et al., 2019), we and others consider lipidized PrRP analogs with dual agonist effects on both GPR10 and NPFF-R2 necessary for full *in vivo* PrRP potency (Alexopoulou et al., 2022; Karnosova et al., 2021).

In this study, we further extended our previously published studies with lipidized PrRP analogs by designing and characterizing three new series of PrRP31 analogs lipidized by dodecanoic, myristic, and palmitic acids or hexadecanedioic acid in positions 1 and/or 11 attached *via* various linkers and/or modified at phenylalanine 31. To find dual GPR10 and NPFF-R2 agonists with a potent and long-lasting anorexigenic effect, lipidized PrRP31 analogs were tested and compared. First, the binding affinities of lipidized PrRP31 analogs not only to GPR10 and NPFF-R2 but also to NPFF-R1 as well as activation of relevant signaling pathways were determined in cells overexpressing relevant receptors in order to select the best analog(s). An unchanged or even lowered affinity to NPFF-R1 was a necessary condition for eligible analogs. Then, food intake in lean mice and rats after acute or repeated peripheral administration of lipidized PrRP31 analogs and their stability in rat plasma were tested. Finally, the body weight-lowering effects of the most potent lipidized analog was proven in mice with diet-induced obesity (DIO).

2. Materials and methods

2.1. Peptide synthesis

Human PrRP31, 1DMe, stable analog of NPFF (DYL (N-Me) FQPQRF-NH_2) and lipidized human PrRP31 analogs (for sequences see Table 1) were synthesized and purified as described previously at the Institute of Organic Chemistry and Biochemistry, CAS (Maixnerova et al., 2011; Prazienkova et al., 2017). Lipidization of PrRP31 analogs was performed as shown in (Maletinska et al., 2015; Prazienkova et al., 2017) on fully protected peptide on resin as a final step. The purity and identity of all peptides were determined by analytical HPLC and by using a Q-TOF micro MS technique (Waters, MA, USA). HPLC was carried out on a C18 Vydac reversed phase column; elution with the gradient 2–80% CH_3CN in 0.1% TFA (in 25 min) was used (Table 1).

2.2. Iodination of peptides

Human PrRP31 and 1DMe were iodinated as described previously in (Maixnerova et al., 2011; Maletinska et al., 2013) at Tyr^{20} and D-Tyr^{1}, respectively, with $Na^{125}I$ purchased from Izotop (Budapest, Hungary) using IODO-GEN™ (Pierce, Rockford, IL, USA) according to the manufacturer's instructions (Elbert and Vesela, 2010). Peptides were purified, and the specific activity of all ^{125}I-labeled peptides was approximately 2100 Ci/mmol. The radiolabeled peptides were kept in aliquots at −20 °C and used for binding studies within 1 month.

2.3. Cell cultures and binding assays

Binding or signaling assays were performed on GPR10, NPFF-R2 and NPFF-R1 receptors. CHO-K1 cells stably overexpressing GPR10 (#K1732) were all obtained from Thermo Fisher Scientific Inc. Brand (Waltham, MA, USA). CHO-K1 cells overexpressing human NPFF-R2 (#ES-490-A) or NPFF-R1 (#ES-491-C) were both purchased from Perkin Elmer (Waltham, MA, USA). All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Growth and assay media were prepared according to the respective manufacturer's protocols. The medium was changed every 2–3 days, and the cells were cultured as needed.

The binding experiments with CHO-K1 cultured cells overexpressing GPR10 were performed as described in (Maletinska et al., 2015). Preparation of cell membranes from NPFF-R2 or NPFF-R1 receptor-overexpressing cells and binding with cell membranes was performed as described in (Karnosova et al., 2021). Bound radioactivity was determined by a γ-counter Wizard 1470 Automatic Gamma Counter (Perkin Elmer, Waltham, MA, USA). Experiments were carried out in duplicate at least three times.

In addition, binding experiments with wild-type CHO-K1 cells were carried out in order to exclude possible background binding. No specific binding to GPR10 or both NPFF receptors was found (results not shown).

2.4. Signaling experiments in vitro

2.4.1. Cell signaling determined by western blotting

Activation of the ERK, CREB and Akt signaling pathways was studied in cells incubated with either PrRP31 or lipidized PrRP31 analogs, and subsequently, SDS electrophoresis and western blotting were performed. These experiments were carried out using CHO-K1 cells overexpressing GPR10 or NPFF-R2. Cells were seeded in 24-well plates at 30000 cells/well in assay medium (growing medium without selective antibiotics) and were grown for 2 days. The day before the experiment, the medium was changed to serum-free medium for 20 h. The next morning, the cells were incubated with PrRP31 or lipidized PrRP31 analogs at final concentrations ranging from 10^{-11} to 10^{-5} M for 5 min at 37 °C and then

Table 1
Sequences and physicochemical properties of lipidized human PrRP31 analogs.

Analog	Sequence	% Purity	Retention time [min]	Calculated mass	Measured mass
PrRP31	SRTHRHSMEIRTPDINPAWYASRGIRPVGRF-NH_2	99	14,8	3661,9	3662,0
palm-PrRP31	(N-palm) SRTHRHSMEI R TPDINPAWYASRGIRPVGRF-NH_2	99	21,5	3902,6	3902,5
palm^{11}-PrRP31	SRTHRHSMEI K (N-γE(N-palm)) TPDINPAWYASRGIRPVGRF-NH_2	99	19,4	4001,3	4001,2
palm^{11}-TTDS-PrRP31	SRTHRHSMEI K (N-TTDS(N-palm)) TPDINPAWYASRGIRPVGRF-NH_2	97	22,8	4173,7	4173,8
analog 1	((N-palm)γE) SRTHRHSMEI K TPDINPAWYASRGIRPVGRF-NH_2	96	20,9	4029,3	4029,3
analog 2	SRTHRHSMEI K (N-palm) TPDINPAWYASRGIRPVGRF-NH_2	97	22,3	3872,3	3872,1
analog 3	SRTHRHSMEI K (N-GABA(N-palm)) TPDINPAWYASRGIRPVGRF-NH_2	98	22,3	3957,4	3957,4
analog 4	SRTHRHSMEI K (N-γE(N-dodec)) TPDINPAWYASRGIRPVGRF-NH_2	98	18,8	3945,2	3945,2
analog 5	SRTHRHSMEI K (N-γE(N-myr)) TPDINPAWYASRGIRPVGRF-NH_2	97	19,9	3973,3	3973,2
analog 6	SRTHRHSMEI K (N-TTDS(N-myr)) TPDINPAWYASRGIRPVGRF-NH_2	95	20,9	4146,2	4146,1
analog 7	N-Ac-SRTHRHSMEI K (N-TTDS(N-myr)) TPDINPAWYASRGIRPVGRF-NH_2	98	21,0	4188,3	4188,2
analog 8	SRTHRHSMEI K (N-TTDS(N-myr)) TPDINPAWYASRGIRPVGRPheCl_2-NH_2	97	21,3	4214,2	4214,1
analog 9	N-Ac-SRTHRHSMEI K (N-TTDS(N-myr)) TPDINPAWYASRGIRPVGRPheCl_2-NH_2	96	21,4	4256,3	4256,5
analog 10	SRTHRHSMEI K (N-γE(N-myr)) TPDINPAWYASRGIRPVGRPheCl_2-NH_2	98	20,0	4041,3	4041,4
analog 11	N-Ac-SRTHRHSMEI K (N-γE(N-myr)) TPDINPAWYASRGIRPVGRPheCl_2-NH_2	98	20,3	4083,3	4083,5
analog 12	N-Ac-SRTHRHSMEI K (N-TTDS(N-palm)) TPDINPAWYASRGIRPVGRPheCl_2-NH_2	95	23,4	4284,3	4284,3
analog 13	((N-palm-COOH)γE) S RTHRHSMEIRTPDINPAWYASRGIRPVGRF-NH_2	94	18,2	4058,9	4059,0
analog 14	((N-palm-COOH)γEγE) S RTHRHSMEIRTPDINPAWYASRGIRPVGRF-NH_2	98	18,1	4188,4	4188,7
analog 15	SRTHRHSMEI K (N-γE(N-palm-COOH)) TPDINPAWYASRGIRPVGRF-NH_2	95	18,4	4030,9	4030,7
analog 16	SRTHRHSMEI K (N-γEγE(N-palm-COOH)) TPDINPAWYASRGIRPVGRF-NH_2	92	18,4	4160,3	4160,2
Scrambled PrRP31	GHFTHSIrMIKTPrNASVyArPRDwGIRPES-NH_2	93	14,4	3633,9	3633,9

palm: palmitoyl (C16), dodec: dodecanoyl (C12), myr: myristoyl (C14), palm-COOH: hexadecanedioic acid, γE: gamma-glutamic acid, TTDS: 1,13-diamino-4,7,10-trioxatridecan-succinamic acid, GABA: gamma-aminobutyric acid, N-Ac: *N*-acetylated at the N-terminus, PheCl_2: dichlorophenylalanine. HPLC was carried out on a C18 Vydac reversed phase column; elution with the gradient 2–80% CH_3CN in 0.1% TFA (in 25 min) was used.

washed in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4). Lysis, electrophoresis and western blotting were carried out according to (Spolcova et al., 2015). For detection of signaling pathways, the following primary antibodies were used: p44/42 MAPK/ERK1/2 (#9107), phospho-p44/42 MAPK/ERK1/2 (#9106), CREB (#9104), phospho-CREB (#9196), Akt (#4691), phospho-Akt (Ser473) (#4060), and GAPDH (#97166) from Cell Signaling Technology (Danvers, MA, USA); and β-actin (#A5441) from Sigma–Aldrich (St. Louis, MO, USA).

