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Solution Structure and Constrained Molecular Dynamics Study of Vitamin B₁₂ Conjugates of the Anorectic Peptide PYY(3–36)

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Abstract

Vitamin B₁₂–peptide conjugates have considerable therapeutic potential through improved pharmacokinetic and/or pharmacodynamic properties imparted on the peptide upon covalent attachment to vitamin B₁₂ (B₁₂). There remains a lack of structural studies investigating the effects of B₁₂ conjugation on peptide secondary structure. Determining the solution structure of a B₁₂–peptide conjugate or conjugates and measuring functions of the conjugate(s) at the target peptide receptor may offer considerable insight concerning the future design of fully optimized conjugates. This methodology is especially useful in tandem with constrained molecular dynamics (MD) studies, such that predictions may be made about conjugates not yet synthesized. Focusing on two B₁₂ conjugates of the anorectic peptide PYY(3–36), one of which was previously demonstrated to have improved food intake reduction compared with PYY(3–36), we performed NMR structural analyses and used the information to conduct MD simulations. The study provides rare structural insight into vitamin B₁₂ conjugates and validates the fact that B₁₂ can be conjugated to a peptide without markedly affecting peptide secondary structure.

Graphical abstract

Solution structures of vitamin B₁₂ (B₁₂)–peptide conjugates and constrained molecular dynamics associated with such structures are reported, focusing on the appetite-suppressing peptide hormone PYY(3–36). Developing an understanding and model of the effects of B₁₂ conjugation on a peptide's secondary structure may allow, a priori, rational design of such conjugates in the future.

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Keywords

GPCR; molecular dynamics; NMR spectroscopy; PYY(3–36); vitamin B₁₂

Introduction

Peptide YY (PYY), a member of the pancreatic polypeptide family,^[1–8] was first isolated from porcine intestinal tissue extracts in 1980^[9] and was later shown to be a critical enteroendocrine hormone involved in appetite regulation.^[10–12] PYY has two main circulating forms: PYY(1–36) and a truncated form, PYY(3–36).^[13] PYY(1–36) is released in concert with caloric intake or exercise and is cleaved by dipeptidyl peptidase IV (DPP-IV)^[14] in the gut to produce PYY(3–36). PYY(1–36) has an appetite-stimulating effect through activation of the orexigenic Y1 receptor (Y1-R) located in the intestines, blood vessels, and brain.^[15] The two-amino-acid N-terminal (Tyr-Pro) truncation to PYY(3–36) results in an approximate 100-fold decrease in activity at the Y1-R,^[5,16] and generates an agonist of the anorexigenic Y2 receptor (Y2-R) located in the intestines (vagal afferent sensory neuron signaling)^[17,18] and brain,^[19,20] which exerts a G-protein-coupled receptor (GPCR) G_i-mediated anorectic effect.

We recently reported a B₁₂–PYY(3–36) conjugate that demonstrated similar activity to native PYY(3–36) at the Y2-R in vitro, but improved function over PYY(3–36) upon subcutaneous (s.c.) administration in vivo in a lean rat model (conjugate **3** as described herein below).^[21] In earlier work, we focused on conjugating to B₁₂ through the ribose hydroxy group, as it is well established in the field that this is an optimal site for such conjugation, as it does not hinder recognition of B₁₂ by its carrier proteins.^[22–25] Likewise,

we focused on an N-terminal region of PYY(3–36) for conjugation (specifically the K₄ residue), because again it has been well documented that modifications at (or indeed complete loss of) this area do not significantly affect Y2-R agonism.^[26–28] These assumptions bore out, as the EC₅₀ values at the Y2-R obtained for the conjugate produced (and noted as conjugate **3** herein) were similar to that of unconjugated PYY(3–36) used for comparison. These were established using a Fura-2 assay that monitors intracellular Ca²⁺ mobilization under conditions in which the Y2-R signals through a promiscuous G_q GTP binding protein.^[21] Questions that remained from this work, however, were what affect, if any, does B₁₂ conjugation actually have on the PYY(3–36) secondary structure and whether MD simulations could be used to better understand, and possibly predict, any structural modifications observed. To investigate these questions, two conjugates located at the same coupling sites (ribose on B₁₂ and K₄ on PYY(3–36) but with a slightly varying spacer length (one methylene unit difference) were assayed for agonism of the Y2-R using a new fluorescence resonance energy transfer (FRET)-based assay that faithfully reports the normal signal transduction process by which the Y2-R signals through G_i proteins to lower levels of intracellular cyclic adenosine monophosphate (cAMP).^[29] NMR structures were solved for these conjugates, and their in-solution NMR structures were compared with those previously reported by Keire et al.^[30] and Nygaard et al.^[7] Subsequent unconstrained and NMR constrained MD simulations were then also performed.

