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Enhanced Peptide Stability Against Protease Digestion Induced by Intrinsic Factor Binding of a Vitamin B_{12} Conjugate of Exendin-4

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Supporting Information

ABSTRACT: Peptide digestion from proteases is a significant limitation in peptide therapeutic development. It has been hypothesized that the dietary pathway of vitamin B_{12} (B_{12}) may be exploited in this area, but an open question is whether B_{12}-peptide conjugates bound to the B_{12} gastric uptake protein intrinsic factor (IF) can provide any stability against proteases. Herein, we describe a new conjugate of B_{12} with the incretin peptide exendin 4 that demonstrates picomolar agonism of the glugacon-like peptide-1 receptor (GLP1-R). Stability studies reveal that Ex-4 is digested by pancreatic proteases trypsin and chymotrypsin and by the kidney endopeptidase meprin β. Prebinding the B_{12} conjugate to IF, however, resulted in up to a 4-fold greater activity of the B_{12}-Ex-4 conjugate relative to Ex-4, when the IF-B_{12}-Ex-4 complex was exposed to 22 μg/mL of trypsin, 2.3-fold greater activity when exposed to 1.25 μg/mL of chymotrypsin, and there was no decrease in function at up to 5 μg/mL of meprin β.

KEYWORDS: *vitamin B_{12}, exendin-4, intrinsic factor, trypsin, AKAR3*

INTRODUCTION

The human vitamin B_{12} (B_{12}) dietary uptake pathway is a complex process that facilitates access in humans to a vital cofactor of methionine synthase and methyl malonyl CoA mutase enzymes.[1] This pathway involves three major binders, two of which, intrinsic factor (IF) and haptocorrin (HC), being critical for oral uptake (the third, transcobalamin II (TCII), facilitates entry into cells upon enterocyte passage).[2] HC primarily protects B_{12} against acid digestion in the stomach and is enzymatically digested upon entry of the HC-B_{12} complex into the duodenum, whereupon the B_{12} is bound by IF. While IF is produced in gastric parietal cells and can bind B_{12} in the stomach, HC binding is preferred at the lower pH here and it is only upon digestion of HC and a rise in pH in the intestine that IF binding of B_{12} occurs naturally.[3,4] Concomitant with the rise in pH is the release of pancreatic proteases, and it is critical to note that IF, unlike HC, is resistant to pancreatic protease digestion.[5] IF is critical then for delivery of B_{12} through the intestinal tract to the ileum where cubilin-amnionless based receptor mediated enterocyte passage occurs.[6] Employing this pathway for oral peptide delivery, for example, requires conjugation of the peptide to B_{12} in such a way that IF recognition of B_{12} is not critically hindered and that B_{12} conjugated peptide can still exhibit the desired pharmacological function. Such concerns are typically readily addressed, however, and there are now several significant examples of B_{12}-peptide conjugates that meet the above criteria.[7−11] What is not understood, but is no less important, is whether such peptide function is maintained when the conjugate is bound to IF and whether IF, so effective at protecting B_{12}, can provide any protection to a B_{12} conjugated peptide upon exposure to a protease. To investigate these questions we decided to focus on a highly potent peptide (Ex-4) that is the basis of a pharmaceutical (exenatide) currently approved for treatment of diabetes mellitus.[12]

Ex-4 was discovered in the venom of the Gila monster in 1992 by Eng et al. and is an incretin mimetic, sharing 53% homology with glucagon-like peptide-1 (GLP-1). Like GLP-1, Ex-4 stimulates the release of insulin through agonism of the GLP-1 receptor (GLP-1R) (EC_{50} 33 pM), effectively lowering blood glucose levels. Unlike GLP-1, Ex-4 is resistant to the enzyme dipeptidyl peptidase IV (DPP-IV), which rapidly cleaves and inactivates GLP-1 in vivo.[13,14] Since DPP-IV cleaves any peptide with an alanine or proline at the second position from the N-terminus, substituting a glycine for the alanine in GLP-1 results in the resistance seen in Ex-4. This resistance allows Ex-4 to have a half-life of 2.4 h compared to <2 min as seen for GLP-1.[15] Such resistance to DPP-IV does not, however, translate to other proteases, and exenatide therefore must be administered subcutaneously.