2.4.2. *GPR10 receptor activation determined using a beta-lactamase reporter system*

CHO-K1 cells overexpressing GPR10 containing a beta-lactamase reporter gene under the control of diverse response elements combined with a fluorescence-resonance energy transfer (FRET)-enabled substrate (according to the manufacturer's protocol) were used to study the agonist/antagonist properties of PrRP31 and lipidized PrRP31 analogs. Cells were seeded at 40000 cells/well in assay medium, and experiments were performed according to Thermo Fisher's protocol and according to our previous study (Prazienkova et al., 2016). Fluorescence was detected using a Tecan Infinite M1000 fluorometric plate reader (Tecan Group Ltd., Mannedorf, Switzerland) at 409 nm excitation and 460 or 530 nm emissions.

2.5. *Food intake experiments in fasted mice*

All animal experiments followed the ethical guidelines of the EU (86/609/EU) and the Act of the Czech Republic law 246/1992 and were approved by the Committee for Experiments with Laboratory Animals of the Academy of Science of the Czech Republic. Acute food intake (FI) was monitored in male C57BL/6 J mice from Charles River Laboratories (Sulzfeld, Germany), twelve to fourteen weeks old, fed a standard chow diet and housed at a temperature of 23 °C and a daily cycle of 12 h light and 12 h dark (light on 6:00 AM). The day before the experiment, the mice were fasted overnight (16 h). They were injected subcutaneously (SC) with saline or a lipidized PrRP31 analogs at a dose of 5 mg/kg (dissolved in saline) (n = 5–6). Weighed pellets were given to mice fifteen minutes after injection and weighed every 30 min for at least 6 h.

2.6. *Chronic effects of lipidized PrRP analogs on body weight and adiposity in mice with high-fat diet-induced obesity*

From 4 weeks of age, C57BL/6 J mice were fed a high-fat diet (HFD) (13% protein kcal, 60% fat kcal and 27% carbohydrate) and given water *ad libitum* for 16 weeks. Mice resistant to the HFD were withdrawn from the experiment. At the age of 20 weeks, the mice were divided into groups of 10 animals and placed into separate cages with free access to a HFD and water.

Three groups were injected SC either with saline, palm^{11}-PrRP31 or analog 12 at a dose of 5 mg/kg dissolved in saline twice a day for 21 days. The body weight of the mice was monitored daily. At the end of the experiment, overnight-fasted mice were killed by decapitation starting at 8:00 AM, and the inguinal white adipose tissue (iWAT), gonadal white adipose tissue (gWAT), and perirenal white adipose tissue (pWAT) of all mice were dissected, weighed and stored at −70 °C.

2.7. *In vitro stability of lipidized PrRP31 analogs*

The *in vitro* stability of selected PrRP31 analogs was determined in blank rat plasma by liquid chromatography–mass spectrometry (LC–MS). For LC, an Ultimate 3000 (Thermo, USA) was used. The LC system consisted of a DGP-3600SD pump, WPS-3000 T SL autosampler and a TCC-3000SD column compartment. For MS, a 3200 Q-TRAP was employed (AB Sciex, Canada).

The LC method utilized a Chromolith RP-18e (Merck, Darmstadt, Germany) silica-based monolithic column (size 50 × 3 mm ID). Mobile phase A was 0.5% HCOOH in water, and mobile phase B consisted of 0.5% HCOOH in acetonitrile. The gradient profile was as follows: 0–2 min 100% A, 2–3 min from 100% A to 100% B, 3–3.5 min 100% B, 3.5–3.55 from 100% B to 100% A, 3.55–20 min 100% A. The flow rate was 0.5 mL/min during the first seven minutes of the analysis. From the seventh to the eighteenth minute, the flow rate was increased to 1.0 mL/min. For the last two minutes, the flow rate was decreased back to 0.5 mL/min. Extensive reconditioning of the column was necessary to obtain a reproducible retention of the analytes. The column temperature was 25 °C, and the samples were stored at 15 °C in the autosampler. The injection volume was 50 µl. Analyst version 1.6 (AB Sciex) was used for

data acquisition.

The MS instrument consisted of a Turbo-V ion source containing electrospray ionization (ESI) in positive mode. The voltage of the ion spray was 5500 V, the curtain gas was 15 psig, the source temperature was 450 °C, ion source gas 1 was 50 psig and ion source gas 2 was 50 psig. Specific mass transitions were found and used for individual lipidized peptides, and multiple reaction monitoring (MRM) methods were developed with the appropriate standards and then applied for the stability measurements.

Prior to the analysis, all plasma samples were stored at −80 °C in LoBind Eppendorf tubes (Eppendorf AG, Hamburg, Germany). Stock solutions of all analogs were prepared by dissolving the relevant peptide in 0.5% HCOOH in H_2O to a final concentration of 1 mg/mL. After conditioning plasma to 37 °C (Incubator HOTCOLD-S, BioGrade, Czech Republic) overnight, the plasma (at 37 °C) was spiked with a solution of a specific lipopeptide to a final concentration of 100 ng/ml. Then, samples were collected at 0.5, 1, 2, 4, 8, 24 and 48 h after spiking. Fifty microliters of taken-out blank/spiked plasma samples was diluted with 950 µl of a solution consisting of 0.1% HCOOH in acetonitrile/H_2O, 2/8 (*v*/v). Then, the samples were transferred to a 1.8 mL glass vial for subsequent LC–MS analysis. Blank plasma samples were removed from the incubator before spiking with peptides. All samples were collected in triplicate.

2.8. Statistical analysis

The data are presented as the means ± SEMs and were analyzed in GraphPad Software (CA, USA). The saturation and competitive binding experiments were analyzed using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Inhibition constants (K_i) were calculated from IC_{50} values, dissociation constants (K_d) and the concentrations of radioligands.

In cell signaling experiments using western blots, dose–response curves and half-maximal effective concentrations for agonists (EC_{50}) were obtained from the nonlinear regression analyses. The results from signaling assays determined by the beta-lactamase reporter system were analyzed by nonlinear regression by log agonist *versus* response using GraphPad software, and their EC_{50} values were determined. Experiments using immunoblotting were analyzed using one-way ANOVA followed by Dunnett's *post hoc* test; $p < 0.05$ was considered statistically significant.

Acute FI in fasted mice and body weight in DIO mice were analyzed by two-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. FI at 45 min after injection was determined by one-way ANOVA followed by Dunnett's post-hoc test, where the most significant decrease was observed. Finally, weights of adipose tissue in DIO mice were evaluated by one-way ANOVA followed by Dunnett's post-hoc test. $P < 0.05$ was considered statistically significant.

For stability experiments, targeted compounds were quantitated using appropriate mass transitions. Data were acquired and managed utilizing Analyst software version 1.6 (AB Sciex).

3. Results

3.1. Structure of lipidized PrRP31 analogs

Sequences and physicochemical properties of all lipidized PrRP31 analogs are shown in Table 1. Lipidization was performed by attachment of monocarboxylic (dodecanoic, myristic or palmitic) or dicarboxylic (hexadecanedioic) fatty acid at the N-terminus of Ser^1 of the peptide or at the secondary amino group of Lys^{11}, the substitute for the original Arg^{11}. Four series of analogs were designed and synthesized.

The first series contains previously published palm-PrRP31, palm^{11}-PrRP31 and palm^{11}-TTDS-PrRP31 with palmitic acid attached to Ser^1 by an amide bond or to Lys^{11} through gamma glutamic acid (γE) or 1,13-diamino-4,7,10-trioxatridecan-succinamic acid (TTDS) as linkers, respectively (Karnosova et al., 2021; Maletinska et al., 2015; Prazienkova et al., 2017).

Natural PrRP31 and its lipidized analogs of the first series served as comparators in the second, third, and fourth series.

The second series contains analogs with structure similar to those in the comparative first series, but with different attachment of palmitic acid (analog 1 and 2), then palmitoylated PrRP31with a new linker, gamma-aminobutyric acid (GABA) (analog 3) and PrRP31 lipidized with shorter fatty acids than palmitic attached through γE in position 11 (analogs 4 and 5). In the third series, analogs 7, 9, 11, and 12 contain N-terminal acylation and analogs 8–12 have C-terminal dichlorophenylalanine ($PheCl_2$) that was previously found as the most efficient modification of Phe^{31} (Prazienkova et al., 2016). The TTDS or γE linkers, respectively, were used for attachment of myristic (analogs 6–11) or palmitic (analog 12) acid to position 11. Analogs 9 and 12 differ only in fatty acid. In the fourth series, analogs 13–16 have attached hexadecanedioic acid, HOOC-$(CH_2)_n$-COOH, $n = 14$, at Ser^1 or Lys^{11} with either the γE or γE γE linker (Table 1).

Finally, scrambled peptide based on PrRP31 was synthesized and used as a negative control in *in vitro* experiments (Table 1).

3.2. Binding affinities of lipidized PrRP31 analogs for GPR10, NPFF-R2 and NPFF-R1 receptors

In the first comparative series, all palmitoylated analogs showed affinity for GPR10 similar to and affinity for NPFF-R2 and NPFF-R1 increased (palm-PrRP31 even about 50-fold for both receptors) compared to natural PrRP31 (Table 2), which was consistent with our previously published results (Karnosova et al., 2021).

The binding affinities of analogs of the second, third, and fourth series for GPR10, NPFF-R2 and NPFF-R1 were determined in cells overexpressing the particular receptor and compared to natural PrRP31 and lipidized analogs of the first comparatory series (Table 2).

