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# Long-Acting Lipidated Analogue of Human Pancreatic Polypeptide Is Slowly Released into Circulation

Kathrin Bellmann-Sickert,[†] Christian E. Elling,[‡] Andreas N. Madsen,[∥] Paul B. Little,[‡] Karsten Lundgren,[‡] Lars-Ole Gerlach,[‡] Ralf Bergmann,[§] Birgitte Holst,[∥] Thue W. Schwartz,[∥] and Annette G. Beck-Sickinger*[,†]

[†]Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, Leipzig University, Brüderstrasse 34, D-04103 Leipzig, Germany

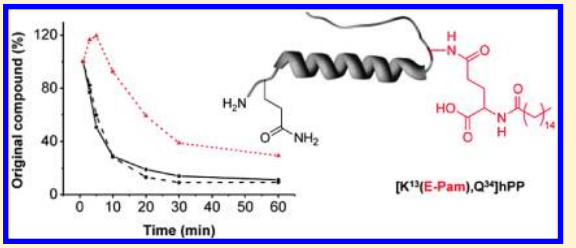
[‡]7TM Pharma, Fremtidsvej 3, DK-2970 Hørsholm, Denmark

[§]Helmholtz-Zentrum Dresden-Rossendorf, Institute of Radiopharmacy, Bautzner Landstrasse 400, D-01328 Dresden, Germany

[∥]Laboratory for Molecular Pharmacology, Department of Neuroscience and Pharmacology, The University of Copenhagen, The Panum Institute, Blegdamsvej 3, DK-2200 Copenhagen, Denmark

Supporting Information

**ABSTRACT:** The main disadvantages of peptide pharmaceuticals are their rapid degradation and excretion, their low hydrophilicity, and low shelf lifes. These bottlenecks can be circumvented by acylation with fatty acids (lipidation) or polyethylene glycol (PEGylation). Here, we describe the modification of a human pancreatic polypeptide analogue specific for the human (h)$Y_2$ and h$Y_4$ receptor with PEGs of different size and palmitic acid. Receptor specificity was demonstrated by competitive binding studies. Modifications had only a small influence on binding affinities and no influence on secondary structure. Both modifications improved pharmacokinetic properties of the hPP analogue in vivo and in vitro, however, lipidation showed a greater resistance to degradation and excretion than PEGylation. Furthermore, the lipidated peptide is taken up and degraded solely by the liver but not the kidneys. Lipidation resulted in prolonged action of the hPP analogue in respect of reducing food intake in mice after subcutaneous administration. Therefore, the lipidated hPP analogue could constitute a potential new therapeutic agent against obesity.




## INTRODUCTION

One of the major health problems predominantly in the Western world is the abnormal accumulation of body fat, referred to as obesity. Not only the overweight itself but most of all its relation to other life threatening diseases constitutes the severity of this issue including diabetes mellitus, cardiovascular diseases, and several kinds of cancer. The risk of a reduced life expectancy as well as public economic problems is considered to already increase with a body mass index (BMI) of 25 kg/m$^2$. This is further emphasized by the fact that in the U.S., 300 000 deaths per year are attributed to obesity. Especially individuals between an age of 25 and 70 years are affected, and obesity is more prevalent in women.[1] Up to now, the only treatment, besides behavioral changes considering food intake and exercise, is bariatric surgery, which however is accompanied with severe side effects.[2] Therefore, pharmaceuticals that regulate appetite and food intake are urgently required. Especially, peptide drugs derived from peptide hormones that already exist within the metabolism are considered to be preferable in contrast to small molecules, as they are believed to be less toxic and more predictable in their in vivo behavior.[3] Among those, the peptide hormone pancreatic polypeptide (PP) is of special interest. PP has first been isolated about 40 years ago from chicken pancreatic extracts and is a member of the neuropeptide Y (NPY) family that consists of three distinct peptides: NPY, peptide YY (PYY), and PP.[4] It specifically binds to and activates the G protein-coupled receptor (GPCR) $Y_4$. PP is mainly expressed in endocrine cells of the pancreas, especially the duodenal portion.[5] Plasma concentrations of PP are regulated by food intake, and its levels rise proportionally to the caloric intake of a meal.[6] Furthermore, PP concentration depends on an intrinsic circadian rhythm because its secretion stays at a quite low level after the first meal of the day and increases with every further meal. After excretion, it rapidly and persistently reduces food intake, delays gastric emptying, and stimulates sympathetic activity and oxygen consumption, thus increasing energy expenditure.[7] These anorectic effects are probably mediated by $Y_4$ receptors in the area postrema, a BBB deficient region, from where it sends anorectic signals via brainstem pathways, hypothalamic neuropeptides, and by modulating the expression of other gut hormones.[8] Because of these qualities, PP constitutes an interesting compound in drug development against obesity and e.g. the Willi—Prader syndrome.[9] The $Y_2$ receptor, that is selectively activated by the

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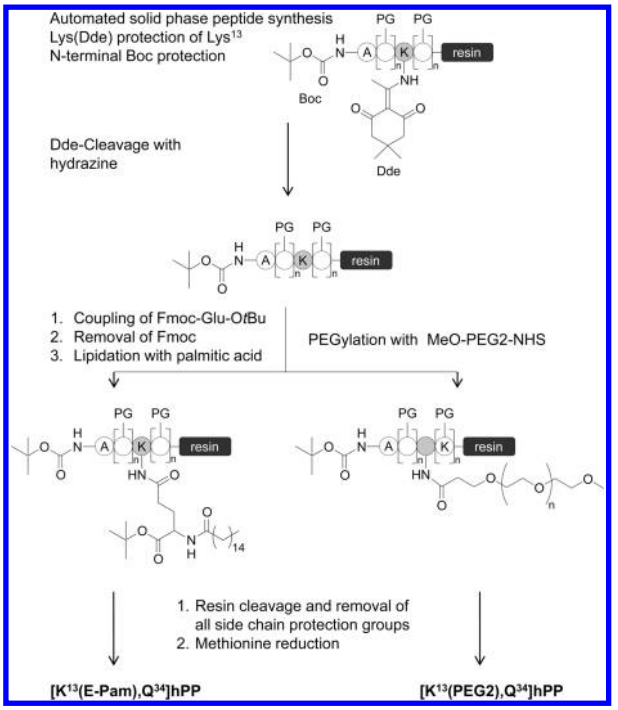
**Scheme 1. Synthesis of [K$^{13}$(E-Pam),Q$^{34}$]hPP and [K$^{13}$(PEG2),Q$^{34}$]hPP[a]**

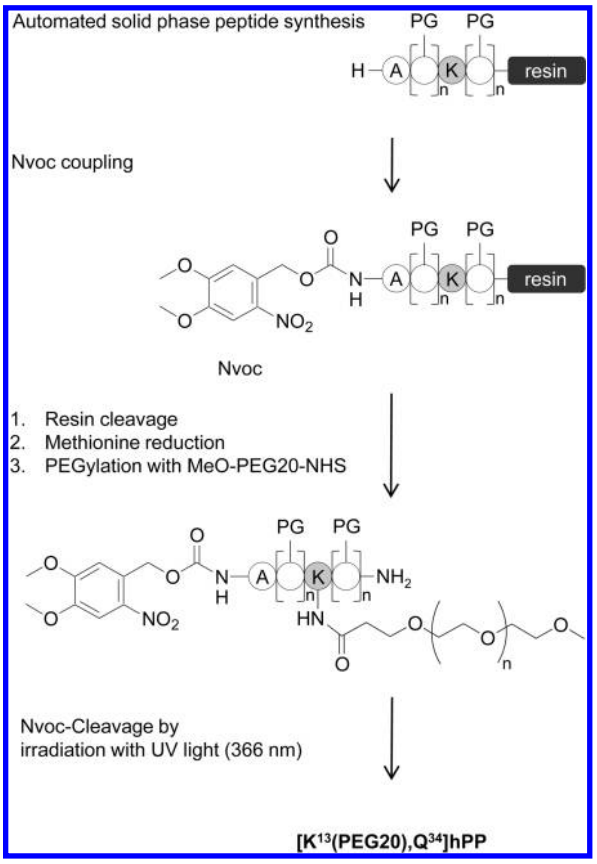



[a] PG, acid labile protection group.

