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Brief Article

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Oral Delivery of the Appetite Suppressing Peptide hPYY(3−36) through the Vitamin B_{12} Uptake Pathway

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Supporting Information

ABSTRACT: hPYY(3−36) injections have shown positive effects on appetite regulations, sparking increased interest in hPYY(3−36) research. Of great interest is oral delivery of hPYY(3−36) that can achieve clinically relevant weight-loss outcomes in what would be a highly patient compliant route. Successful oral delivery of other peptides utilizing the vitamin B_{12} pathway has been shown but below clinically relevant levels. Herein, we present clinically relevant in vivo oral delivery of B_{12}−hPYY(3−36) conjugates.

INTRODUCTION

Over the past 30 years, a drastic increase in the prevalence of obesity has made it one of the greatest public health challenges facing the world today.[1,2] A great deal of work has focused on causes and effects of obesity with considerable interest shown in studying hormones, such as human PYY(3−36) (hPYY(3−36)), that regulate appetite and energy expenditure.[3]

hPYY(3−36) is a 34 amino acid hormone (see Figure 1 for sequence) that together with pancreatic polypeptide (PP) and neuropeptide Y (NPY) belongs to the pancreatic polypeptide family.[4] hPYY(3−36) is derived from the 36 amino acid precursor peptide PYY, which is mainly present in the ileum and is expressed by large intestine endocrine cells,[5] where it is released in response to nutrient ingestion, proportional to the calories consumed,[6] and in response to exercise.[7] The truncated form of PYY, hPYY(3−36), has received the greatest attention because of its putative effects on appetite control,[4] although the mechanisms of action of hPYY(3−36) remain under debate.[8]

IKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY

Figure 1. Amino acid sequence of hPYY(3−36). Target conjugation sites used herein are the N-terminal isoleucine (I3) and lysine residue 4 (K4) as underlined. Note hPYY(3−36) used in these studies was C-terminally amidated as is the case in the natural peptide.[3]

While central administration of hPYY(3−36) has been shown to increase food intake,[4] possibly via the activation of the lower affinity receptors Y_1 and Y_5,[9] peripheral (or systemic) administration of hPYY(3−36) has been shown to inhibit food intake in rodents,[10] primates,[11] and humans.[12] In addition to appetite suppressing effects, hPYY(3−36) has also been shown to improve glycemic control in rodent models of diabetes.[13]

In obese humans, fasting plasma concentrations of hPYY(3−36) are reduced, and overweight subjects have a relative deficiency of postprandial hPYY(3−36) release associated with reduced satiety.[12] However, despite lower basal levels of hPYY(3−36) in obese humans, obesity does not appear to be associated with resistance to the effects of hPYY(3−36). Infusion of hPYY(3−36) into a group of obese volunteers resulted in a comparable reduction in calorie intake when compared with lean controls.[12] Moreover, hPYY(3−36) levels and postprandial rise are also restored in obese individuals who lose weight[14] and who undergo gastric bypass surgery.[15]

Although studies have shown that injection of hPYY(3−36) holds great promise, patient compliance and quality of life also need to be taken into consideration. As is often the case, both suffer with the use of intravenous delivery. An oral delivery system is expected to solve both of these issues. However, there are two major barriers that must be overcome for successful delivery: (1) proteolytic degradation in the stomach and intestine; (2) inadequate absorption due to inability to cross the ileal epithelium.[16]

Humans have developed a highly efficient uptake and transport mechanism in the gastrointestinal tract for the absorption and cellular uptake of the vitamin B_{12} (B_{12}) molecule (~1350 Da).[17] To adapt this uptake pathway for peptide delivery, the recognition of, and affinity for, the various B_{12} binding proteins must not be lost or grossly diminished. Using insulin as the lead compound, we have previously demonstrated in vivo glucose reduction upon oral administration of a B_{12}−insulin conjugate.[18−21] Conjugation to B_{12} takes advantage of the natural intrinsic factor (IF) mediated uptake mechanism facilitating peptide internalization and transport into blood serum. Given that brain delivery is required for the function of neuropeptides such as hPYY(3−36), it is important to note that B_{12} can cross the blood−brain barrier in a process most likely mediated by transcobalamin II (TCII).[22]