Results and Discussion

In vitro evaluation of conjugates **3** and **4**

All assays were performed *at least* in triplicate. Conjugates **3** and **4** were tested for their abilities to lower cAMP levels. Figure 1 shows the dose–response relationships for **3** and **4** relative to K₄PYY, showing both conjugates are less active than K₄PYY and that **4** is more active than **3**. Both conjugates are, however, within one-half log order of K₄PYY.

This difference in EC₅₀ values in comparing conjugates with K₄PYY was initially presumed to be a compromise between steric hindrance and/or unfavorable flexibility resulting in modifications/interactions that negatively affect the peptide structure–activity profile. At this point we decided to pursue NMR and molecular dynamics (MD) studies to further explore these in vitro observations.

NMR analysis of **3** and **4**

Proton chemical shifts for **3** were assigned by analyzing TOCSY, DQF-COSY, and 2D NOE spectra at 25 °C. The experiments were also performed at 20 and 30 °C, at which slight shifts of some proton signals resolved overlapping peaks. The NMR spectra of **3** have some similarity to those of PYY(3–36), such as line broadening of many signals,^[7] indicating increased dynamics, and a decrease in the chemical shift range of the backbone amide protons relative to full-length PYY. All backbone amide protons were assigned with the exception of Leu24, which could not be definitely determined due to overlap. Figure S17 (Supporting Information) shows the proton chemical shift difference between **3** and PYY(3–36). There are chemical shift differences for residue Lys4, which is not surprising, as this is the attachment site for B₁₂. Figure 2 shows an overlay of **3** after MD calculations and

PYY(3–36). The first chemical shift difference to consider is that for the methyl and α protons of Ala7. In PYY(3–36), the methyl group of residue Ala7 is pointing toward the α helix, whereas in conjugate **3** the methyl group is oriented away from the α helix. A 2D NOE cross-peak between the Ala7 methyl group and the ring protons of Tyr20 was observed in PYY(3–36),^[7] but in **3** only a very weak cross-peak was observed in the longest mixing time 2D NOE experiment. This difference in orientation would also put the Ala7 α proton in distinct environments. The backbone amide protons have different chemical shifts for the β turn residues and residues in the N-terminal side of the α helix, specifically residues Gly9, Ala12, Ser13, Glu15, Leu17, and Arg19. This can be explained by the unraveling of the α helix and increased dynamics at the N-terminal side in the PYY(3–36) structure. Conjugate **3** maintains an α -helical structure similar to that of full-length PYY, and the residue with the largest chemical shift difference for the amide proton, Leu17, has α chemical shift value closer to that of the full-length peptide: PYY Leu17 NH 8.40 ppm,^[7] **3** Leu17 NH 8.36 ppm, PYY(3–36) 7.95 ppm.^[7] Nygaard et al. suggest that the Pro2–Tyr27 interaction is important for the stability of the PP fold, and that loss of this interaction in PYY(3–36) creates both conformational and dynamic changes in the structure, especially around the turn region.^[7] Close inspection of **3** indicates possible hydrogen bonds from Glu6 to Tyr27 and Ser23, which may stabilize the PP fold in the conjugate.^[7]

To elaborate on the structural studies, we decided to also investigate the solution structure of **4** to allow a direct comparison with **3**. Based on the NMR spectra, the conjugate structures appear very similar with only minor differences in the proton chemical shift assignments (Supporting Information Figure S18) and greater than 95% similarity in the 2D NOE spectra (Supporting Information Figure S19).