N-terminally truncated PYY analogue, PYY (3−36), is also involved in the regulation of food consumption.[10] No endogenous peptide binds to both the hY$_2$ and the hY$_4$ receptor, however, by replacing Pro$^{34}$ with Gln, a dual specific single digit nanomolar agonist on both the hY$_2$ and the hY$_4$ receptor was obtained, obinepitide.[11] Position 34 had previously been found to be crucial in differential receptor recognition through the design of the prototype hY$_1$ selective ligand [Pro$^{34}$]NPY.[12]

However, the usage of peptides and proteins as therapeutic agents bears severe drawbacks, as there is rapid degradation and excretion and bad water solubility. Additionally, they often cause allergic reactions following strong immune responses.[13] Therefore, strategies to minimize the above-mentioned bottlenecks need to be developed. Among those strategies, formation of peptide analogues, drug delivery systems, and acylation with fatty acids (lipidation) or polyethylene glycol (PEGylation) have evolved during the past decades. The lipidation approach focuses on a covalent binding of fatty acids to the peptide backbone to allow the peptide to noncovalently bind to plasma proteins, which protects the peptide from proteolytic attacks.[3] An alternative approach is the covalent attachment of PEG chains to the peptide, thus mimicking the effects of the plasma proteins as in the case of lipidation.[14] Furthermore, PEGylation provides higher solubility in water and hinders the immune system from a strong response to the peptide and thereby prevents allergic reactions.[15] To elongate the actions of Obinepitide, we introduced a Lys at position 13 that enabled site-specific modification with a 2 or 20 kDa PEG (further referred to as PEG2 and PEG20) or a palmitoyl (Pam) moiety. We further investigated the impact of these modifications on receptor binding specificity, stability, bioavailability, biodistribution, and duration of action in vivo of the peptide.

**Scheme 2. Synthesis of [K$^{13}$(PEG20),Q$^{34}$]hPP[a]**




[a] PG, acid labile protection group.

## RESULTS

**Peptide Synthesis.** Preparation of all analogues was performed as illustrated in Schemes 1 and 2. For peptide synthesis, the 4-(2′,4′-dimethoxyphenyl)-fluorenylmethoxycarbonyl-aminomethyl-phenoxy-acetoamido-norleucylaminomethyl (Rink amide) resin was used in order to obtain an amidated C-terminus. Site specific modification by palmitic acid and PEG2 was achieved by using the 2-acetyl-5,5-dimethyl-1,3-cyclohexanedionyl (Dde) protection group at the N$^{\varepsilon}$ of Lys at position 13. This group could specifically be cleaved off by hydrazine, thereby releasing the free amino group. To prevent a loss in affinity and to increase water solubility, the Pam group was linked to the peptide by a glutamyl spacer, coupled to Lys$^{13}$ via the $\gamma$-carboxyl group of an N-terminally fluorenylmethoxycarbonyl (Fmoc) and C-terminally *tert*-butyl (*t*Bu) protected Glu. PEGylation was achieved with a α-methoxy-$\omega$-carboxy succinimidyl ester PEG2 (MeO-PEG2-NHS) on resin by using 4-(dimethylamino)pyridine (DMAP) and *N*,*N*′-diisopropylcarbodiimide (DIC) as catalysts for the acylation reactions (Scheme 1).[16]

[K$^{13}$(PEG20),Q$^{34}$]hPP was prepared in solution by coupling MeO-PEG20-NHS to an N-terminally 6-nitroveratryloxycarbonyl (Nvoc)-protected [K$^{13}$,Q$^{34}$]hPP followed by UV induced cleavage of Nvoc (Scheme 2).[17] Peptides were analyzed by matrix assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry and reversed-phase high-performance liquid chromatography (RP-HPLC). Purification was carried out by RP-HPLC. Yields and analytical data are presented in Table 1. By

**Table 1. Analytical Data of hPP and Its Analogues**

| compd | elution at % B in A[a] | | purity[b] (%) | yield (%) | | molecular mass (Da) | |
|---|---|---|---|---|---|---|---|
| | $C_{18}$[c] | $C_8$[d] | | raw | purified | calcd | obsd |
| hPP | 46.7 | 42.7 | >95 | 71 | 18 | 4181.8 | 4183.1 |
| hPYY | 38.9 | 37.5 | >95 | 69 | 17 | 4309.9 | 4311.0 |
| [$K^{13}$,$Q^{34}$]hPP | 45.6 | 41.0 | >95 | 70 | 17 | 4237.1 | 4238.4 |
| [$K^{13}$(E-Pam),$Q^{34}$]hPP | 64.2 | 52.2 | >95 | 61 | 16 | 4607.4 | 4607.9 |
| [$K^{13}$(PEG2),$Q^{34}$]hPP | 49.0 | 44.7 | >95 | 54 | 19 | ~6500 | 6304.1 |
| [$K^{13}$(PEG20),$Q^{34}$]hPP | 50.8 | 51.6 | >95 | 85 | 13 | ~26500 | 26040 |

[a] Concentration of B in A at the point of peptide elution from analytical RP-HPLC. Eluants were 0.1% TFA in $H_2O$ (A) and 0.08% TFA in ACN (B). [b] Purity was determined by two different RP-HPLC systems as described under [c] and [d]. [c] Analytical data were obtained from a Vydac RP18-column (4.6 mm × 250 mm; 5 μm). [d] Analytical data were obtained from a Vydac MS C8 column (4.6 mm × 250 mm; 5 μm).

using this technology, selective modification in the presence of multiple amino groups was obtained.

**Binding Assays.** Affinity of the peptide analogues to their receptors was examined by competitive binding assays in which $^{125}$I-labeled specifically binding ligands were incubated at a constant concentration together with the analogues in varying concentrations on COS7 cells transfected with the receptor to be investigated. Our goal was to maintain the specificity of the new ligand [$K^{13}$,$Q^{34}$]hPP for both $hY_2$ and $hY_4$ receptors as well as the influence of the stabilizing modifications on affinity. Table 2 shows that hPYY and hPP had affinities in the subnanomolar range for their respective receptors, whereas [$K^{13}$,$Q^{34}$]hPP showed nanomolar affinities for both $hY_2$ as well as for the $hY_4$ receptor. Modification of [$K^{13}$,$Q^{34}$]hPP with palmitic acid resulted in a retention of binding capacity in the case of the $hY_2$ receptor and in a modest loss on the $hY_4$ receptor. In contrast, PEGylation with PEG2 decreased affinity only marginally compared to the unmodified ligand with 2.26 and 4.45 nM for $hY_2$ and $hY_4$, respectively, while increase in PEG size dramatically decreased affinity to the receptors. In conclusion, modification with palmitic acid and PEG2 resulted in ligands that still bound with appropriate affinities to their receptors.