A critical component to the success of this delivery strategy is that the uptake capacity of the B_{12} pathway is sufficient to meet the necessary increase in plasma hPYY(3−36) levels required to suppress appetite. By using the B_{12} uptake pathway, we

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expect to be able to deliver up to 1.1 nmol of peptide per dose into the systemic circulation (at an efficiency/bioavailability of ~25%).[23] With respect to hPYY(3−36), obese and lean subjects receiving a total dose of 2 nmol/m^2 of body surface area during a 90 min infusion period decreased their caloric intake by 30% and 31%, respectively.[12] These studies indicate that the anticipated rise in plasma hPYY(3−36) concentrations using the oral B_{12} uptake pathway is comparable to rates of infusion adopted within previous studies.

CONJUGATE SYNTHESIS

Cyanocobalamin (B_{12}) was covalently conjugated to hPYY(3−36) in a two-step coupling reaction (Figure S1). First, the 5′-OH group of the α ligand of B_{12} was activated by the coupling agent 1,1′-carbonyl-di(1,2,4-triazole) (CDT) in dry dimethylsulfoxide (DMSO) for 30 min at 40 °C under nitrogen atmosphere. The activated B_{12} was precipitated from solution by the addition of diethyl ether and acetone (5:1 v/v) and isolated by centrifugation at 3000*g* for 10 min. The activated B_{12} was then coupled to the N terminus (of isoleucine 3) of hPYY(3−36) in 50 mM HEPES buffer (pH 8) with gentle rotation overnight at 4 °C. Two expected minor secondary products of this coupling reaction include a conjugate with B_{12} attached to the side chain of lysine 4 (K4; this is the second amino acid of the truncated form hPYY(3−36); see Figure 1 for sequence) and a conjugate with B_{12} attached to the N terminus and the side chain of K4. As the activated B_{12} will only react with amine groups, only the three products mentioned above are expected. Furthermore, because the reaction proceeded buffered at a pH of 8, conjugation to the N terminus is expected to be the primary product based on the predicted pK_a of 7.93 and 10.36 for the N terminus and K4 side chain, respectively.[24,25] For detailed synthesis procedures, see the Supporting Information. Purity of conjugates used was over 95% as assayed by RP-HPLC.

PURIFICATION AND CHARACTERIZATION

The crude reaction mixture was purified on a C_{18} analytical column using reverse-phase high performance liquid chromatography (RP-HPLC). Unreacted B_{12} was separated from hPYY(3−36) and three conjugate products (Figure S2).

B_{12} was identified as the peak at a retention time (t_R) of 18 min by ^{1}H NMR and comparison against the HPLC chromatogram of commercial B_{12}. On the basis of comparison against commercial hPYY(3−36), the remaining peaks at t_R = 32−34 min were assumed to be the conjugates and unreacted hPYY(3−36). To quickly identify unreacted hPYY(3−36), the crude mixture was separated on an HPLC instrument with detection at two different wavelengths, 254 and 360 nm. While B_{12} absorbs at 360 nm, hPYY(3−36) does not. In comparing the chromatograms at the varied detection wavelengths, the B_{12}−hPYY(3−36) conjugates were identified as peaks 1, 2, and 4 at t_R = 32.3, 32.8, and 33.8 min, respectively. Unreacted hPYY(3−36) was identified as peak 3, t_R = 33.2 min (Figure 2).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) was used to characterize the HPLC purified peaks 1−4. Peaks 1 and 2 had nearly identical spectra with a representation shown in Figure 3. The prominent peak at 5407 *m*/*z* represents a 1:1 B_{12}−hPYY(3−36) conjugate. The spectrum for peak 3 shows a prominent peak at 4049 *m*/*z* representing unreacted hPYY(3−36) (Figure S3). Peaks 1 and 2 were identified as 1:1 B_{12}−hPYY(3−36)