Analogs 1, 2 and 3 of the second series had binding affinities for GPR10 comparable to and analogs 4 and 5 even higher than natural PrRP31 (Table 1, Fig. 1). Except of analog 4, all analogs of the second series had affinity for NPFF-R2 several-fold higher than natural PrRP31. However, analogs 1–3 showed also several-fold increased affinity for NPFF-1R (Table 2).

Analogs 6–12 of the third series showed similar or even increased binding affinity for both GPR10, and NPFF-R2 compared to natural PrRP31. Except for analogs 6 and 7, binding affinity of the analogs for NPFF-R1 stayed similar to or was even lower than that of natural PrRP31 (Table 2).

The fourth series of analogs 13–16 with hexadecanedioic acid showed lower affinity for GPR10 and NPFF-R2, with Ki values one and to two orders of magnitude higher, respectively, than analogs of the previous series (Table 2). Their binding affinity for NPFF-R1 was even lower. It is obvious that modification with dicarboxylic fatty acids decreased PrRP affinity for all three receptors.

Scrambled peptide did not show any affinity for GPR10, NPFF-R1 and NPFF-R2 (Table 2).

3.3. Lipidized PrRP31 analogs activate the ERK, Akt, and CREB pathways in GPR10- and NPFF-R2-overexpressing cells

Using western blotting, the activation of diverse signaling pathways was tested in cells overexpressing either GPR10 or NPFF-R2 (Fig. 1). Natural PrRP31 and all tested lipidized PrRP31 analogs significantly increased the phosphorylation of MAPK/ERK 1/2 in GPR10 or NPFF-R2 cells (Fig. 1A and B). Besides, most of the tested analogs significantly increased phosphorylation of Akt on Ser473 in GPR10- and NPFF-R2 cells (Fig. 1A and B). Analog 15 did not induce an increase in p-Akt in GPR10 (Fig. 1A) but in NPFF-R2 cells, it phosphorylated Akt very significantly and the most powerfully of all analogs activated CREB (Fig. 1B). Scrambled peptide did not activate any pathway tested

Table 2
Binding affinities to CHO-K1 cell line expressing GPR10 or NPFF-R2.

Analog	GPR10 ^{125}I-hP31 K_i [nM]	% of PrRP31	NPFF-R2 ^{125}I-1DMe K_i [nM]	% of PrRP31	NPFF-R1 ^{125}I-1DMe K_i [nM]	% of PrRP31
PrRP31	4.6 ± 0.7	100	19 ± 2.3	100	40 ± 4.2	100
palm-PrRP31	4.0 ± 0.01	113	0.5 ± 0.04	4171	0.8 ± 0.11	5176
palm^{11}-PrRP31	3.5 ± 0.7	130	9.1 ± 1.1	207	21 ± 2.9	192
palm^{11}-TTDS-PrRP31	3.8 ± 0.5	121	2.1 ± 0.2	901	17 ± 2.5	238
analog 1	6.9 ± 1.0	66	2.9 ± 0.3	650	10 ± 1.5	396
analog 2	6.2 ± 0.8	74	6.3 ± 0.5	301	14 ± 0.3	300
analog 3	6.6 ± 1.4	69	3.6 ± 0.2	530	9.1 ± 0.9	443
analog 4	2.0 ± 0.4	231	28 ± 3.5	68	63 ± 4.4	64
analog 5	1.9 ± 0.07	246	1.6 ± 0.2	1171	73 ± 7.0	56
analog 6	1.8 ± 0.4	257	4.2 ± 0.5	449	11 ± 1.1	381
analog 7	2.1 ± 0.9	218	10 ± 0.3	182	15 ± 0.8	267
analog 8	1.6 ± 0.7	278	3.6 ± 0.3	525	61 ± 4.9	67
analog 9	1.8 ± 0.7	253	12 ± 1.0	161	36 ± 2.8	113
analog 10	3.2 ± 1.1	145	19 ± 2.6	98	149 ± 17	27
analog 11	3.1 ± 1.0	146	17 ± 1.9	108	157 ± 12	26
analog 12	1.5 ± 0.6	309	2.9 ± 0.4	643	28 ± 3.9	142
analog 13	31 ± 5.0	15	130 ± 9.2	14	780 ± 75	5
analog 14	28 ± 4.9	16	140 ± 17	13	523 ± 66	8
analog 15	23 ± 5.4	20	313 ± 46	6	933 ± 110	4
analog 16	15 ± 2.8	30	320 ± 25	6	2158 ± 82	2

The data are presented as the means ± SEM and were analyzed in Graph-Pad Software. Experiments were performed in duplicates at least in three independent experiments.

(Fig. 1A,B).

Dose–response experiments revealed activation of pERK with EC_{50} in a nanomolar range after stimulation with either natural PrRP31 or selected lipidized PrRP31 analogs (3, 9, 12, 15) in GPR10-overexpressing cells (Fig. 1C). In cells overexpressing NPFF-R2, natural PrRP31 induced phosphorylation of ERK at a millimolar concentration, and so did analog 15 (Fig. 1D). Analogs 9 and 12 shifted the EC_{50} to a nanomolar range and thus revealed stronger agonist properties to NPFF-R2 than natural PrRP31 (Fig. 1D).

3.4. Lipidized PrRP31 analogs are strong agonists of GPR10

Using the beta-lactamase reporter gene response with a FRET substrate, GPR10 activation was studied to confirm the agonist properties of PrRP31 and lipidized PrRP31 analogs. All lipidized analogs behaved as agonists of the GPR10 receptor, and their EC_{50} values were in the picomolar range (Table 3). The most potent GPR10 agonists were palm-PrRP31, palm^{11}-PrRP31, palm^{11}-TTDS-PrRP31, analogs 1–3 and analog 12, whose GPR10 activation was 3-fold higher than that of natural PrRP31.

3.5. Lipidized PrRP31 analogs with different linkers strongly reduced food intake after acute administration

All PrRP31 analogs were tested in rodents for their anorexigenic effect in fasted lean mice after a single acute injection (Table 3, Fig. 2). Lipidized PrRP31 analogs or saline were SC injected into fasted mice, and FI was measured for at least 6 h. FI 45 min after injection, when the maximal effect of compounds is observed, is presented in Table 3. As we have already reported, SC administration of natural PrRP31 had no anorexigenic impact and was similar to that in the saline-treated group (Maletinska et al., 2015).

Analogs of the first comparative series with different linkers and positions of fatty acids palm-PrRP31, palm^{11}-PrRP31, palm^{11}-TTDS-PrRP31 and similar analogs of the second series 1–3 showed strong, long-lasting anorexigenic effects (Table 3, Fig. 2A). Analogs 4 and 5 with dodecanoyl and myristoyl groups at position 11, respectively, did not cause a significant decrease in FI (Table 3, Fig. 2B).

All analogs (6–12) of the third series, with $PheCl_2$ on the C-terminus, behaved as potent anorexigenic compounds. The strongest FI-lowering effect was observed after injection of analogs 6, 8, 9 and 12 (Table 3, Fig. 2C, D). In contrast, the fourth series of analogs showed anorexigenic effects neither in fasted mice (Table 3, Fig. 2E).

Based on high affinity for both GPR10 and NPFF-R2 and agonist properties at both receptors, together with strong anorexigenic effect in acute FI test, analogs 9 and 12 were selected as the most potent substances. Both analogs revealed increased affinity for GPR10 and NPFF-R2 but unchanged affinity for NPFF-R1 compared to natural PrRP31. Besides, analog 9 activated GPR10 similarly as and analog 12 even more powerfully than natural PrRP. Therefore analog 12 was selected as the best for chronic administration to mice with DIO.

3.6. Lipidized PrRP31 analogs decreased body weight and adiposity in DIO mice after chronic administration

Analog 12 was as the most potent lipidized PrRP31 selected from *in vitro* and acute FI experiments in fasted and free-fed rodents for long-term administration in DIO mice. Palm^{11}-PrRP31 served as a comparator, as its strong anorexigenic effect and body weight-lowering effect were reported previously (Prazienkova et al., 2017).

The body weight and weights of adipose tissues in DIO mice were significantly lowered after palm^{11}-PrRP31 and analog 12 treatment, and analog 12 was even more potent in lowering body weight than palm^{11}-PrRP31 (Fig. 3).

3.7. Lipidized PrRP analogs have a prolonged half-life in rat plasma

Lipidization of the natural/original peptides significantly improved their stability in blood plasma (Fig. 4). The concentration of human PrRP31 decreased quickly below the detection limit during the first four hours of incubation. The highest stabilities of the measured lipidized analogs were detected in analogs 1, 2, palm^{11}-PrRP31, and palm-PrRP31. Lipidized peptides with the TTDS linker provided significantly lower plasma stability than those with other linkers. In general, lipidization proved to be an efficient way of protecting the plasma stability of PrRP31.

4. Discussion

In this study, we aimed to design and test various lipidized PrRP31 analogs with an increased binding affinity for GPR10 and NPFF-R2 and a reduced or at least unchanged affinity for NPFF-R1 to suppress possible unwanted side effects, with prolonged stability and preserved anorexigenic effects. As reported previously by us (Maletinska et al., 2015; Prazienkova et al., 2017) and recently by Alexopoulou et al.