**Circular Dichroism (CD) Spectroscopy.** All CD spectra showed a spectrum characteristic for the PP-fold structure.[18] They correlate to an α-helix with minima at 222 and 208 nm and a maximum at 190 nm,[19] revealing no alterations in secondary structure caused by the modifications (Figure 1).

**Proteolytic Stability Studies in Human Blood Plasma in Vitro.** N-Terminally 5(6)-carboxyfluorescein (CF)-labeled analogues were examined in citrate stabilized human blood plasma at 37 °C for 144 h in order to evaluate their stability against proteases and peptidases in a life-near system. Metabolites were examined by RP-HPLC with fluorescence detection and MALDI-ToF mass spectrometry. Degradation profiles are shown in Figure 2A. While CF-hPP and CF-[$K^{13}$,$Q^{34}$]hPP were rapidly metabolized with half times around 50 h each, especially the lipidated as well as the PEG20 modified analogue revealed a strong stabilization, as at the end of the observation time about 90% of intact peptide were still found. The analogue modified with the smaller PEG moiety also showed an increased resistance to proteolytic digestion, however, this effect was less pronounced than for the other derivatives. Analysis of degradation products of CF-[$K^{13}$,$Q^{34}$]hPP (Figure 2B) revealed most labile peptide bonds between $Thr^{32}$ and $Arg^{33}$ as well as $Q^{34}$ and $Arg^{35}$ and to a weaker extent between $Leu^{24}$ and $Arg^{25}$ as well as $Arg^{25}$ and $Arg^{26}$.

**In Vivo Bioavailability and Biodistribution Studies on Rats.** For examination in vivo, [$K^{13}$,$Q^{34}$]hPP, [$K^{13}$(E-Pam),$Q^{34}$]hPP, and [$K^{13}$(PEG2),$Q^{34}$]hPP were selected, while [$K^{13}$-(PEG20),$Q^{34}$]hPP was excluded due to its low affinity to the receptors. Furthermore, it showed a pronounced adherence to reaction vessels and syringes that did not allow us to obtain enough material for injection. Peptides were N-terminally coupled to the chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) that noncovalently binds charged metal ions. In our case, $^{68}$Ga was utilized as a probe to follow the metabolism of the peptides.[20] Radiolabeled peptides were injected into rats via their tail vein and blood samples were taken after 1, 3, 5, 10, 20, 30, and 60 min. Samples were then centrifuged, and plasma proteins were precipitated. Radioactivity was measured after every step. Metabolites in the supernatants were examined by RP-HPLC after precipitation. Furthermore, positron emission tomography (PET) studies were conducted during the incubation time of 60 min. Parallel to these studies biodistribution studies were performed.

**Table 2. Competitive Binding Assay of hPP and Its Analogues on Human Y Receptors**

| compd | $IC_{50}$[a] (nM) | | | |
|---|---|---|---|---|
| | $hY_1$ | $hY_2$ | $hY_4$ | $hY_5$ |
| hPP | nd[b] | nd | 0.64 ± 0.13 | nd |
| hPYY | nd | 0.23 ± 0.01 | nd | nd |
| [$K^{13}$,$Q^{34}$]hPP | 967 ± 327 | 1.07 ± 0.08 | 2.09 ± 0.22 | 24.2 ± 3.6 |
| [$K^{13}$(E-Pam),$Q^{34}$]hPP | 2649 ± 563 | 0.64 ± 0.04 | 25.9 ± 1.9 | 28.5 ± 2.6 |
| [$K^{13}$(PEG2),$Q^{34}$]hPP | 2672 ± 597 | 2.26 ± 0.31 | 4.45 ± 0.4 | 45.1 ± 3.9 |
| [$K^{13}$(PEG20),$Q^{34}$]hPP | nd | 33.6 ± 0.4 | 231 ± 45 | nd |

[a] Values are means of at least two experiments ± SEM, max concentration of competitor 10 μM. [b] Not determined.

*The Bioavailability of the Lipidated Peptide Is Increased.* In Figure 3A, a plot of the intact peptide concentration (which means the amount of original substance in respect to the injected radioactivity) against time is shown. As can be seen, $^{68}$Ga-DOTA-[$K^{13}$,$Q^{34}$]hPP was excreted rapidly from the organism with a half residence time of 3.6 min, a value typical for peptides of that size.[21] From the RP-HPLC chromatograms (data not shown) can be concluded that mainly the fast clearance rate rather than the metabolizing of the peptide was responsible for its low bioavailability. Astonishingly, the PEGylated analogue showed a very similar profile with a half residence time of 4.5 min. Here again, the low bioavailability was especially due to the fast clearance through the kidneys but not due to the degradation by proteases, which was slightly prevented in comparison to the unmodified analogue. $^{68}$Ga-DOTA-[$K^{13}$(E-Pam),$Q^{34}$]hPP in

contrast showed a 6-fold increased body residence half time of 25 min. Furthermore, it reached a plateau at a higher concentration of the intact peptide than both the other peptides.

*Lipidation Keeps Peptide Longer in the Blood Circulation and Directs It to the Liver.* In Figure 3B, biodistribution data of the compounds in those tissues and fluids that showed the most prominent effects are shown. As can be clearly seen, $^{68}$Ga-DOTA-[K$^{13}$,Q$^{34}$]hPP and $^{68}$Ga-DOTA-[K$^{13}$(PEG2),Q$^{34}$]hPP accumulated already after 5 min in the kidneys with standard uptake values (SUV) of 32.31% and 44.24%, respectively. This content even increased to 80.13% and 73.78%, respectively, within 60 min. In contrast to that, $^{68}$Ga-DOTA-[K$^{13}$(E-Pam),Q$^{34}$]hPP revealed the highest values after 5 min in liver, with 7.86% increasing to 13.68% after 60 min. Furthermore, it showed a very high blood concentration of 9.3% at the beginning but a low overall percentage in the rat body, indicating a much slower uptake of the peptide within the organism than for the control and the PEGylated peptide.

Observations made in the biodistribution studies are supported by the PET pictures shown in Figure 3C. Clearly, for $^{68}$Ga-DOTA-[K$^{13}$,Q$^{34}$]hPP and $^{68}$Ga-DOTA-[K$^{13}$(PEG2),Q$^{34}$]hPP accumulation in the kidneys is shown while $^{68}$Ga-DOTA-[K$^{13}$(E-Pam),Q$^{34}$]hPP assembled in the heart and the liver quite rapidly and was transferred to the liver during the next 60 min.

*Fatty Acyl Moieties Bind the Peptide to Plasma Proteins.* The ability of the peptides to bind to plasma proteins is documented in Figure 4. Herein the solid columns show radioactivity in the plasma after centrifugation of the blood samples, while hatched columns represent the radioactivity in the supernatant after precipitation of the plasma proteins. All compounds showed a decrease in radioactivity after precipitation, however, in case of the lipidated analogue, this effect was considerably more pronounced than for the other analogues, indicating a strong binding to plasma proteins.