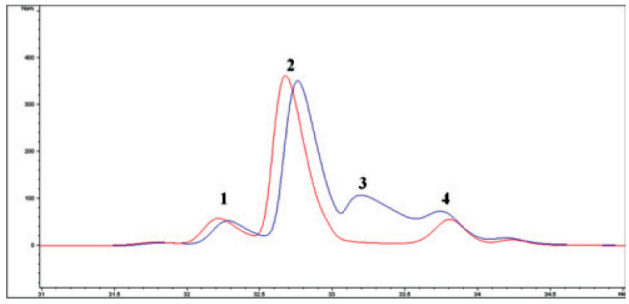

Figure 2. RP-HPLC chromatogram showing separation of four species from the crude reaction mixture with detection at 254 nm (blue) and 360 nm (red). Absence of B_{12} absorption at 360 nm indicates that peak 3 is unreacted hPYY(3−36), while peaks 1, 2, and 4 are B_{12}−hPYY(3−36) conjugates.

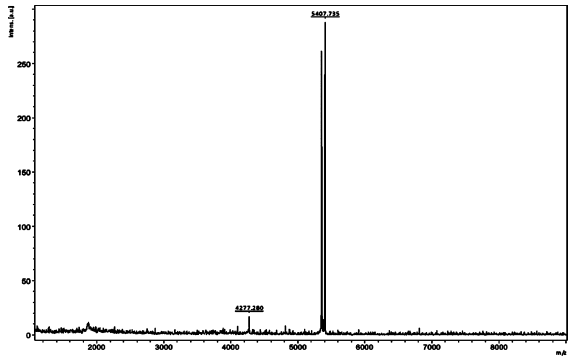

Figure 3. Representative MALDI-ToF MS spectrum of peaks 1 and 2 from the HPLC showing the presence of a prominent peak at 5407 *m*/*z* representing a 1:1 B_{12}−hPYY(3−36) conjugate.

conjugates (Figure 3), while peak 4 showed evidence of a 2:1 B_{12}−hPYY(3−36) conjugate (Figure S4).

Peaks 1 and 2, which were both identified as 1:1 B_{12}−hPYY(3−36), were combined for further testing. Because the goal of this study was to show uptake over function, complete separation of the 1:1 conjugates was not deemed necessary.

Indeed peaks 1 and 2 were combined for subsequent in vitro and in vivo assays (1/2). On the basis of the experimental conditions and the pK_a of the N-terminal amine versus the K4 amine, it is expected that peak 1 represents the conjugate with B_{12} coupled to the K4 side chain and peak 2 represents the conjugate with B_{12} coupled to the N terminus. Conjugation at such sites were also chosen, since neither position will interfere with recognition of hPYY(3−36) by enzyme-linked immunosorbent assay (ELISA), used for detection following in vivo testing. Confirmation of N-terminal conjugation was achieved through use of MALDI-MS on tryptically digested 1:1 B_{12}−hPYY(3−36) (Figure S5).

Circular dichroism (CD) spectroscopy was performed to determine if the hPYY(3−36) had retained its α-helical secondary structure following conjugation to B_{12}. The CD spectrum of 1/2 exhibited a positive peak at 192 nm and negative peaks at 210 and 222 nm indicative of the expected α-helical structure (Figure 4). The overall percent helicity of the conjugates was calculated to be 33%. Further melting studies at 222 nm showed that the conjugates can be reversibly unfolded yielding a melting temperature of ~51 °C (Figure S6). The percent helicity and the melting temperature recorded are in line with previously reported values for hPYY(3−36),[26]

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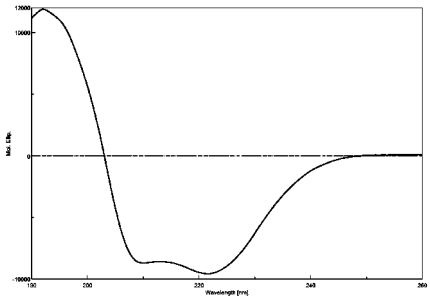

Figure 4. CD spectrum of combined 1:1 conjugate fractions 1/2 run between 190 and 260 nm at 45 μM. Percent helicity was calculated to be 33%, consistent with hPYY(3−36).[26]

indicating that B_{12} conjugation has not significantly affected folding/stability.