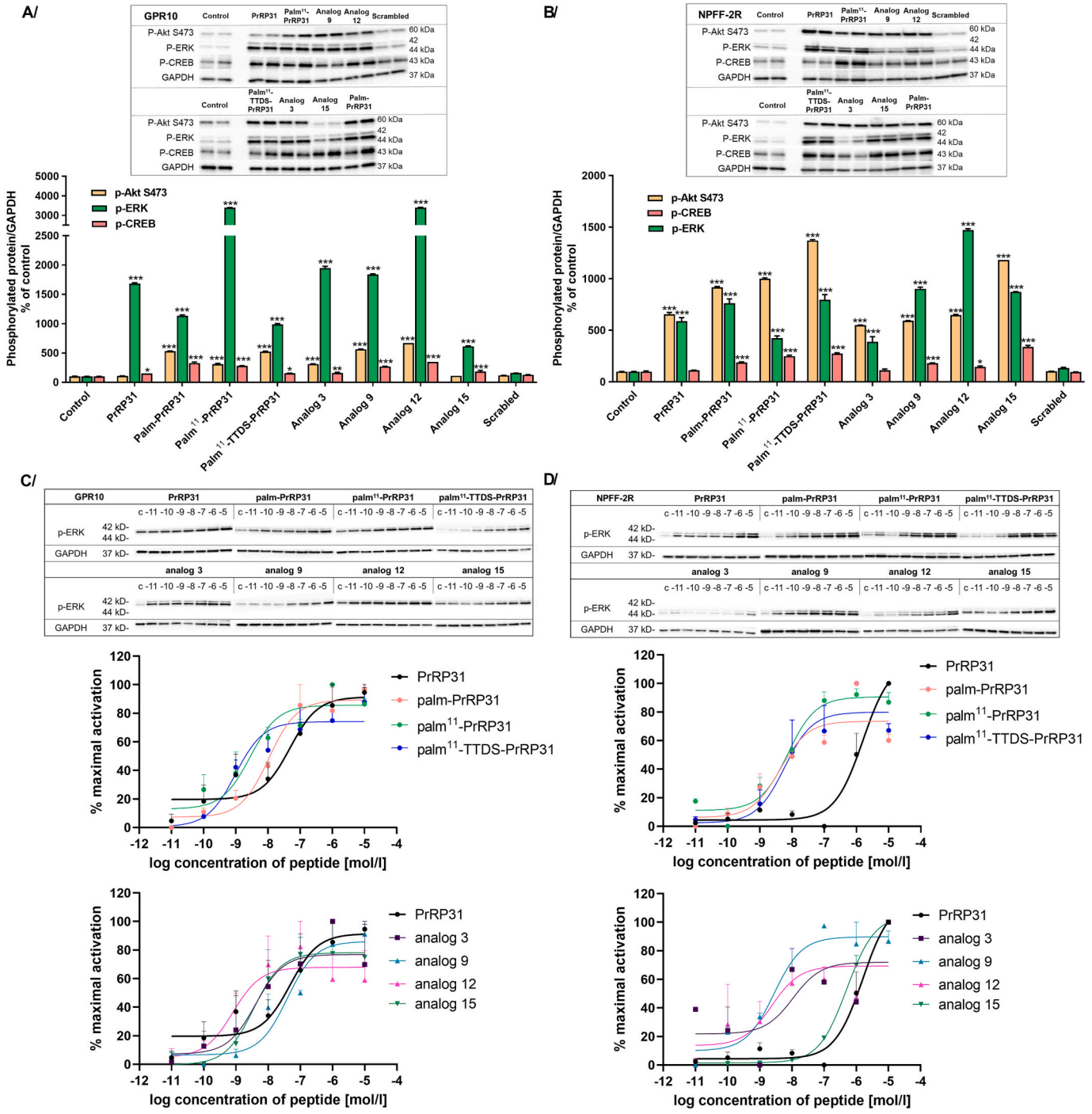


Fig. 1. Activation of signaling pathways using western blots in cells expressing GPR10 or NPFF-R2 receptor.
Induction of Akt (S473), ERK and CREB phosphorylation after 5 min of incubation at 37 °C with peptides at final concentrations of 10^{-6} M in CHO-K1 cells expressing receptors A/ GPR10, B/ NPFF-R2. Dose-response phosphorylation of ERK in CHO-K1 cells expressing C/ GPR10 and D/ NPFF-R2. Densitometric quantification was normalized to GAPDH, and the phosphorylation level in the untreated control was standardized as 100%.
Data are presented as means ± SEM and were analyzed in Graph-Pad Software using one-way ANOVA followed by Dunnett's *post hoc* test. Experiments were performed in duplicates and repeated two (GPR10) or three (NPFF-R2) times. The curves show means ± SEM of all experiments. Significance is * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ lipidized PrRP31 analog *vs* PrRP31.

(Alexopoulou et al., 2022), the dual agonism of lipidized PrRP analogs to GPR10 and NPFF-R2 receptors could be a critical determinant for the desired body-weight lowering effect. In our recent *in vitro* study, we showed that the lipidized PrRP analogs palm-PrRP31 and palm^{11}-PrRP31 had higher affinity for NPFF-R1 than natural PrRP31 (Karnosova et al., 2021). Conversely, the activity of the mentioned analogs at other potential off-target receptors involved in food intake regulation was minimal or negligible (Karnosova et al., 2021). Based on our previous studies, we concluded that a strategy to find selective dual agonists of GPR10 and NPFF-R2 with acceptable low affinity for NPFF-R1 might be desirable for the potential successful treatment of metabolic diseases such as obesity and type 2 diabetes mellitus.

The neuropeptides PrRP and NPFF recently arouse particular interest due to their pronounced expression levels in areas of the hypothalamus

Table 3
Activation of GPR10 receptor and acute food intake in fasted lean mice.

Analog	EC_{50} [pM]	% of PrRP31	Acute FI (45 min) [g]	% of saline treated group
saline	–	–	0.43 ± 0.01	100
PrRP31	530 ± 71	100	0.41 ± 0.04	95
palm-PrRP31	72 ± 6	409	0.05 ± 0.02	12
palm^{11}-PrRP31	39 ± 5	272	0.13 ± 0.02	30
palm^{11}-TTDS-PrRP31	84 ± 18	340	0.05 ± 0.02	12
analog 1	78 ± 19	367	0.08 ± 0.04	19
analog 2	77 ± 2	371	0.06 ± 0.03	14
analog 3	81 ± 13	353	0.16 ± 0.04	37
analog 4	332 ± 28	86	0.33 ± 0.03	77
analog 5	213 ± 22	134	0.35 ± 0.04	81
analog 6	401 ± 48	71	0.23 ± 0.03	53
analog 7	294 ± 37	97	0.26 ± 0.04	60
analog 8	378 ± 60	76	0.13 ± 0.04	30
analog 9	338 ± 57	85	0.06 ± 0.02	14
analog 10	278 ± 26	103	0.21 ± 0.04	49
analog 11	300 ± 37	95	0.18 ± 0.04	42
analog 12	109 ± 13	262	0.10 ± 0.04	23
analog 13	336 ± 16	85	0.44 ± 0.03	102
analog 14	316 ± 36	91	0.41 ± 0.04	95
analog 15	262 ± 33	109	0.37 ± 0.04	86
analog 16	343 ± 80	83	0.45 ± 0.04	105

The data are presented as the means ± SEM and were analyzed in Graph-Pad Software. EC_{50} is the concentration of peptide at 50% of maximal effect. Experiments were performed in duplicates at least in three experiments. Food intake experiments were analyzed by One-way ANOVA followed by Dunnett's *post hoc* test at 45 min after SC injection of saline or PrRP31 analogs. The data are expressed as food intake consumed and percent of food intake of saline-treated group.

and brain stem, respectively. Other RF-amide family peptides, for example, PrRP-expressing neurons in the brainstem, have already been shown to mediate satiety (Koller et al., 2021). GPR10 is involved in the regulation of metabolism, including energy expenditure and fat storage, and GPR10-deficient mice display late-onset obesity (Bjursell et al., 2007; Gu et al., 2004; Prazienkova et al., 2021). Both PrRP and GPR10 are expressed in the hypothalamus and brainstem, areas connected to the regulation of food intake and body weight, as reviewed in Prazienkova et al. (Prazienkova et al., 2019a). High *Npffr2* mRNA expression was found in the amygdala, hypothalamus and medulla oblongata (Gouarderes et al., 2004; Koller et al., 2021). The role of NPFF-R2 in the regulation of energy expenditure is less studied; however, NPFF-R2 seems to be involved in body weight regulation and thermogenesis. NPFF-R2-deficient mice showed accentuated HFD-induced obesity associated with failure to activate brown adipose tissue thermogenesis and lowered expression of uncoupling protein-1 (Zhang et al., 2018). The idea of PrRP dual agonists for a potential treatment of obesity could be further supported by regional distribution of PrRP and its receptors GPR10 and NPFF-R2 in human brain areas connected with energy homeostasis. High level of prolactin-releasing peptide (Takahashi et al., 2000), GPR10 (Bhattacharyya et al., 2003) (Jhamandas and Goncharuk, 2013) and NPFF-R2 (Ubuka et al., 2009) immunoreactivity was confirmed in hypothalamus and brain stem.

The effects of RF-amide peptides such as kisspeptins (Kp), RF amide-related peptides (RFRPs) or PrRPs, are not only mediated through their receptors, but they also act through NPFF-R1 and NPFF-R2 (Quillet et al., 2016). From our study it is clear that PrRP analogs prefer to act through NPFF-R2 rather than NPFF-R1. On the other hand, Kps, together with RFRPs, prefer to act through NPFF-R1 (Quillet et al., 2016). NPFF-R1 was found to be weakly expressed in Gonadotropin-releasing hormone (GnRH) neurons in mice of both sexes. Kps and RFRPs were found to affect food intake and control the reproductive system by regulating GnRH neurons and gonadotropin secretion possibly (Poling et al., 2012). However, the involvement of NPFF-R1 signaling in the regulation of energy homeostasis has not been fully investigated. One study with NPFF-R1-deficient mice reported unaffected body weight and total energy expenditure in males fed a standard diet or HFD despite their reduced food intake and impaired glucose tolerance and insulin sensitivity (Leon et al., 2018). Conversely, NPFF-R1-deficient female mice displayed increased BW after HFD or ovariectomy, and those on HFD showed the decrease in energy expenditure while their glycemic homeostasis was unaffected (Leon et al., 2018).