The hypothesis herein then is that this pancreatic degradation and general protease limitation may be overcome, at least to some degree above unmodified peptide, by conjugating B_{12} to Ex-4 and subsequently adding IF, assuming the necessary maintenance of B_{12} binding by IF and Ex-4 agonism are controlled. To test these hypotheses we

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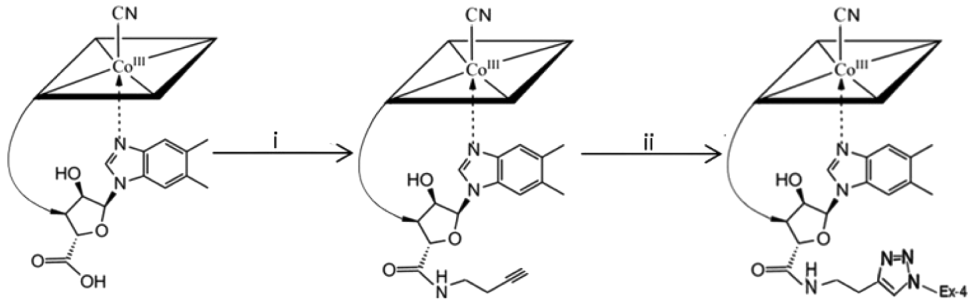
Scheme 1. Synthesis of B_{12}-Ex-4 Conjugate 4[a]



2 3 4

[a]Reagents and conditions: (i) EDCI, HOBt, 1-amino-3-butyne, rt, 16 h; (ii) **1**, $CuSO_4$, sodium ascorbate, 1 h.

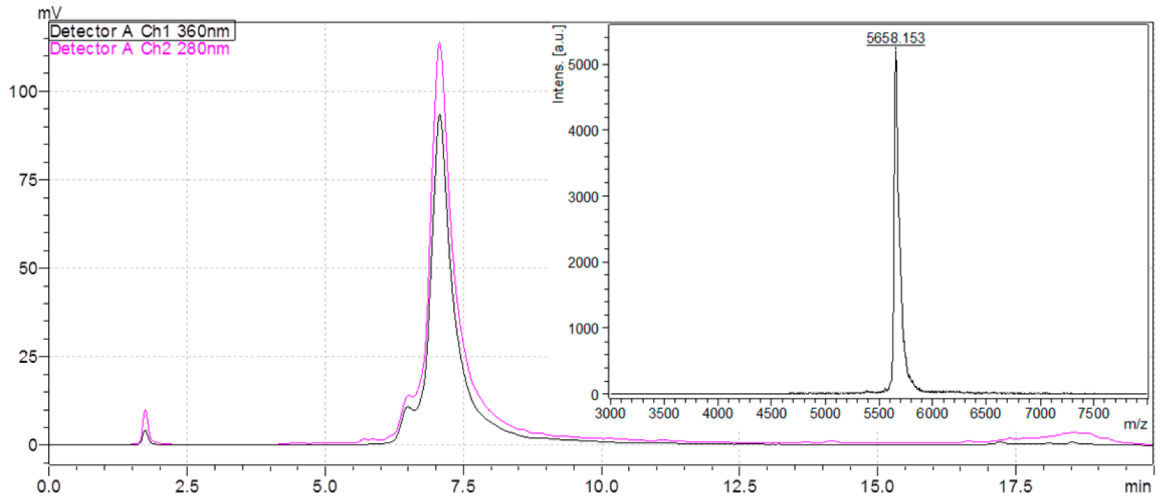


Figure 1. LC trace showing purified **4** as a monomer (~7 min) and dimer (~6.5 min) and MALDI-Tof MS (inset) of **4** showing m/z of 5658.153 Da, which corresponds to the +1 of **4**.

synthesized a B_{12}-Ex-4 conjugate focusing on the lysine 12 (K12) position of Ex-4 and the ribose 5′-hydroxyl group of the B_{12} moiety as sites of conjugation since both sites on the respective moieties had published precedent for allowable modification.[16−18] Binding to IF was confirmed by radioassay, and agonism of the GLP-1 receptor was then established for an azido modified K12-Ex-4 (**1**), B_{12}-Ex-4 (**4**), and IF-B_{12}-Ex-4 (IF-**4**). With such establishing parameters controlled for stability against the abundant intestinal endopeptidases, trypsin, chymotrypsin, and the kidney protease meprin β[19] were compared for **1**, **4**, and IF-**4**.

EXPERIMENTAL SECTION

(AzidoK12)-Ex-4 (**1**) was conjugated to B_{12} at the K12 position using Huisgens/Sharpless click chemistry,[20] using Ex-4 modified at the lysine 12 ε-amine with an azido group during solid-phase synthesis. The 5′ hydroxyl group of B_{12} was also modified prior to coupling, being selectively oxidized to a carboxylic acid (**2**) using 2-iodoxybenzoic acid, as previously described by us.[21] Subsequent coupling of 1-amino-3-butyne to **2** with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDCI) and 1-hydroxybenzo-triazole (HOBt) produced B_{12} with a terminal alkyne at the ribose 5′-position (**3**) (see Scheme 1). Compound **3** was purified using a Shimadzu Prominence HPLC on an Eclipse XDB C18 5 μm 4.6 mm × 150 mm column with a mobile phase of 0.1% TFA water and elution with acetonitrile on a gradient starting at 15% acetonitrile increasing to 35% over 20 min (NMR for **3** is provided as Supplementary Figure S1).