**Acute Feeding Studies.** *Palmitoylation Prolongs the Anorectic Effect of the hPP Analogue.* To determine the effect of the palmitoylated conjugate on the time course of the anorectic effect of the hPP analogue, we conducted an initial food intake study in male C57BL/6J mice. The animals were fasted, randomized according to their body weight, and then either given vehicle, [K$^{13}$,Q$^{34}$]hPP or [K$^{13}$(E-Pam),Q$^{34}$]hPP, 3 mg/kg administered subcutaneously. The peptides were dosed half an hour before beginning of the dark phase, and food intake was measured for 48 h. Figure 5 shows the accumulated food intake for the first 3 h (Figure 5A) as well as for the first 12 h (Figure 5B). A significant reduction in food intake was observed already after 0.5 h in the case of [K$^{13}$,Q$^{34}$]hPP as compared to vehicle, however, after 7.5 h, the accumulated food intake had returned back to that observed in the vehicle treated animals. In contrast, although the food intake in the animals treated with the palmitoylated peptide was lower from the first time point, it first reached statistical significance after 4.5 h compared to vehicle treated animals (Figure 5B). Importantly, the accumulated food intake remained to be lower than that of the vehicle treated mice throughout the observation period (shown for the first 12 h in

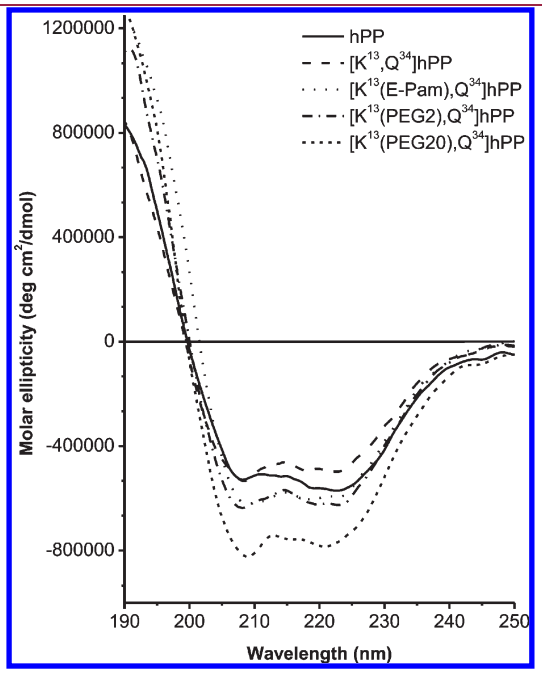
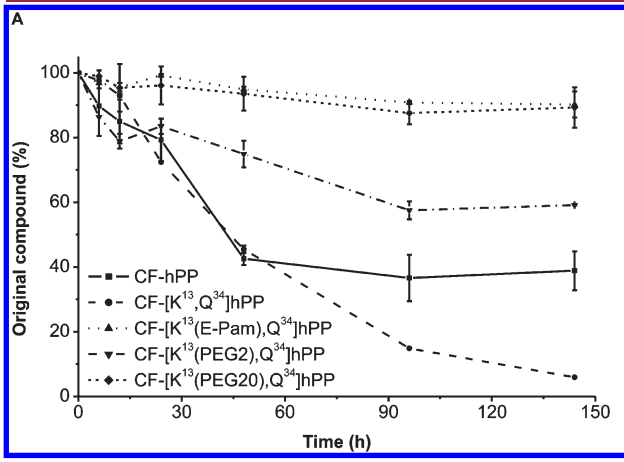



**Figure 1.** CD spectroscopy of hPP and its analogues. CD spectra were recorded at concentrations of 5 $\mu$M in 10 mM phosphate buffer pH 7.0.




B

CF-APLEPVYPGDNAKPE-QM-AQ-YAADL-R-RYINMLT-RQ-R-Y-NH$_2$

| cleavage products of CF-[K$^{13}$,Q$^{34}$]hPP | molecular mass (Da) calcd. | obsd. |
|---|---|---|
| CF-APLEPVYPGDNAKPE-OH | 1955 | 1959 |
| CF-APLEPVYPGDNAKPEQM-OH | 2214 | 2219 |
| CF-APLEPVYPGDNAKPEMAQ-OH | 2413 | 2418 |
| CF-APLEPVYPGDNAKPEQMAQYAADL-OH | 2947 | 2952 |
| CF-APLEPVYPGDNAKPEQMAQAADLR-OH | 3103 | 3108 |
| CF-APLEPVYPGDNAKPEQMAQYAADLRRYINMLT-OH | 3995 | 3997 |
| CF-APLEPVYPGDNAKPEQMAQYAADLRRYINMLTRQ-OH | 4280 | 4282 |
| CF-APLEPVYPGDNAKPEQMAQYAADLRRYINMLTRQR-OH | 4436 | 4434 |

**Figure 2.** Degradation of hPP and its analogues in human blood plasma. (A) N-terminally CF-labeled analogues were incubated in human blood plasma at 37 °C for indicated time points. Quantification of original compound in samples occurred by RP-HPLC. Data are presented as means ± SEM. (B) Cleavage pattern and analytical data of cleavage products of CF-[K$^{13}$,Q$^{34}$]hPP. Cleavage products were separated by RP-HPLC and analyzed by MALDI-ToF mass spectrometry.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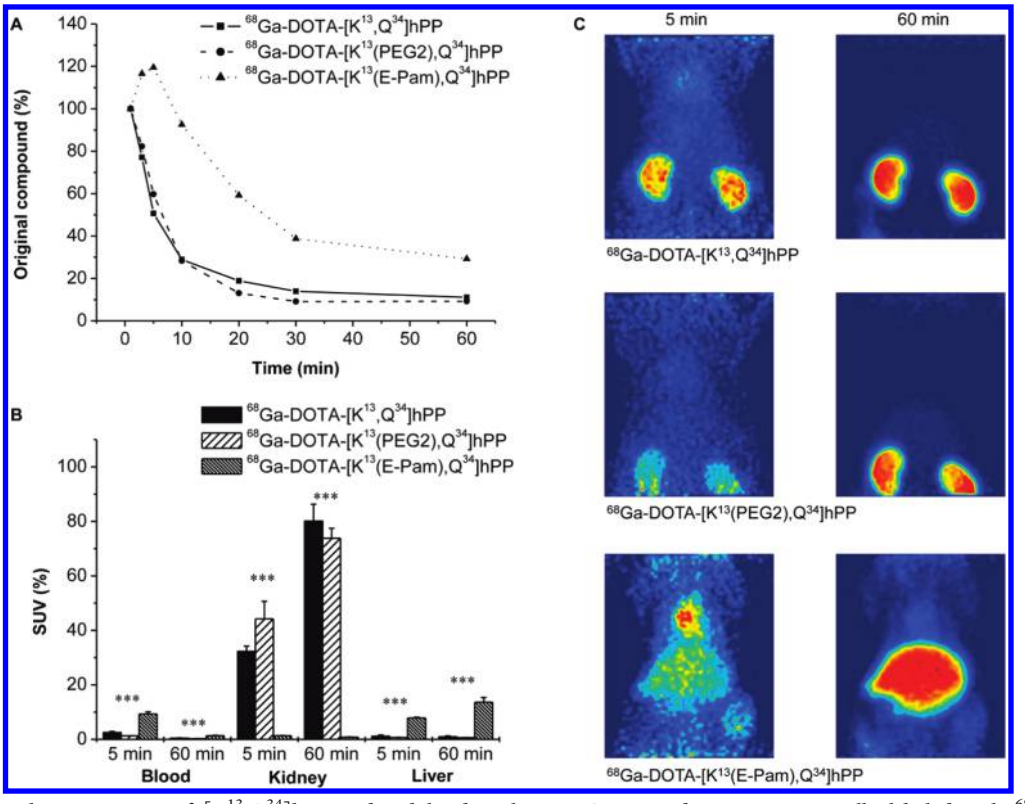


**Figure 3.** In vivo characterization of $[K^{13},Q^{34}]$hPP and stabilized analogues. Compounds were N-terminally labeled with $^{68}$Ga-DOTA, and biodistribution and kinetics were studied by small animal PET after single intravenous injection. (A) Availability of original compound in rat plasma. Blood samples were taken at indicated time points, centrifuged, and precipitated. Radioactivity of supernatants was measured. The content of the original compound was quantified by RP-HPLC. (B) SUV (Activity concentration in the tissue [Bq/g]/injected activity [Bq]/body weight [g]) of radioactivity in blood, kidney, and liver 5 and 60 min after injection. SUV are presented as means ± SEM for four animals. Significance: *** $P < 0.0001$ (C) Small animal PET studies on rats 5 and 60 min post injection.