As we have previously reported, lipidized PrRP analogs palmitoylated at Ser^{1} or Lys^{11} bind with high affinity to both GPR10 and NPFF-R2 receptors, revealing strong anti-obesity, antidiabetic (Maletinska et al., 2015; Prazienkova et al., 2017) and neuroprotective effects in relevant rodent models (Holubova et al., 2019; Popelova et al., 2018; Prazienkova et al., 2019b). Based on our previous structure-activity studies with lipidized PrRP31 analogs (Maletinska et al., 2015; Prazienkova et al., 2016; Prazienkova et al., 2017), three new series of palmitoylated and myristoylated PrRP31 analogs were designed, synthesized and tested in this study and compared with the first series containing natural PrRP31 and previously published analogs.

The second series analogs presented in this study are actually analogs of the first series with modified sites of lipidization and linkers as palmitoylation at Lys in position 11 *via* γE and TTDS linkers [15] in palm^{11}-PrRP31 and palm^{11}-TTDS-PrRP31 of the first comparative series resulted in dual GPR10 – NPFF-R2 agonism with strong anorexigenic properties. Binding affinities determined in the first and second series suggested that a linker could influence the affinity of analogs for receptors. Interestingly, there was no difference between palm^{11}-PrRP31 with the γE linker and analog 2 with no linker in affinity for all receptors; however, the change in γE for the TTDS or GABA linker in palm^{11}-TTDS- or analog 3, respectively, enhanced binding affinity for NPFF-R2 2- to 3-fold.

In the third series, we modified previously published PheCl$_2^{31}$ PrRP31 analogs palmitoylated or myristoylated at the N-terminus that showed a strong long-lasting anorexigenic effect in fasted mice, most likely owing to a higher stabilization due to a noncoded C-terminal amino acid without a negative impact on their biological effect (Prazienkova et al., 2016). Here we combined the substitution of Phe^{31} for PheCl$_2^{31}$ with N-terminal acetylation to enhance the stability of the molecule against proteolytic degradation.

In the fourth series, attachment of hexadecanedioic acid instead of palmitic acid was applied, aiming again at increased stability in the last series of analogs. From our *in vitro* data, several PrRP31 lipidized analogs were selected as potent dual agonists of GPR10 and NPFF-R2, namely analogs 1–3 of the second series with fatty acids attached through different linkers and analogs 8–12 of the third series with C-terminal PheCl$_2^{31}$, whose *in vitro* biological activity was comparable with palm-PrRP31 or palm^{11}-PrRP31. ERK and Akt activation induced by analogs 3, 9, 12 in GPR10 and NPFF-R2 containing cells confirmed that these analogs acted as agonists at both receptors and their agonism to

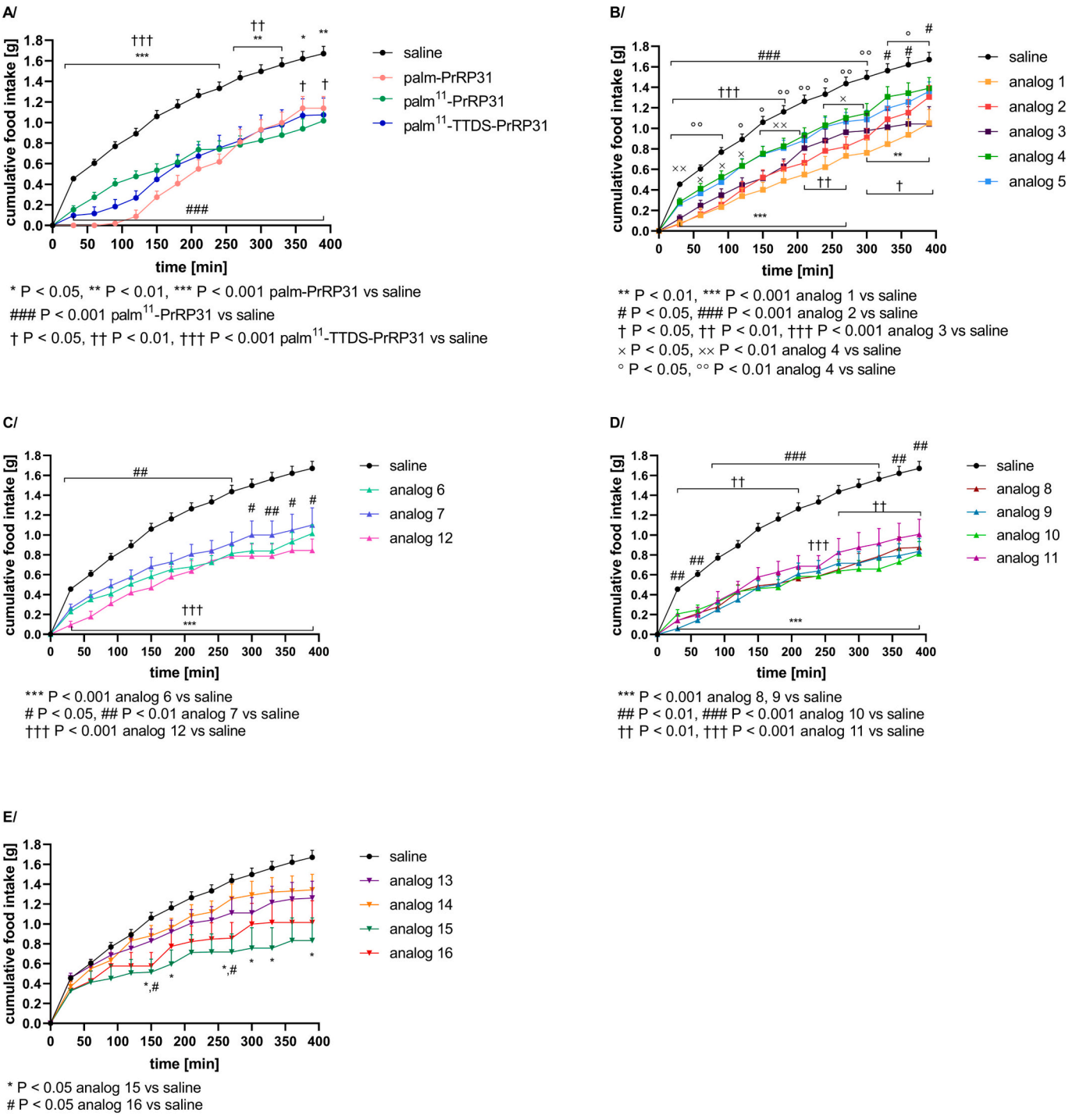


Fig. 2. Acute food intake in fasted lean mice.
Cumulative food intake was monitored in fasted mice every 30 min after subcutaneous injection of peptides at a dose 5 mg/kg for 6 h. Data are presented as means ± SEM and were analyzed by Graph-Pad Software using two-way ANOVA followed by Dunnett's *post hoc* test. All data are compared to food intake of saline-treated group. Experiments were carried out in three independent experiments (n = 5–6 in each experiment). Significance as described in the Figure.

GPR10 was further approved by receptor activation assay. Analog 9 activated GPR10 similarly as and analog 12 even more powerfully than natural PrRP31.

On the other hand, analogs of the fourth series with dicarboxylic acids (13–16) showed substantially decreased affinity for both GPR10 and NPFF-R2 compared to natural PrRP31. Regardless of the way of attachment to the peptide, hexadecanedioic acid lowered affinity for both GPR10 and NPFF-R2 and proved as an unsuitable modification. See Fig. 5 for structure-activity relationship of the tested analogs.

In this study, we also aimed to develop analogs of similar or even suppressed affinity to NPFF-R1 to avoid possible unknown side effects. From all analogs with high affinity to both GPR10 and NPFF-R2, analogs 4, 5, and 6–12 had lower or at least similar affinity for NPFF-R1 as natural PrRP31.