IN VIVO UPTAKE STUDIES

The in vivo uptake profile of the 1:1 conjugates was subsequently studied in Sprague−Dawley rats (375 ± 84 g, mean ± SD). hPYY(3−36) was administered at 1×10^{-5} M by oral gavage (1 mL) and peripherally by intraperitoneal (ip) injection (1 mL). As a control, free hPYY(3−36) was administered under identical conditions using the same concentration and volume. The change in the concentration of hPYY(3−36) from baseline was then calculated over time from 0−4 h. Following administration of B_{12}−hPYY(3−36) by oral gavage, the concentration of hPYY(3−36) increased to 184 ± 81 pg/mL by 1 h, with the trend demonstrating increasing concentrations of hPYY(3−36) over time (Figure 5). In

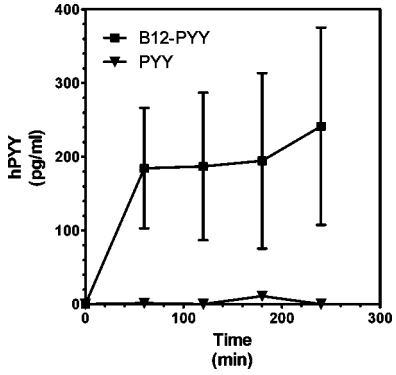

Figure 5. B_{12}−hPYY(3−36) and free hPYY(3−36) were administered by oral gavage, and the presence of hPYY(3−36) in rat blood serum was monitored by ELISA over a period of 4 h: B_{12}−hPYY(3−36) gavage, n = 12; hPYY(3−36) gavage, n = 4.

contrast, plasma hPYY(3−36) concentration was only slightly elevated (mean peak concentration, 11 ± 6 pg/mL) in response to oral administration of the free hPYY(3−36) (Figure 5).

The peak plasma hPYY(3−36) concentration (241 pg/mL) observed following oral administration of B_{12}−hPYY(3−36) is comparable to that observed in total plasma PYY following meal ingestion in this animal model (Sprague−Dawley rat, 165 ± 25 pg/mL).[27] In humans, the plasma response of total PYY is similar to that seen in other animal models, with one recent study observing a mean Δ of 10 pg/mL following ingestion of 75 g of glucose.[28] Given that humans have a far greater B_{12} uptake capacity than rats (rat is about 9−13 pmol per dose, with humans about 0.7−1.4 nmol per dose),[29] this work then suggests that the system described herein may be suitable for delivering clinically relevant doses of hPYY(3−36) in humans (with suitable dose delivery studies performed to maximize/optimize delivery). To compare the plasma profile of B_{12}−hPYY(3−36) against hPYY(3−36), ip studies with identical concentration and volume (1×10^{-5} M, 1 mL) were performed.

Following injection of both B_{12}−hPYY(3−36) and free hPYY(3−36), the peptide was detected in rat blood serum, peaking at 1 h and decreasing significantly over the subsequent 3 h time course. The profile for B_{12}−hPYY(3−36) matches that of free ip administered hPYY(3−36) (Figure 6). The matching