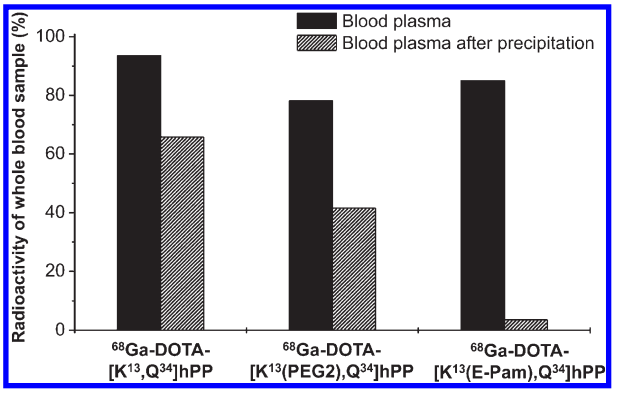


**Figure 4.** Content of original compound in whole rat blood plasma and plasma supernatant after precipitation of plasma proteins. Whole blood samples were centrifuged. Plasma proteins were precipitated with ACN/TFA/$H_2O$ (50:5:45). Radioactivity of whole blood sample, plasma, and supernatant after precipitation was measured.

Figure 5). Thus lipidation resulted in a prolonged duration of action of the anorectic effect of the hPP analogue.

## DISCUSSION

The main goal of this work was to evaluate the effects of PEGylation and lipidation on the in vivo behavior of a peptide in terms of bioavailability, biodistribution, and duration of action. Therefore, we synthesized and biologically characterized a $hY_2$ and $hY_4$ selective hPP analogue that has been stabilized against proteolytic degradation and premature clearance from the body by lipidation and PEGylation.

For about three decades and especially during the last 10 years, PEGylation has been the method of choice to overcome severe drawbacks of the usage of peptides as pharmaceuticals as there are low stability against proteases, low hydrophilicity, and immunogenicity. To a lower extent, lipidation has been conducted for a similar purpose. In the cases of both modifications, inhibition of proteolysis is achieved by shielding through steric hindrance. While the PEG chains are discussed to collect water molecules thereby extending their size and to wriggle around the peptide, fatty acids are believed to noncovalently bind to serum proteins that protect the peptide. This last hypothesis has been studied extensively by Kurtzhals et al. Already in 1995, that group investigated the binding of acylated insulin derivatives on immobilized human serum albumin and found that binding occurs with association constants of $10^4-10^5$ $M^{-1}$ and mostly depends on nonpolar and ionic interactions with the protein but only to a small extent on the chain length of the fatty acid. Furthermore, they observed binding at more than one class of sites and a binding capacity of human serum albumin (HSA) for acylated insulins of more than 5 mol/mol.[22] These data account for our observations of a pronounced coprecipitation of the radioactively labeled lipidated peptide with plasma proteins, indicating a strong binding between both of these compounds. In the case

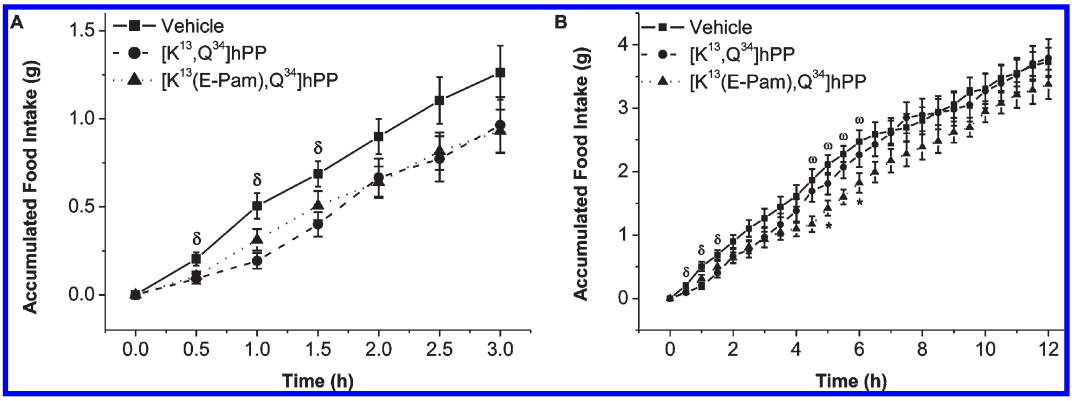


**Figure 5.** Accumulated food intake in C57BL/6J mice. (A) Food intake in the initial 3 h of the study. $\delta$: $P < 0.05$ for vehicle and $[K^{13},Q^{34}]$hPP. (B) Food intake over a time period of 12 h. $\omega$: $P < 0.05$ for vehicle and $[K^{13}(E\text{-Pam}),Q^{34}]$hPP, * $P < 0.05$ for $[K^{13},Q^{34}]$hPP and $[K^{13}(E\text{-Pam}),Q^{34}]$hPP.

of the PEGylated analogue, this effect is slightly reduced because PEG is supposed to minimize the interactions with other proteins due to its qualities as a synthetic polymer.[23]

Despite the predicted tremendous effects on protein stabilization of the two applied modifications, both bear drawbacks in synthesis and handling. In the case of lipidation, decreased water solubility of the peptide needs to be circumvented. Therefore we introduced a glutamate linker between the peptide and the fatty acid. This idea was inspired by a study conducted by Knudsen et al. Herein they also introduced the lipophilic moiety together with a linker, which could have been $\gamma$-glutamyl, $\gamma$-aminobutyroyl, $\beta$-alaninyl, or piperidyl, to glucagon-like peptide (GLP)-1. For $\gamma$-glutamyl, they observed best results concerning potency and stabilization because this spacer is predicted to enhance binding of plasma albumins to the peptide.[24] Furthermore, it adds one charge to the peptide sequence, thus enhancing its hydrophilicity. For PEGylation, the size of the molecule forms the major challenge, leading to the fact that mostly conjugation of PEG to peptides occurs in solution, which often is attenuated by unspecificity and low yields. In comparison to this conventional PEGylation reaction, our protection group based method for the coupling of small PEG moieties to Lys side chains on solid phase guarantees a precise site-specificity. Furthermore, it is more feasible and efficient by virtue of utilization of much less solvent in relation to the yield. Modification on the resin is applicable for PEG moieties of a molecular weight up to 2 kDa. For modification with larger PEGs, however, we needed to switch to reactions in solution. Here again, we used a protection group based strategy by application of the photolabile Nvoc group at the N-terminal position. After successful coupling this group could easily be removed by UV light.[17]

It was early on appreciated that the Y receptors recognize either the combined N- and C-terminal ends of the PP-fold peptides as in the $hY_1$ and $hY_4$ receptors or only the C-terminal amino acid sequence TRQRY-$NH_2$ of PYY and NPY as in the $hY_2$ receptor.[25,26] The C-terminal sequence is very important also for the selective recognition as exchange of $Gln^{34}$ against $Pro^{34}$ in either NPY or PYY led to a peptide that lost its $hY_2$ receptor affinity and consequently was highly selective for the $hY_1$ receptor.[12] The opposite exchange in hPP, i.e. the exchange of $Pro^{34}$ with a Gln residue, led to the development of obinepitide, a single digit nanomolar dual specific $hY_2$ and $hY_4$ receptor selective agonist,[11] as confirmed in the present study. For modification we chose a Thr against Lys substitution at position 13, which is located in the $\beta$-turn region of the PP-fold, i.e. far from the receptor recognition epitope.[25] This position served as the best compromise for selectivity and binding efficiency. In the case of lipidation, a selective decrease in binding affinity at $hY_4$ was observed, which might be due to the reversible complexation of bovine serum albumin (BSA) used in the assay that blocks the central region of hPP (residues 5−20), which is known to be involved in binding.[18] Because BSA is about 30 times larger than PEG2, this could explain why modification with smaller PEGs does not result in dramatically decreased affinity. The attachment of PEG20, in contrast, led to a significant loss in affinity for both receptors, which could be due to the irreversibility of the conjugation in opposition to the reversible complexation of BSA in case of the lipidation.[27]

It should be emphasized that the N-terminally CF- and DOTA-labeled compounds were not tested for selectivity because the labels were only introduced for following degradation in vitro and in vivo, respectively.