The most important biological activity of lipidized PrRP31 analogs determined in this study was their anorexigenic effects measured by food intake decrease in fasted mice. In addition to three analogs from the first series used as comparators with known strong anorexigenic effects

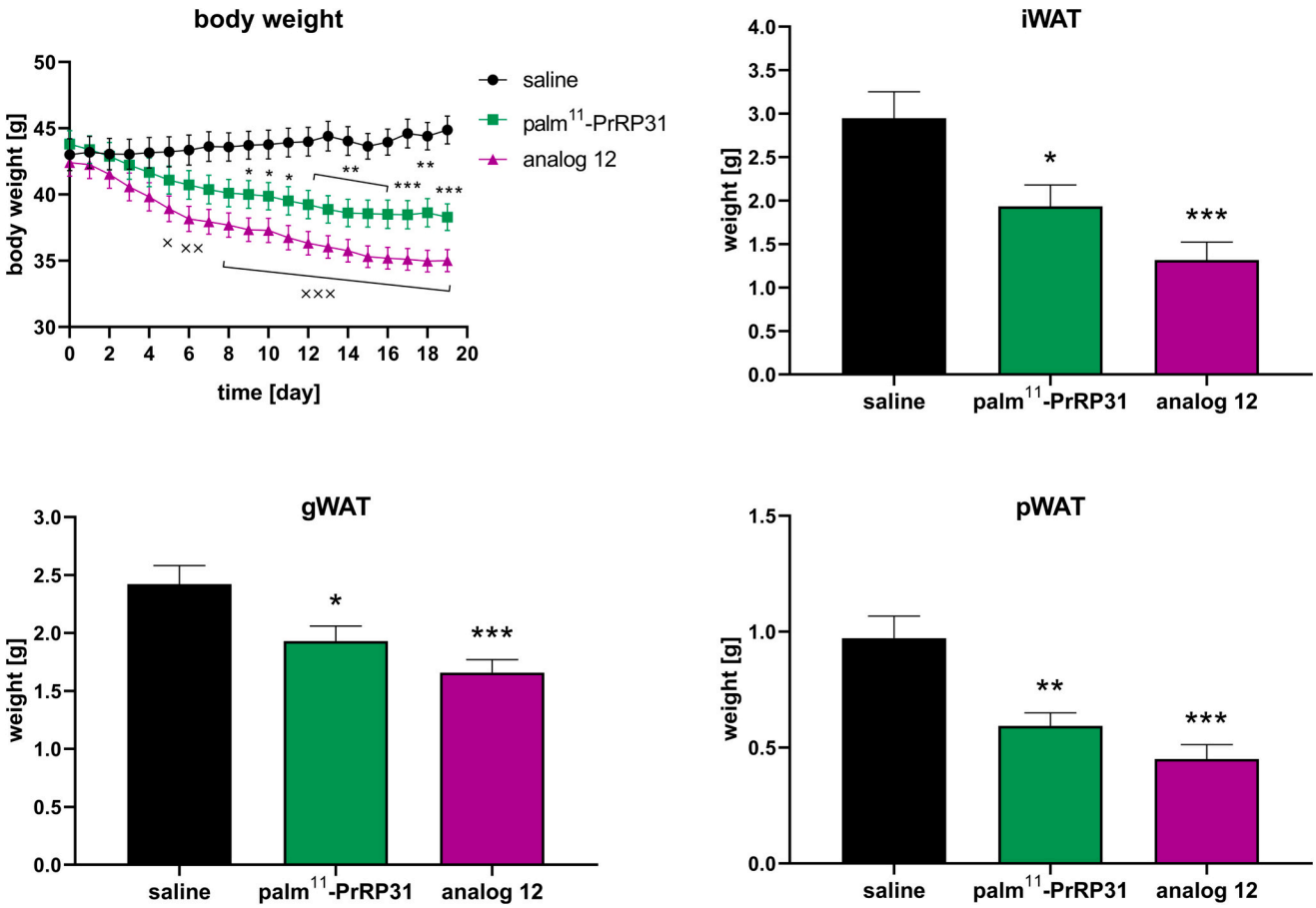


Fig. 3. Chronic effect of palm^{11}-PrRP31 and analog 12 on body and adipose tissue weight in DIO mice.
Saline or peptides at a dose of 5 mg/kg ($n = 10$) were injected twice daily for 21 days. Body weights were monitored daily and weights of adipose tissue were measured at the end of the experiment. Data are presented as means ± SEM and were analyzed in Graph-Pad Software using two-way ANOVA for body weight and one-way ANOVA followed by Dunnett's *post hoc* test for weights of adipose tissue. All data are compared to saline-treated group. Significance is ** $P < 0.01$, *** $P < 0.001$ of palm^{11}-PrRP31 or analog 12 *vs* saline, ^{x} $P < 0.05$, ^{xxx} $P < 0.001$ of analog 12 *vs* palm^{11}-PrRP31. iWAT – inguinal white adipose tissue, gWAT – gonadal white adipose tissue, pWAT – perirenal white adipose tissue.

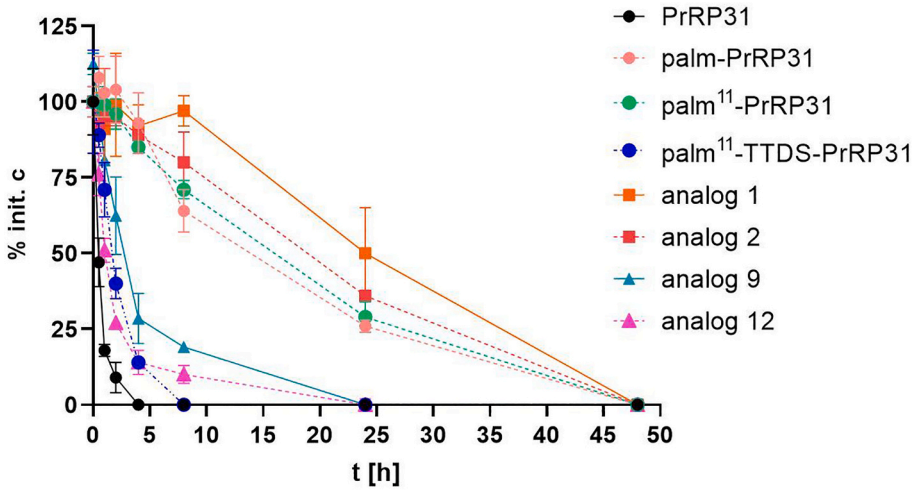
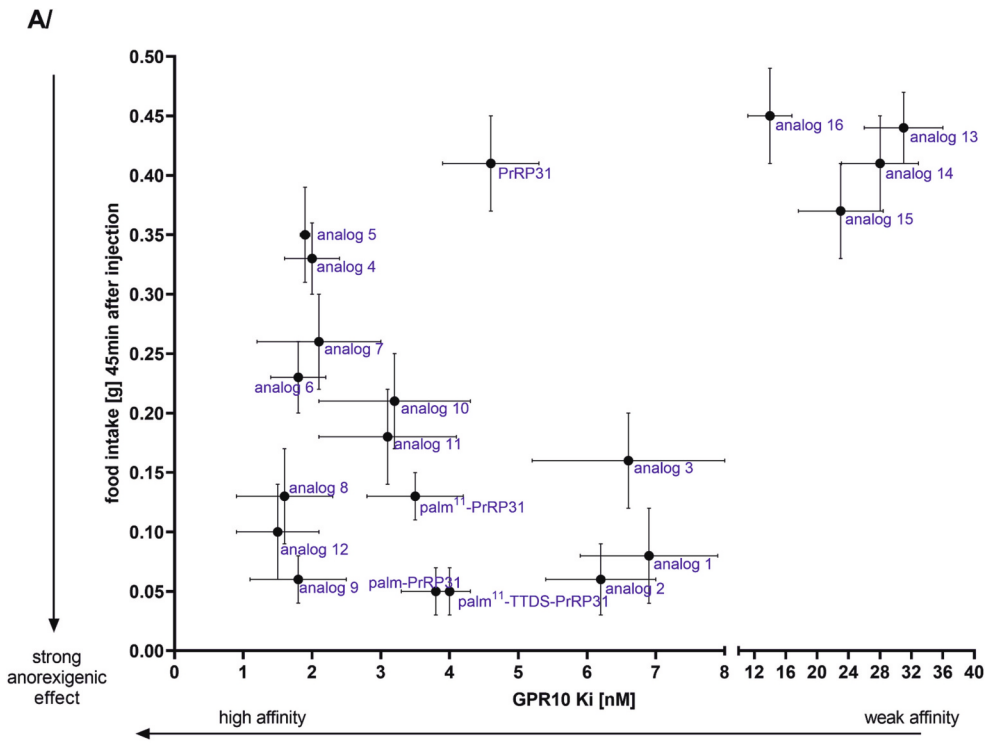


Fig. 4. Stability of lipidized PrRP analogs in rat plasma monitored by LC-MS.
Experiments in all time points were accomplished in triplicate. The data are presented as means ± SD.

(Prazienkova et al., 2017), analogs 1 and 2 and analogs 8, 9 and 12 with PheCl$_2^{31}$showed the most potent anorexigenic properties (Fig. 5). Among all the tested analogs, analog 12 acetylated at the N-terminus with PheCl$_2^{31}$ and palmitoyl attached through the TTDS linker to Lys^{11} showed the strongest anorexigenic effects resulting probably from its high affinity for both GPR10 and the NPFF-R2. Analog 12 had also had not increased affinity for NPFF-R1 and prolonged anorexigenic effect was also observed in the fasted mice.



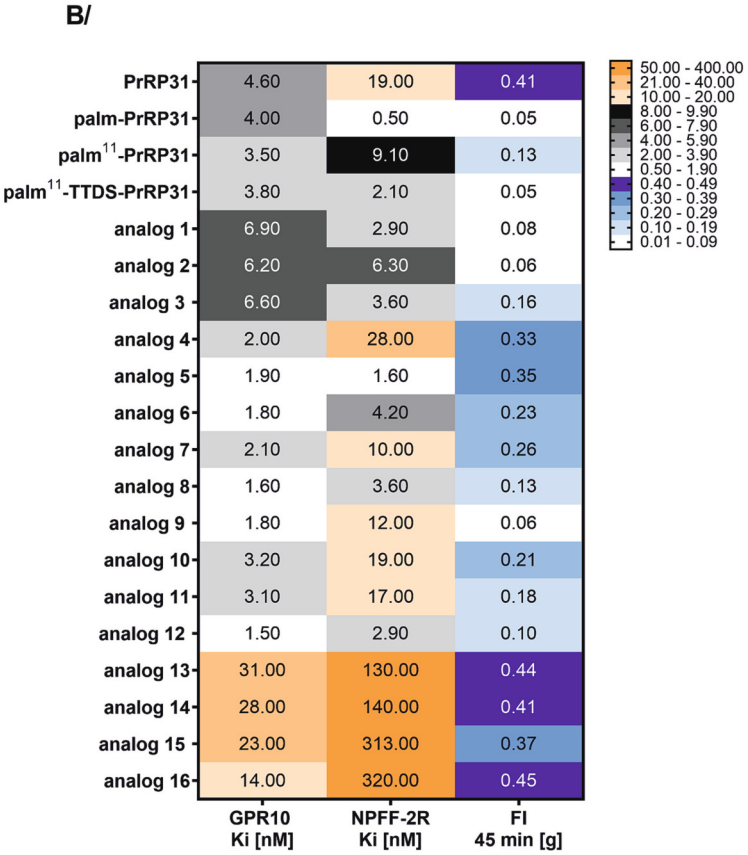


Fig. 5. Structure-activity of analogs in the study.
A/ Relationship between affinity of analogs to GPR10 and anorexigenic effect, B/ Heatmap of affinity of analogs to GPR10 and NPFF- 2R compared to anorexigenic effect.