The major difference observed between **3** and **4** is the proton chemical shift change at B7 and B2 on the dimethylbenzimidazole (DMB) ligand (see Supporting Information Figure S12 for B₁₂ atom numbering scheme). The C20 methyl protons are closer to the B4 and B2 protons in **4** based on the presence of a weak cross-peak between the C20 methyl protons and B2, which is only seen in the longest mixing time 2D NOE spectra of **3**, and a cross-peak between the methyl protons and B4, which is stronger for **4**. Weak cross-peaks are observed in **4** between a propionamide proton of the *g* side chain of B₁₂ (Supporting Information Figure S12) and the H α of D11 as well as the methyl protons of A12. The α helix motif as a whole is critical for association and subsequent agonism.^[26] This is consistent with reported Y2-R interactions, as the C-terminal pentapeptide region is well established as the critical region, or “address”, of the main interactions with Y2-R, while the α helix is considered the “message”, indicating that both areas are critical in Y2-R agonism.^[26]

NMR constrained MD studies of **3** and **4**

Molecular dynamics simulations of **3** and **4** were performed both with and without the NMR constraints defined for **3** to consider differences in behavior and potential alternative structures in the simulations. The observed structural changes across the simulations identified intra-PYY(3–36) interactions that might, through their stabilization in the isolated conjugate, promote the decreased activity of **3** relative to **4**. The unconstrained MD

simulation data then provide an additional set of structures for considering accessible geometries beyond the restrained set. The average structures from representative MD time ranges for **3** and **4** are shown in Figures 3 (front/side view) and 4 (top view). Apparent from these views, and the full simulations in general, are the persistence of 1) much of the α helical structure and 2) localization of the B₁₂ fragment itself to the unstructured region approaching the loop into the α helix (in the images, this loop is at the base of all structures, including residues Glu10, Asp11, and Ala12). Across all of the simulations, several hydrogen bonding motifs are found to persist at the onset of the simulations and over the time evolution of the structure dynamics that serve to effectively anchor the B₁₂ at this loop region. Those which specifically anchor the B₁₂ to this region in all simulations are shown in Figure 5, visualizing the two most persistent motifs for these structures: a pair of hydrogen bonds from a single B₁₂ amide side chain to Pro8 and Glu16 (left, Motif 1) and an amide side chain to Pro8 hydrogen bond and coordination of a hydroxy group H atom on Ser13 to the B₁₂ cyano nitrogen atom (right, Motif 2). The structural basis for preservation of the loop region itself across all simulations is evident in Figure 6, which shows that (left) Glu16 is engaged in several persistent hydrogen bonding interactions with Gly9, Glu10, and Asp11, whereas at the far end of the loop (right), Glu6 is in close proximity to hydrogen bond acceptors on Tyr27 and Ser23. The NMR distance lists, ranges, and time-averaged MD structures for **3** and **4** are provided in the Supporting Information.

The differences between structures **3** and **4**, both with and without NMR constraints, are largely localized to the C-terminal side of the α helix and occur to varying degrees in all of the simulations. With the B₁₂ largely predicted to be confined to the PYY(3–36) loop due to several strong hydrogen bonding interactions, and with the well-known attenuation of PYY(3–36) activity with modifications to or removal of the C-terminal region, the most logical explanation for any change in behavior to come from these single conjugate simulations is some structural change at the C-terminal region of PYY(3–36). This may occur from B₁₂ binding-induced conformational changes at the PYY N terminus, causing changes in activity, meaning any observed interactions between the B₁₂ binding-constrained N terminus and concomitantly proximal C-terminal regions are of great interest. Despite the small change in tether length for these two cases, RMSD analyses and average structure generation produced two distinct structures that, for each case, revealed binding interactions deemed consistent with the trends in activity.

MD simulations of **3** and **4** highlight hydrogen bonding interactions that may govern the differences observed in Y2-R agonism and subsequent calcium mobilization and inhibitory cAMP effects. PYY(3–36) does not tolerate any interaction at the C terminus with respect to Y2-R stimulation.^[32] If the answer to the decreased activity of **3** lies solely on some response internal to the conjugate, one might argue from the MD simulations that the shortened tether length enhances the stiffness of the unstructured region (residues 3–9) by decreasing its conformational flexibility upon hydrogen bonding between the B₁₂ and the near-loop region. This result would provide a less flexible N-terminal region and a more persistent hydrogen bonding pocket for the C-terminal region with which to interact. By the loss of flexibility, necessary at the C-terminal region for biological activity, reduced activity would be predicted (and is observed). Kaiser et al. recently reported data showing that