After the successful synthesis and characterization by binding assays and CD spectroscopy, we investigated the proteolytic stability of the peptides in human blood plasma. Conclusions from our in vitro stability studies reveal a strong prevention from proteolysis by fatty acid attachment and to a much lesser extent by PEGylation with small PEGs. In contrast to the first, the latter observation is rather surprising because a literature review would predict a much higher grade of stabilization in case of PEGylation. For example, Youn et al. investigated proteolytic attacks on growth hormone releasing factor (GRF) (1−29) carrying a 5 kDa PEG moiety. In comparison to the unmodified GRF(1−29) they could observe a 7-fold increase in proteolytic stability in rat blood plasma.[28] Even more strikingly, studies on PEGylated salmon calcitonin (sCT), conducted by Na et al., revealed no degradation at all after incubation in nasal mucosa, while the unmodified sCT rapidly degraded to 50% within approximately 70 min.[29] These peptides all were about 1000 kDa smaller than the peptide we modified; therefore, we introduced a larger PEG moiety, resulting indeed in increased prevention of proteolytic digestion. However, a significant loss in receptor affinity limits the future applicability of this peptide. In respect to the lipidation, Yamamoto et al. acylated a tripeptide with butyric, hexanoic, and octanoic acid and observed dramatic stabilization in skin homogenates increasing with the size of the fatty acid moiety.[30] This supports our observations, as there does not occur any distinct degradation during the time of the experiment with the lipidated peptide.

Our main goal was now to evaluate if the in vitro data could be translated into in vivo conditions as well as the effects of the modifications on clearance behavior and biodistribution. Therefore, we injected radioactively labeled peptides into rats. Our first aim was to ascertain the effects of the modifications on the bioavailability of the peptide. Similarly to the stability studies in human blood plasma, the results from the bioavailability studies were rather surprising with respect to PEGylation. While lipidation clearly and strongly increased bioavailability, PEGylation did not have any significant effect despite the fact that glomerular filtration through the kidneys usually excludes blood constituents larger than 5000 kDa. Therefore, a longer residence of the PEGylated peptide with a size of approximately 6500 Da should have been assumed, particularly as PEG is known to increase the hydrodynamic volume of peptides by assembling water molecules. Furthermore, in many studies during the last decades, researchers stated that PEGylation significantly enhanced bioavailability. On the one hand, the failure of protection by PEG surely could be due to the size of the PEG chain, but on the other hand, Lee et al. observed a 10-fold increase in plasma half-life after intravenous injection in mice for a peptide containing 29 amino acids and a 2 kDa PEG on a Lys side chain of glucagon-like peptide (GLP)-1 (7−36), which indicates that PEGylation in our case should at least show a small effect.[31] A study conducted by Yamaoka et al. could partly enlighten this discrepancy. They investigated the clearance rates of PEGs of different molecular weights and observed a fast elimination from the body for PEGs smaller than 30 kDa and a significantly slower clearance for those larger than 30 kDa. Especially PEGs smaller than 8 kDa revealed an unrestricted ultrafiltration through the kidneys.[32] Therefore, here again a dramatic increase in PEG chain length would probably yield better bioavailability data, however, at the cost of activity.

In contrast to that, the data found for the lipidated peptide are in good agreement with literature data. Studies on GLP-1 acylated with fatty acids differing in length revealed increases in plasma residence half-lives at an average of 12-fold in pigs,[24] which is in the range of our observations. This leads to the conclusion that lipidation in our case decelerates the circulation of the peptide in the bloodstream.

Furthermore, we determined the influence of the modifications on the biodistribution in vivo. The obtained data showed two main conclusions: First, there is more evidence on the observation that lipidation retains the peptide longer in the bloodstream. This can be concluded from the high blood contents at the beginning and the very low overall radioactivity measured in the body fluids and organs after 5 and 60 min in respect to the injected radioactivity. The quantitative data received from the bioavailability studies are well supported by the PET pictures that show a rapid accumulation of unmodified and PEGylated peptide in the kidneys, while the lipidated peptide first is found throughout the whole body but mainly in the heart and later solely in the liver.

Second, lipidation directs the peptide to the liver and prevents excretion through the kidneys. This is even more astonishing, as the peptide is nearly solely translocated to the liver and shows actually no absorption by the kidneys. In contrast to that, the unmodified and the PEGylated peptide are exclusively collected in the kidneys, coincidently with their size and hydrophilicity, respectively. From these data, it can be concluded that the modification, especially in the case of lipidation, directs the peptide to the place where it becomes degraded. It should be stated that modification with DOTA increases the hydrophilicity of a peptide, and therefore kidney clearance of the unmodified and the PEGylated peptide could also result from the chelator moiety.[33] However, because the lipidated peptide is found nearly solely in the liver after 60 min, the influence of DOTA seems to be less strong than that of the stabilizing moiety. With respect to the liver, it is commonly known that it functions as a storage organ for fat and conducts degradation of fatty acids and proteins. Its vasculature consists of discontinuous capillary walls through which substances ranging from small size to large macromolecules can diffuse freely from the blood to the extravasal district. Furthermore, it is known that liver cells can internalize several proteins by receptor mediated or passive mechanisms. That way the fatty acyl modified peptide can be recognized and taken up by the fat and vitamin A storing Ito and Kupffer cells that are macrophages and located directly at the inner sinusoidal wall next to the bloodstream. The storage in Kupffer cells and successive excretion into the bile has been reported for polymers like PEG larger than 50 kDa but is also conceivable for lipidated peptides noncovalently bound to serum albumins.[34] In addition, lipidated peptides can also be internalized into endothelial cells. This hypothesis is emphasized by a study conducted by Nelson et al. They investigated the transport of myristoylated cargos into B lymphocyte cells BA/F3 and observed a profound association and entry of the cargos into these cells compared to loading with the help of TAT-peptides.[35] These data generally indicate that lipidation is an appropriate way to increase uptake of peptides by cells and that way the overall bioavailability of these cargos.[36]

Most importantly, the anorectic effect of the dual $hY_2$ and $hY_4$ receptor specific hPP analogue was significantly prolonged by lipidation. Whereas the reduction in food intake observed after subcutaneous administration of the unlipidated peptide only lasted for a few hours, the anorectic effect of the lipid modified peptide was prolonged for more than 12 h (Figure 5). Although, for example novel N-terminally branched and thereby stabilized $hY_2$ selective PYY isoforms showed a similar decrease in food intake their duration of action was short-lived similar to the nonlipidated hPP analogue of the present study.[37]

## CONCLUSIONS

We succeeded in the synthesis of a dual $hY_2$ and $hY_4$ receptor specific hPP analogue that was stabilized against proteolysis and rapid clearance by lipidation. For the lipidated peptide, we achieved excellent in vitro stabilization as well as a longer in vivo half time and therefore higher bioavailability. Furthermore, we observed a more persistent and retarded uptake of the lipidated peptide in the whole body and a direction to the liver instead of the kidneys. Initial studies on food intake demonstrated that lipidation prolonged the anorectic effect as compared to the unmodified analogue. Therefore, this lipidated hPP analogue should be further investigated as a potential anti-obesity agent.