However, measurement of the stability of analogs in rat plasma revealed unexpected results. Surprisingly, all analogs containing the TTDS linker showed longer stability than natural PrRP31 but much shorter than analogs with no or γE linker. This feature was observed despite the fact that all analogs with no linker (analog palm-PrRP31, analog 2), analogs with a TTDS linker (analogs palm^{11}-TTDS-PrRP31, 6–9 and 12) and analogs with a γE linker (palm^{11}-PrRP31, 10–11) are very potent anorexigenic dual agonists. This could be a result of the fast central action of the TTDS analogs after peripheral administration, or of their biologically active fragments. It is possible that analogue 12 has very fast central effect despite a relatively short half-life in the plasma. Furthermore, analog 12 can be partially enzymatically digested providing bioactive fragments (*i.e.*, fragments longer than 20 amino acids at the C-terminus). Such fragments were not measured by the LC-MS method targeted specifically at intact analog 12. This phenomenon should be further studied.

Analog 12 was further tested in a chronic study with DIO mice. This analog, despite its moderate stability, was shown to be more potent in body and adipose tissue weight reduction than palm^{11}-PrRP31 used as a comparator. Both analogs 12 and palm^{11}-PrRP31 were able to significantly decrease body weight and adipose tissue weight after repeated administration, and analog 12 had a significantly more pronounced effect than palm^{11}-PrRP31. In a recent study (Alexopoulou et al., 2022), PrRP31 was lipidized by octadecanedioic acid (C18), which was previously used in the long-lasting GLP-1 analog semaglutide (Lau et al., 2015). Even though this modification resulted in unaffected binding affinity to GPR10, this analog had less affinity to NPFF-R2 and showed no effect on FI and body weight in DIO mice. However, a recent study by others also showed that stearoyl acid (C18) lipidized PrRP analogs bound to not only GPR10 but also NPFF-R2 with high affinity and had long-lasting anti-obesity effects in DIO mice (Alexopoulou et al., 2022). Furthermore, shorter lipidized PrRP fragments (decapeptide) with attached C18 fatty acid with 8-amino-3,6-dioxaoctanoic acid as a linker were demonstrated to maintain strong affinity for GPR10 and NPFF-R2 with a robust weight-lowering effect (Alexopoulou et al., 2022).

It is well known that both NPFF receptors are involved also in central cardiovascular regulation particularly within the brainstem and hypothalamic paraventricular nucleus (reviewed (Jhamandas and Goncharuk, 2013). Therefore, possible side effects after the intervention with our dual PrRP agonists could be expected. However, all current knowledge on the role of PrRP and NPFF in the regulation of blood pressure relies on studies from acute effects in experimental animal models. Moreover, our study with conscious rats did show no change of arterial blood pressure after intraperitoneal or subcutaneous acute or chronic administration (our unpublished results).

This study also strongly supports conclusions from our previous studies that dual GPR10-NPFF-R2 agonism is needed for efficient attenuating effects on FI and body weight in DIO mice. In contrast, lipidized PrRP analogs with a selective affinity to GPR10 even proclaimed desirable for FI and body weight decrease (Pflimlin et al., 2019), caused only modest effect (4% of initial body weight after chronic two-week administration) compared to the body weight-lowering effects of our analogs in the DIO model (12% of initial body weight for palm^{11}-PrRP31 and 17% for analog 12) or the study of Alexopoulou (10% of body weight) (Alexopoulou et al., 2022). To summarize, in our study, analog 12 as a dual GPR10/NPFF-R2 agonist revealed the most significant anorexigenic effect and could be a potential anti-obesity compound.

In conclusion, our study and others confirmed that dual GPR10-NPFF-R2 agonism is necessary for full anorexigenic activity. Our newly designed analogs in this study were compared with previously published analogs, and most of them revealed strong dual agonism with partially suppressed NPFF-R1 affinity. The most potent dual agonist with stabilized N- and C-termini and palmitoylated through the TTDS linker to Lys^{11} showed strong anti-obesity properties and is a potential compound for further studies.

Author contributions

V.S. measured and evaluated the data, prepared the figures and wrote the manuscript. A.K. measured the data, wrote the manuscript and prepared the figures; M.B. synthetized analogs, B.N. measured FI in rats and performed rat plasma preparation, L.H. assisted in the preparation of the figures and measured the data. A.M. assisted in the preparation of the figures and wrote the manuscript. A.F. performed the analytics, D.S. performed the LC–MS analyses and wrote the manuscript. B.Ž. revised the manuscript. J.K. designed the experiments, measured FI in rats and revised the manuscript. L.M. designed the analogs and experiments, measured FI, evaluated the data and wrote and revised the manuscript. All authors have read and approved the published version of the manuscript.

Funding

This work was supported by the Czech Science Foundation (GACR 21-03691S), Czech Academy of Sciences (RVO:67985823 and RVO:61388963) and by the project National Institute for Research of Metabolic and Cardiovascular Diseases (Programme EXCELES, ID Project No. LX22NPO5104) - Funded by the European Union - Next Generation EU.

Declaration of Competing Interest

There is no conflict of interest.

Data availability

The data that has been used is confidential.

Acknowledgments

We would like to thank Hedvika Vysušilová (Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic) for her excellent technical assistance and animal handling, along with Aleš Marek and his team (Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic) for iodination of peptides.

References

Alexopoulou, F., Bech, E.M., Pedersen, S.L., Thorbek, D.D., Leurs, U., Rudkjaer, L.C.B., Fosgerau, K., Hansen, H.H., Vrang, N., Jelsing, J., et al., 2022. Lipidated PrRP31 metabolites are long acting dual GPR10 and NPFF2 receptor agonists with potent body weight lowering effect. Sci. Rep. 12, 1696.

Anjum, S., Khattak, M.N.K., Tsutsui, K., Krishna, A., 2021. RF-amide related peptide-3 (RFRP-3): a novel neuroendocrine regulator of energy homeostasis, metabolism, and reproduction. Mol. Biol. Rep. 48, 1837–1852.

Bhattacharyya, S., Luan, J., Challis, B., Schmitz, C., Clarkson, P., Franks, P.W., Middelberg, R., Keogh, J., Farooqi, I.S., Montague, C., et al., 2003. Association of polymorphisms in GPR10, the gene encoding the prolactin-releasing peptide receptor with blood pressure, but not obesity, in a U.K Caucasian population. Diabetes 52, 1296–1299.

Bjursell, M., Lenneras, M., Goransson, M., Elmgren, A., Bohlooly, Y.M., 2007. GPR10 deficiency in mice results in altered energy expenditure and obesity. Biochem. Biophys. Res. Commun. 363, 633–638.

Boyle, R.G., Downham, R., Ganguly, T., Humphries, J., Smith, J., Travers, S., 2005. Structure-activity studies on prolactin-releasing peptide (PrRP). Analogues of PrRP-(19-31)-peptide. J. Pept. Sci. 11, 161–165.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Deluca, S.H., Rathmann, D., Beck-Sickinger, A.G., Meiler, J., 2013. The activity of prolactin releasing peptide correlates with its helicity. Biopolymers 99, 314–325.

Elbert, T., Vesela, I., 2010. Conditions for Succsesful labeling of oxidation sensitive peptides by Na[I-125] - Iodo-gen (tm) system. J. Label. Compd. Radiopharm. 53, 288–291.

Engstrom, M., Brandt, A., Wurster, S., Savola, J.M., Panula, P., 2003. Prolactin releasing peptide has high affinity and efficacy at neuropeptide FF2 receptors. J. Pharmacol. Exp. Ther. 305, 825–832.