unwinding of C-terminal residues of neuropeptide Y (NPY) is critical for Y2 receptor binding and activation.^[32] Solution NMR experiments showed that the ligand is tethered to the second extracellular loop by hydrophobic contacts and revealed NPY to undergo remarkable structural changes within the C terminus. The C-terminal pentapeptide plays a role in extensive and susceptible interactions in NPY; a network that is also relevant for PYY(3–36) in regards to Y2-R agonism. Changes in the C-terminal amino acids can easily disturb receptor binding or switch receptor selectivity for both NPY and PYY(3–36) as observed in numerous earlier structure-activity studies.^[33] The ultimate conclusion from Kaiser et al. directly relates to our work, as the binding mode of NPY [and in our case, B₁₂ conjugates of PYY(3–36)] might have more general implications for peptide binding GPCR systems.

The MD simulations from this study do indicate that if the origin of the decreased activity of **3** is entirely due to factors internal to the B₁₂–PYY(3–36) conjugate itself, then constraint of the C-terminal region by hydrogen bonding interactions with the N-terminal region could explain it—and that this kind of internal mechanism may have its origin in the reduction of tether length.

Conclusions

B₁₂–PYY(3–36) conjugates **3** and **4** with various methylene spacer lengths between the B₁₂ and PYY(3–36) show similar Y2-R agonism to that of PYY(3–36). Both intra- and intermolecular interactions between B₁₂ and the peptide and small changes in the secondary structure of the peptide brought on by conjugation were observed. Based on the information collected from the NMR constrained MD studies, it would have been possible to offer a detailed assessment of the potential function of both conjugates. These observations suggest that MD could be used a priori to guide conjugate rational design and minimize the number of conjugates that would need to be screened—information of considerable benefit in development terms. Conjugates and modifications of B₁₂ have garnered much interest in recent years for their clinical and medicinal applicability.^[34] Based on the studies described herein, an ideal B₁₂–peptide conjugate would be one with an appropriate linker length to allow optimal function of both the peptide and B₁₂, which could be predicted by MD via inter- and intramolecular interactions that are known to be useful and/or harmful to the overall function of each component.

Experimental Section

Synthesis of alkyne precursors (1 and 2) and conjugates (3 and 4)

Two B₁₂–alkyne precursors were prepared by activation of a B₁₂–carboxylic acid (B₁₂–CA) derivative^[35] with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazole (HOBt) in anhydrous DMSO under argon (Scheme 1). Full characterization of the alkyne precursor **1** and **2**, including RP-HPLC, MALDI-ToF MS, and NMR can be found in the Supporting Information (Figure S1–11, Table S1). For conjugate synthesis, click chemistry^[36] was implemented using a copper iodide (CuI) and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) method, adapted from Gryko et al.^[37,38] Copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC) synthesis of conjugates **3** and **4**

via the alkyne precursors **1** and **2** and a K₄-azido PYY(3–36) (K₄PYY) is described in Scheme 2 (spacer length $n=2, 3$ for precursors **1** and **2**). K₄PYY was initially tested against PYY(3–36) amide (Sigma–Aldrich), and there was no observed difference in Y2-R agonism. Subsequently, K₄PYY was used as the control for all assays. Characterization of the B₁₂-PYY(3–36) conjugates **3** and **4**, including HPLC and MALDI-ToF MS, can be found in the Supporting Information (Figure S13–16).