## EXPERIMENTAL SECTION

Peptides were synthesized on a Syro peptide synthesizer from Multisyntech as described in the following section. Purity was controlled by analytical reversed phase high performance liquid chromatography on a Vydac RP18-column (4.6 mm × 250 mm; 5 $\mu$m) as well as on a Vydac MS C8 column (4.6 mm × 250 mm; 5 $\mu$m). Purity was >95% for all compounds. Identity was confirmed by MALDI/ToF mass spectrometry on a Voyager RP mass spectrometer (Perseptive Biosystems).

**Peptide Synthesis.** The peptides were synthesized by automated multiple Fmoc/*t*Bu-based solid-phase peptide synthesis (SPPS) (Syro, MultiSynTech) and manual coupling on a Rink amide resin (resin loading: 0.45 mmol/g) as already described in literature.[38] Lys with Dde-protection on its side chain at position 13 and Glu at the side chain of $Lys^{13}$ were introduced for 3 h at rt by manual coupling of 5 equiv of Fmoc-protected amino acid, DIC, and 1-hydroxybenzotriazole (HOBT) dissolved to a concentration of 0.5 M in *N*,*N*-dimethylformamide (DMF). The Dde protection group was removed by washing 10 times for 10 min with 2% hydrazine in DMF at rt. Success of removal was examined by measuring the absorption of the wash solution at 301 nm.[17]

CF was coupled N-terminally for 1 h at rt by applying 1.5 equiv of CF, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIPEA) dissolved to a concentration of 0.5 M in DMF. CF polymers were cleaved by applying a solution of 20% piperidine in DMF for 40 min at rt. Hydroxyl groups of CF were then trityl-protected for 16 h at rt by using 4 equiv of trityl chloride and DIPEA dissolved to a concentration of 0.1 M in dichloromethane (DCM).[39] Tri-*tert*-butyl protected 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was coupled N-terminally for 3 h at rt by manual coupling of 1.5 equiv DOTA, HATU, and DIPEA dissolved to a concentration of 0.5 M in DMF. Nvoc was coupled N-terminally for 3 h at rt by applying 3 equiv of Nvoc-Cl, HOBT, and DIPEA dissolved to a concentration of 0.25 M in DMF.[17] The Pam side chain was introduced for 3 h at rt by manual coupling of 5 equiv of palmitic acid, HOBT, and DIC dissolved to a concentration of 0.5 M in DMF. The PEG2 side chain was introduced for 16 h at rt by manual coupling of 2 equiv of MeO-PEG2-NHS (Nektar Therapeutics or Iris Biotech), DMAP, and DIC dissolved to a concentration of 0.1 M in DMF. The PEG20 side chain was coupled in solution by dissolving 1 $\mu$mol of Nvoc-[$K^{13}$,$Q^{34}$]hPP in DMF to a concentration of 500 $\mu$M and addition of 2 equiv of MeO-PEG20-NHS (Nektar Therapeutics or Iris Biotech), DMAP, and DIC dissolved in DCM to a concentration of 4 mM. The solution was shaken for 16 h at rt. The raw product was precipitated from ice-cold diethyl ether ($Et_2O$) and washed 10 times with $Et_2O$. The precipitate was resolved in 0.1% trifluoroacetic acid (TFA) in $H_2O$. Nvoc cleavage occurred as described in literature[17] by irradiation with UV light at 366 nm.

Cleavage of the peptides occurred as described in literature.[38] A cleavage mixture of TFA/thioanisole (TA)/ethanedithiol (EDT) (90:3:7) was utilized. Reduction of partially oxidized methionines occurred as described in literature.[17] Purification of the peptides was achieved by preparative RP-HPLC on an RP18 column (Waters, 5 $\mu$m, 25 mm × 300 mm) for [$K^{13}$,$Q^{34}$]hPP, [$K^{13}$(E-Pam),$Q^{34}$]hPP, and [$K^{13}$(PEG2),$Q^{34}$]hPP or semipreparative RP-HPLC on a Vydac RP8 column (5 $\mu$m, 10 mm × 250 mm) for [$K^{13}$(PEG20),$Q^{34}$]hPP by using a flow rate of 10 mL/min or 4 mL/min, respectively. Eluants consisted of 0.1% TFA in $H_2O$ (A) and 0.08% TFA in acetonitrile (ACN) (B). Gradients were for [$K^{13}$,$Q^{34}$]hPP and [$K^{13}$(PEG2),$Q^{34}$]hPP 20−60% B in A in 40 min, for [$K^{13}$(E-Pam),$Q^{34}$]hPP 40−80% B in A in 40 min, and for [$K^{13}$(PEG20),$Q^{34}$]hPP 20−70% B in A in 40 min. Identification was performed by MALDI/ToF mass spectrometry (Voyager RP, Perseptive Biosystems), and purity was confirmed by analytical RP-HPLC on a Vydac RP18-column (4.6 mm × 250 mm; 5 $\mu$m) as well as on a Vydac MS C8 column (4.6 mm × 250 mm; 5 $\mu$m). Gradients were for [$K^{13}$,$Q^{34}$]hPP and [$K^{13}$(PEG2),$Q^{34}$]hPP 10−60% B in A in 30 min, for [$K^{13}$(E-Pam),$Q^{34}$]hPP 30− 80% B in A in 30 min, and for [$K^{13}$(PEG20),$Q^{34}$]hPP 20−70% B in A in 40 min. Yields and analytical data are given in Table 1. RP-HPLC chromatograms and MALDT-ToF mass spectra are supplied in the Supporting Information.

**Competitive Binding Assay.** Competitive binding was determined as described in literature.[40] COS7 cells transfected with the respective receptor subtype were incubated with 15 pM $^{125}$I-labeled porcine NPY (Perkin-Elmer) and different concentrations of unlabeled ligand. Half maximal inhibitory concentrations ($IC_{50}$) were determined in duplicates and are quoted as mean ± standard error of the mean (SEM) for two independent experiments.

**CD Spectroscopy.** The CD spectra were recorded on a JASCO model J715 spectropolarimeter. Peptide solutions were measured at a concentration of 5 $\mu$M, dissolved in 10 mM phosphate buffer. The chosen response time was 4 s, and the step resolution was 0.2 nm.

**Metabolism Studies in Human Blood Plasma.** The in vitro stability studies were conducted as described in literature with slight changes.[38] The concentration of the CF-labeled peptides in blood plasma was 10 $\mu$M. Incubation was performed for 144 h at 37 °C with mechanical shaking (300 rpm). After 6, 12, 24, 48, 96, and 144 h, a sample (200 $\mu$L) was withdrawn and mixed with 60 $\mu$L ACN/ethanol (EtOH) (1:1) for plasma protein precipitation. RP-HPLC was conducted using linear gradients depending on the peptide. Degradation was followed by fluorescence detection (MERCK HITACHI FL detector L-7485). Degradation curves were determined in duplicate. For analysis of the cleavage products, the peptides (100 $\mu$M) were incubated for 168 h in human blood plasma and treated as described above. Products were separated by RP-HPLC and identified by MALDI-ToF mass spectrometry.