Fang, Q., He, F., Wang, Y.Q., Guo, J., Zhang, B.Z., Chen, Q., Wang, R., 2007. Pharmacological effects of the dansylated neuropeptide FF analogues on body temperature and morphine analgesia. Neuropeptides 41, 339–347.
Gouarderes, C., Puget, A., Zajac, J.M., 2004. Detailed distribution of neuropeptide FF receptors (NPFF1 and NPFF2) in the rat, mouse, octodon, rabbit, guinea pig, and marmoset monkey brains: a comparative autoradiographic study. Synapse 51, 249–269.
Gu, W., Geddes, B.J., Zhang, C., Foley, K.P., Stricker-Krongrad, A., 2004. The prolactin-releasing peptide receptor (GPR10) regulates body weight homeostasis in mice. J. Mol. Neurosci. 22, 93–103.
Hinuma, S., Habata, Y., Fujii, R., Kawamata, Y., Hosoya, M., Fukusumi, S., Kitada, C., Masuo, Y., Asano, T., Matsumoto, H., et al., 1998. A prolactin-releasing peptide in the brain. Nature 393, 272–276.
Holubova, M., Zemenova, J., Mikulaskova, B., Panajotova, V., Stohr, J., Haluzik, M., Kunes, J., Zelezna, B., Maletinska, L., 2016. Palmitoylated PrRP analog decreases body weight in rats with DIO but not in ZDF rats. J 229, 85–96.
Holubova, M., Hruba, L., Popelova, A., Bencze, M., Prazienkova, V., Gengler, S., Kratochvilova, H., Haluzik, M., Zelezna, B., Kunes, J., et al., 2019. Liraglutide and a lipidized analog of prolactin-releasing peptide show neuroprotective effects in a mouse model of beta-amyloid pathology. Neuropharmacology 144, 377–387.
Hu, K.L., Chang, H.M., Li, R., Yu, Y., Qiao, J., 2019. Regulation of LH secretion by RFRP-3 - from the hypothalamus to the pituitary. Front. Neuroendocrinol. 52, 12–21.
Jhamandas, J.H., Goncharuk, V., 2013. Role of neuropeptide FF in central cardiovascular and neuroendocrine regulation. Front Endocrinol (Lausanne) 4, 8.
Karnosova, A., Strnadova, V., Hola, L., Zelezna, B., Kunes, J., Maletinska, L., 2021. Palmitoylation of prolactin-releasing peptide increased affinity for and activation of the GPR10, NPFF-R2 and NPFF-R1 receptors: in vitro study. Int. J. Mol. Sci. 22.
Koller, J., Herzog, H., Zhang, L., 2021. The distribution of neuropeptide FF and neuropeptide VF in central and peripheral tissues and their role in energy homeostasis control. Neuropeptides 90, 102198.
Lau, J., Bloch, P., Schaffer, L., Pettersson, I., Spetzler, J., Kofoed, J., Madsen, K., Knudsen, L.B., McGuire, J., Steensgaard, D.B., et al., 2015. Discovery of the once-weekly glucagon-like Peptide-1 (GLP-1) analogue Semaglutide. J. Med. Chem. 58, 7370–7380.
Lawrence, C.B., Celsi, F., Brennand, J., Luckman, S.M., 2000. Alternative role for prolactin-releasing peptide in the regulation of food intake. Nat. Neurosci. 3, 645–646.
Lawrence, C.B., Ellacott, K.L., Luckman, S.M., 2002. PRL-releasing peptide reduces food intake and may mediate satiety signaling. Endocrinology 143, 360–367.
Leon, S., Velasco, I., Vazquez, M.J., Barroso, A., Beiroa, D., Heras, V., Ruiz-Pino, F., Manfredi-Lozano, M., Romero-Ruiz, A., Sanchez-Garrido, M.A., et al., 2018. Sex-biased physiological roles of NPFF1R, the canonical receptor of RFRP-3, in food intake and metabolic homeostasis revealed by its congenital ablation in mice. Metabolism 87, 87–97.
Lin, S.H., 2008. Prolactin-releasing peptide. Results Probl. Cell Differ. 46, 57–88.
Maixnerova, J., Spolcova, A., Pychova, M., Blechova, M., Elbert, T., Rezacova, M., Zelezna, B., Maletinska, L., 2011. Characterization of prolactin-releasing peptide: binding, signaling and hormone secretion in rodent pituitary cell lines endogenously expressing its receptor. Peptides 32, 811–817.
Maletinska, L., Spolcova, A., Maixnerova, J., Blechova, M., Zelezna, B., 2011. Biological properties of prolactin-releasing peptide analogs with a modified aromatic ring of a C-terminal phenylalanine amide. Peptides 32, 1887–1892.
Maletinska, L., Ticha, A., Nagelova, V., Spolcova, A., Blechova, M., Elbert, T., Zelezna, B., 2013. Neuropeptide FF analog RF9 is not an antagonist of NPFF receptor and decreases food intake in mice after its central and peripheral administration. Brain Res. 1498, 33–40.
Maletinska, L., Nagelova, V., Ticha, A., Zemenova, J., Pirnik, Z., Holubova, M., Spolcova, A., Mikulaskova, B., Blechova, M., Sykora, D., et al., 2015. Novel lipidized analogs of prolactin-releasing peptide have prolonged half-lives and exert anti-obesity effects after peripheral administration. Int. J. Obes. 39, 986–993.
Mikulaskova, B., Holubova, M., Prazienkova, V., Zemenova, J., Hruba, L., Haluzik, M., Zelezna, B., Kunes, J., Maletinska, L., 2018. Lipidized prolactin-releasing peptide improved glucose tolerance in metabolic syndrome: Koletsky and spontaneously hypertensive rat study. Nutr Diab. 8, 5.
Mouledous, L., Mollereau, C., Zajac, J.M., 2010. Opioid-modulating properties of the neuropeptide FF system. Biofactors 36, 423–429.
Nicklous, D.M., Simansky, K.J., 2003. Neuropeptide FF exerts pro- and anti-opioid actions in the parabrachial nucleus to modulate food intake. Am. J. Phys. Regul. Integr. Comp. Phys. 285, 1046–1054.
Panula, P., Aarnisalo, A.A., Wasowicz, K., 1996. Neuropeptide FF, a mammalian neuropeptide with multiple functions. Prog. Neurobiol. 48, 461–479.
Pflimlin, E., Lear, S., Lee, C., Yu, S., Zou, H., To A, Joseph, S., Nguyen-Tran, V., Tremblay, M.S., Shen, W., 2019. Design of a Long-Acting and Selective MEG-fatty acid stapled prolactin-releasing peptide analog. ACS Med. Chem. Lett. 10, 1166–1172.
Poling, M.C., Kim, J., Dhamija, S., Kauffman, A.S., 2012. Development, sex steroid regulation, and phenotypic characterization of RFamide-related peptide (Rfrp) gene expression and RFamide receptors in the mouse hypothalamus. Endocrinology 153, 1827–1840.
Popelova, A., Prazienkova, V., Neprasova, B., Kasperova, B.J., Hruba, L., Holubova, M., Zemenova, J., Blum, D., Zelezna, B., Galas, M.C., et al., 2018. Novel Lipidized analog of prolactin-releasing peptide improves memory impairment and attenuates hyperphosphorylation of tau protein in a mouse model of Tauopathy. J. Alzheimers Dis. 62, 1725–1736.
Prazienkova, V., Ticha, A., Blechova, M., Spolcova, A., Zelezna, B., Maletinska, L., 2016. Pharmacological characterization of lipidized analogs of prolactin-releasing peptide with a modified C- terminal aromatic ring. J. Physiol. Pharmacol. 67, 121–128.
Prazienkova, V., Holubova, M., Pelantova, H., Buganova, M., Pirnik, Z., Mikulaskova, B., Popelova, A., Blechova, M., Haluzik, M., Zelezna, B., et al., 2017. Impact of novel palmitoylated prolactin-releasing peptide analogs on metabolic changes in mice with diet-induced obesity. PLoS One 12, e0183449.
Prazienkova, V., Popelova, A., Kunes, J., Maletinska, L., 2019a. Prolactin-releasing peptide: physiological and pharmacological properties. Int. J. Mol. Sci. 20.
Prazienkova, V., Schirmer, C., Holubova, M., Zelezna, B., Kunes, J., Galas, M.C., Maletinska, L., 2019b. Lipidized prolactin-releasing peptide agonist attenuates hypothermia-induced tau hyperphosphorylation in neurons. J. Alzheimers Dis. 67, 1187–1200.
Prazienkova, V., Funda, J., Pirnik, Z., Karnosova, A., Hruba, L., Korinkova, L., Neprasova, B., Janovska, P., Benzce, M., Kadlecova, M., et al., 2021. GPR10 gene deletion in mice increases basal neuronal activity, disturbs insulin sensitivity and alters lipid homeostasis. Gene 774, 145427.
Quillet, R., Ayachi, S., Bihel, F., Elhabazi, K., Ilien, B., Simonin, F., 2016. RF-amide neuropeptides and their receptors in mammals: pharmacological properties, drug development and main physiological functions. Pharmacol. Ther. 160, 84–132.
Roland, B.L., Sutton, S.W., Wilson, S.J., Luo, L., Pyati, J., Huvar, R., Erlander, M.G., Lovenberg, T.W., 1999. Anatomical distribution of prolactin-releasing peptide and its receptor suggests additional functions in the central nervous system and periphery. Endocrinology 140, 5736–5745.
Spolcova, A., Mikulaskova, B., Holubova, M., Nagelova, V., Pirnik, Z., Zemenova, J., Haluzik, M., Zelezna, B., Galas, M.C., Maletinska, L., 2015. Anorexigenic lipopeptides ameliorate central insulin signaling and attenuate tau phosphorylation in hippocampi of mice with monosodium glutamate-induced obesity. J. Alzheimers Dis. 45, 823–835.
Takahashi, K., Yoshinoya, A., Arihara, Z., Murakami, O., Totsune, K., Sone, M., Sasano, H., Shibahara, S., 2000. Regional distribution of immunoreactive prolactin-releasing peptide in the human brain. Peptides 21, 1551–1555.
Ubuka, T., Morgan, K., Pawson, A.J., Osugi, T., Chowdhury, V.S., Minakata, H., Tsutsui, K., Millar, R.P., Bentley, G.E., 2009. Identification of human GnIH homologs, RFRP-1 and RFRP-3, and the cognate receptor, GPR147 in the human hypothalamic pituitary axis. PLoS One 4, e8400.
Zhang, L., Ip, C.K., Lee, I.J., Qi, Y., Reed, F., Karl, T., Low, J.K., Enriquez, R.F., Lee, N.J., Baldock, P.A., et al., 2018. Diet-induced adaptive thermogenesis requires neuropeptide FF receptor-2 signalling. Nat. Commun. 9, 4722.