In vitro assay of **3** and **4** at the Y2 receptor coupled to G_i

Conjugates **3** and **4** were tested for their abilities to lower levels of cAMP in an in vitro assay using HEK293 cell monolayers (Figure 7).^[29] These cells were engineered so that they stably express the human GLP-1 receptor (GLP-1R), while also transiently expressing the human Y2-R. Furthermore, these cells were virally transduced with the genetically encoded FRET reporter AKAR3, which is used to monitor cAMP-dependent protein kinase (PKA) activation intracellularly. This assay is unique in that it allows FRET-based detection of the ability of PYY(3–36) to counteract the action of a GLP-1R agonist (Exendin-4; Ex-4) to raise levels of cAMP. When the cells are first treated with Ex-4 (33 pm; injection 1) so that levels of cAMP are elevated, AKAR3 exhibits increased FRET, measured as a decrease in the 485/535 nm emission ratio (Figure 7). This change of FRET occurs after an approximate lag time of 50 s. If PYY(3–36) is then applied at the 180 s time point (injection 2), a functional antagonism of the action of Ex-4 is measured so that the change of FRET is decreased. Note that no change of FRET is measured in response to the administration of a negative control standard extracellular saline (SES; Figure 7). By varying the concentration of added conjugate, it is possible to determine dose–response relationships, and to also determine EC₅₀ values describing inhibitory actions of PYY(3–36) conjugates versus 33 pm Ex-4 in this assay. Figure 7 illustrates these responses to the first injection of either Ex-4 or SES, and the second injection of K₄PYY or SES. To normalize these raw data for subsequent dose–response analysis, “end-point” values of FRET were measured during the last 10 sample intervals (Figure 7). As illustrated in Figure 1, the dose-dependent inhibitory actions of PYY(3–36) conjugates are then quantified relative to a value of 100% that corresponds to the maximal inhibitory effect measured when testing 300 nm PYY(3–36) in this assay.

NMR studies of **3** and **4**

NMR studies were executed initially to observe any structural differences between the conjugates and free peptide in solution. Because in vivo studies had previously established in vivo function for **3**, extensive structural studies were first performed with **3** and a direct comparison made to published PYY(3–36).^[7] Full NMR studies of **4** were then completed to serve as a comparison with **3**. Full descriptions of methods and conditions are provided in the Supporting Information.

MD studies of **3** and **4**

To complement the NMR studies, MD simulations of **3** and **4** were completed in attempts to explain the minor differences of Y2-R agonism between the two conjugates. These MD studies took the form of 50 ns simulations to probe the potential variation in simulation

geometries and time-averaged structures that arise from different tether lengths. MD simulations were performed using the GROMACS (ver. 5.0.4)^[39] software package. The NMR distance lists, ranges, and tabulated distances from the time-averaged MD structure for **3** are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Dose–response curves monitored as percent inhibition of Ex-4 action at AKAR3 by K₄PYY and conjugates **3** and **4** at the Y2-R. EC₅₀ values: K₄PYY: 1 ± 0.2 nM, **3**: 6 ± 2 nM, **4**: 2 ± 0.2 nM. EC₅₀ values are the average ±SEM.

Figure 2. Overlay of PYY(3–36) (blue) versus 3 (tan). The structure of PYY(3–36) was obtained from the RCSB Protein Data Bank (www.pdb.org); PDB ID: 2DF0. The program Chimera (UCSF; www.cgl.ucsf.edu/chimera/)^[31] was used to display the image.

Figure 3. Side-on views (aligned along the α helix) of RMSD average structures (across 10 ns sampling increments) for restrained (left) and unrestrained (right) MD simulations of A) **3** and B) **4**.

Figure 4. Top-down views (aligned along the α helix) of RMSD average structures (across 10 ns sampling increments) for restrained (left) and unrestrained (right) MD simulations of A) **3** and B) **4**.

Figure 5.
Two persistent hydrogen bonding interaction motifs between the B₁₂ and PYY(3–36) unstructured region (residues 3–10) across all simulations.

Figure 6.

Two persistent hydrogen bonding motifs that define the PYY(3–36) loop region (left) and stabilizing interactions between the unstructured N-terminal region and the α helix.

Figure 7.

485/535 FRET ratio through the course of a run. The first injection occurs at 100 s followed by a second injection at 180 s.

Scheme 1.

Synthesis of B₁₂-alkyne precursors **1** and **2**. *Reagents and conditions:* EDC, HOBT, anhydrous DMSO; reactions carried out under argon for 16 h at RT.

Scheme 2.

Copper(I) alkyne-azide cycloaddition synthesis of B₁₂-PYY(3-36) conjugates **3** and **4** via alkyne precursors **1** and **2** with a two- or three-methylene-unit spacer between the 5'-amide on B₁₂ and the triazole linkage to K₄PYY. *Reagents and conditions:* Cu^I, TBTA; reactions were carried out for 16 h at RT. PYY(3-36) adapted from PDB ID: 2DF0.