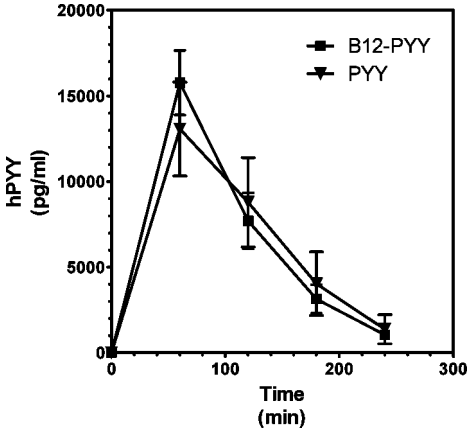

Figure 6. B_{12}−hPYY(3−36) and free hPYY(3−36) were administered by ip, and the presence of hPYY(3−36) in rat blood serum was monitored by ELISA over a period of 4 h: B_{12}−hPYY(3−36), n = 8; hPYY(3−36), n = 4.

ip profile indicates that conjugation of B_{12} did not affect the in vivo residency time of hPYY(3−36) in plasma. Given the picomolar concentrations of TCII in rat serum, this is as expected, since any TCII effect would be overwhelmed by the injected dose.[29] This profile for ip is in contrast to the profile noted following gavage, but the concentration of hPYY(3−36) remains above baseline (>200 pg/mL), at the 4 h time-point for both. This profile for gavage may in part be the result of delayed (compared to ip injection) transport of the hPYY(3−36) through the IF-mediated uptake pathway, but it may also be the result of the conjugate being bound by serum TCII, which may be prolonging in vivo residency. The maximum hPYY(3−36) level observed by gavage (241 pg/mL) corresponds to low picomolar concentrations of hPYY(3−36), within the binding capacity of TCII.[22]

CONCLUSIONS

The road blocks to the use of vitamin B_{12} in peptide/protein delivery have been listed as limited uptake capacity, relatively slow delivery time (several hours), variance across individuals and within individuals as they age, and a question about whether any protection against proteolysis is provided by the conjugated peptides/proteins. With these questions in mind, we have successfully synthesized B_{12}−hPYY(3−36) conjugates and shown oral delivery of a highly significant dose of hPYY(3−36) utilizing the B_{12} uptake pathway. We feel that, rather than attempting to modify the B_{12} pathway (through permeability enhancement for example) or attaching multiple proteins per B_{12} (difficult syntheses and purification and often difficult to quantify protein/B_{12} ratio), careful selection of the

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peptide/protein to be delivered will allow exploitation of the pathway. Peptides such as PYY and GLP-1 have been modified to produce isoforms[26] with improved receptor specificity or protease resistance (in the case of GLP-1, a natural peptidase resistance form, exenatide, was isolated from the Gila monster and is sold commercially as Byetta). By combination of this stability with a delivery need that can theoretically be met orally by the B_{12} uptake capacity and given that the target effect is not as stringently defined by temporal and stoichiometric limits compared to, for instance, insulin delivery for glucose regulation, then the four concerns noted above regarding the B_{12} pathway for drug delivery are addressed.

Herein, we show that, at an oral dose of $\sim 10^{-5}$ M B_{12}–hPYY(3–36), a clinically relevant dose with a peak plasma concentration of 241 pg/mL of hPYY(3–36) was delivered using the Sprague–Dawley rat as the in vivo model. This number also represents a clinically relevant dose should it translate to humans.[29] The hPYY(3–36) level achieved was also sustained over the time course of the experiment. With the dose achieved and lifetime noted, this uptake profile bodes well for future clinical investigations. To explore these highly positive results further, full dose-delivery studies (including with PYY isoforms and GLP-1) and studies pertaining to function now need to be undertaken. Such work is ongoing.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, mass spectra, and melting circular dichroism spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

hPYY(3–36), human PYY(3–36); PP, pancreatic polypeptide; NPY, neuropeptide Y; B_{12}, vitamin B_{12}; IF, intrinsic factor; TCII, transcobalamin II; CDT, 1,1′-carbonyl-di(1,2,4-triazole); DMSO, dimethylsulfoxide; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI-ToF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ELISA, enzyme-linked immunosorbent assay; CD, circular dichroism

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