**In Vivo Studies.** *$^{68}$Ga-Labeling and Injection into Male Wistar Rats.* $^{68}$Ga was generated as described in literature[41] by a $^{68}$Ge/$^{68}$Ga generator based on pyrogallol resin support. $^{68}$Ga-labeling was performed according to literature[42] by dissolving 50 nmol of DOTA-labeled peptide in 0.5 M ammonium acetate and adding approximately 250 MBq $^{68}$Ga within a volume of 190 $\mu$L. pH was adjusted to 3.8−4.2. The solution was incubated at 90 °C for 20 min with mechanical shaking (1000 rpm). Afterward, the incubation reaction was stopped by addition of 10 $\mu$L of 30 mM ethylenediaminetetraacetic acid (EDTA). Aggregates were broken by addition of 500 $\mu$L 2 M NaCl in 0.5 M phosphate buffer, pH 7.4, and shaking at 1000 rpm for 15 min. The solution was then subjected to size exclusion chromatography on a HiTrap desalting column (GE Healthcare). The peptides were eluted with an isotonic NaCl solution. Fractions of 1 mL were collected.

*Animal Experiments.* The animals were maintained at 12/12 L/D cycle in temperature and humidity controlled rooms with free access to standard chow and tap water unless otherwise stated. For in vivo rat studies, six-week-old male Wistar rats with an average weight of 164 ± 22 g were used. The rat experiments complied with the directives of the German law governing the conduct of animal experimentation and were approved by the Regional Council for Animal Care in Dresden and the Landesdirektion Dresden. Anaesthetization of the rats was conducted by inhalation of desfluran (8% in a 30% oxygen/air mixture). For the in vivo mice study, 32 eleven-week-old C57BL/6J mice (Taconic M and B, Ry, Denmark) with an average weight of 23 ± 3 g were used. All animal studies were approved by The Animal Experimentation Inspectorate, The Danish Ministry of Justice. Anaesthetization of the mice was conducted by inhalation of isofluran (1.5% in a 100% oxygen/air).

*Bioavailability and Small Animal PET Imaging Studies.* Maximal 1 mL of the $^{68}$Ga-DOTA-peptide solution in isotonic NaCl with an activity between 15 and 25 MBq were injected in the tail vein with 1 mL/min. Arterial blood samples were withdrawn using a catheter of the right femoral artery. Blood samples of 400 $\mu$L each were taken after 1, 3, 5, 10, 20, 30, and 60 min. Samples were centrifuged at 13 000 rpm for 3 min, plasma proteins were precipitated by addition of 800 $\mu$L of ACN/TFA/$H_2O$ (50:5:45), incubation at −78 °C for 3 min, and centrifugation at 13 000 rpm for 3 min. The supernatant was examined by RP-HPLC on a Zorbax 300SB-C18 column (250 mm × 9.4 mm, 5 $\mu$m) with a gradient of 5−70% eluent B (0.04% TFA in ACN) in eluant A (0.05% TFA in $H_2O$) in 20 min, 90% B in A in 1 min, 90% B in A for 9 min at a flow rate of 2 mL/min. Detection of radioactivity was achieved by the detector system Ramona (raytest Isotopenmessgeräte GmbH).

PET studies (60 min duration) were accomplished by a microPET Primate P4 scanner (Siemens Medical Solutions) with a field of view (FOV) of axial 7.5 cm and transaxial 22 cm. The scanner had a computer-controlled bed and operated in three-dimensional list-mode. The raw list mode data were transformed into 3D sinograms followed by Fourier rebinning and 3D iterative image reconstitution combined with an MAP reconstruction (3D-OSEM+MAP). The intrinsic resolution at the center of FOV was 1.85 mm, and the spatial resolution obtained with this reconstitution ranged from 2.2 to 2.7 mm. Transmission correction was achieved by transmission scans of 18 min ($^{57}$Co source).

*Biodistribution Studies.* For biodistribution studies, activity of 2 MBq in 0.5 mL isotonic NaCl solution was injected intravenously into eight male Wistar rats. Four of the rats were euthanized 5 min and the remaining 4 animals 60 min post injection. Blood and organs (brown adipose tissue, hair, skin, brain, pancreas, spleen, adrenals, kidneys, fat, muscle, heart, lung, thymus, harderian glands, liver, femur, testes) were extracted and wet-weighed, and radioactivity was counted by the Perkin-Elmer Precisely Wizard 3″ Automatic gamma counter. Percent injected dose per gram (% ID $g^{-1}$), and standard uptake values (SUV) (activity concentration in the tissue [Bq/g]/injected activity [Bq]/body weight [g]) were determined for each sample.

*Acute Feeding Study in Mice.* The mice were group housed four mice per cage in the HM2 system (HM2 System, MBRose, Faaborg, Denmark). Three weeks before the study, a subcutaneous (sc) small microtransponder (E-vet, Haderslev, Denmark) was implanted in each mouse to identify individual animals in the HM2 system. On the day of the study, mice were randomized into three groups ($n$ = 10−11) receiving sc injection with vehicle, [K$^{13}$,Q$^{34}$]hPP, and [K$^{13}$(E-Pam), Q$^{34}$]hPP. Before dosing, mice were fasted for 18 h (6 h into the dark phase) and dosed at the beginning of the dark phase. Right after dosing, food was reintroduced to the animals, and food/water intake was monitored for the next 48 h (only food intake data for the first 12 h after dosing is shown). To accustom the mice to the sc dosing procedure, the animals were pricked five times on separate days. Values are quoted as mean ± SEM.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Chromatographic and mass spectrometric characterization of the peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Phone: 0049-341-9736900. Fax: 0049-341-9736909. E-mail: beck-sickinger@uni-leipzig.de.

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## ■ ABBREVIATIONS USED

ACN, acetonitrile; BMI, body mass index; cAMP, cyclic adenosine monophosphate; BBB, blood−brain barrier; Boc, *tert*-butoxycarbonyl; CD, circular dichroism; CF, 5(6)-carboxyfluorescein; CNS, central nervous system; DCM, dichloromethane; Dde, 2-acetyl-5,5-dimethyl-1,3-cyclohexanedionyl; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; EDT, ethanedithiole; EDTA, ethylenediaminetetraacetic acid; $Et_2O$, diethyl ether; EtOH, ethanole; equiv, equivalents; Fmoc, fluorenylmethoxycarbonyl; FOV, field of view; GLP, glucagon like peptide; GPCR, G protein-coupled receptor; GRF, growth hormone releasing factor; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; hNPY, human neuropeptide Y; HOBT, 1-hydroxybenzotriazole; hPP, human pancreatic polypeptide; hPYY, human peptide YY; HSA, human serum albumin; $IC_{50}$, half maximal inhibitory concentration; L/D cycle, light/dark cycle; MALDI-ToF, matrix assisted laser desorption/ionization time-of-flight; MeO-PEGx-NHS, α-methoxy-ω-carboxy succinimidyl ester polyethylene glycol × kDa; Nvoc, 6-nitroveratryloxycarbonyl; Pam, palmitoyl; PEG, polyethylene glycol; PET, positron emmission tomography; RP-HPLC, reversed phase high performance liquid chromatography; sc, subcutaneously; sCT, salmon calcitonin; SEM, standard error of the mean; SPPS, solid-phase peptide synthesis; SUV, standard uptake value; rt, room temperature; rpm, revolutions per minute; TA, thioanisole; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid

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