Compounds **1** and **3** were coupled using copper(II) sulfate and sodium L-ascorbate (see Scheme 1). The new B_{12}-Ex-4 conjugate (**4**) was purified with a Shimadzu HPLC using an Eclipse XDB C18 5 μm 4.6 mm × 150 mm column with a mobile phase of 0.1% TFA water and elution with acetonitrile. A gradient run from 20% acetonitrile to 42% acetonitrile during the first 3 min and then 42 to 47.5% acetonitrile during the next 10 min was used to separate **4** from starting materials. The product was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI-ToF MS) (see Figure 1, inset). Compound **4** was purified to greater than 97% purity by HPLC (see Figure 1). The tendency of Ex-4 to aggregate resulted in a small shoulder at 6.5 min.[22]

DISCUSSION

Initially, IF binding of **4** was confirmed by radiometric chase assay using ^{57}Co-labeled B_{12} and compared to free $B_{12,}$ as cyanocobalamin (see Figure 2).[23] Significant IF binding of **4** (6.8 nM) was maintained, albeit reduced from unmodified B_{12} (0.12 nM).

Once IF binding of **4** (IF-**4**) was confirmed, agonism of the GLP-1R was assayed for **1**, **4**, and IF-**4** using HEK-293 cells stably transfected with the GLP-1R (HEK-GLP-1R).[25] To this

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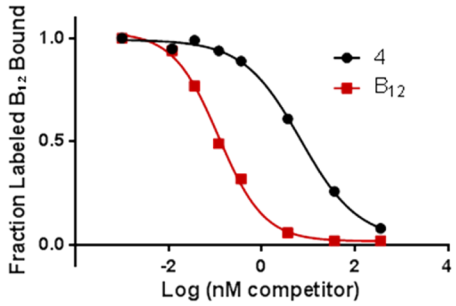

Figure 2. IF binding to B_{12} (0.12 nM) and **4** (6.8 nM). IF used in these assays was produced in the plant *Arabidopsis* in the apo-form and of high purity.[24]

end, we employed a new assay that uses adenoviral transduction to express the genetically encoded FRET reporter AKAR3 that serves as a sensitive readout for cAMP production due to the fact that AKAR3 undergoes a decrease of 485/535 nm emission FRET ratio when it is phosphorylated by cAMP-dependent protein kinase A (PKA) subsequent to GLP-1R activation.[26−28] This is the first instance to our knowledge of a FRET assay for GLP-1R using viral AKAR3 and offers a ready route to sensitive high-throughput screening of the GLP-1R. An EC_{50} for **1**, **4**, and IF-**4** were measured at 26, 68, and 132 pM, respectively (see Figure 3). It is worth noting that the azido modification to

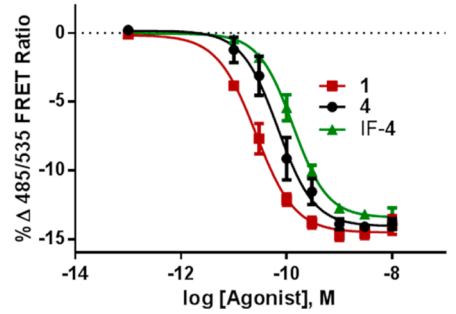

Figure 3. Dose−response analysis of **1**, **4**, and IF-**4** yielded EC_{50} values of 26, 68, and 132 pM, respectively, as determined by monitoring the 485/535 nm FRET emission ratio.

the K12 position of Ex-4 showed no significant reduction in potency compared to unmodified Ex-4 suggesting a useful general route for selective conjugation to Ex-4 through click chemistry approaches.[20] Compounds **4** and IF-**4** show that further conjugation to the K12 position effects function but still demonstrates low picomolar effective concentrations.

Compounds **1**, **4**, and IF-**4** were analyzed for stability against proteolysis by measuring remaining function at the receptor compared to undigested controls. Compounds **1**, **4**, and IF-**4** were tested for function at [100 nM], a concentration at which each had comparable percent change in FRET ratio (see Table 1). Each protease was analyzed separately so that the protective

Table 1. Percent Change in FRET at 100 nM for 1, 4, and IF-4

compd	% change in FRET at 100 nM
1	−12 ± 0.01
4	−13 ± 0.02
IF-4	−12 ± 0.01

nature of B_{12} and IF could be analyzed for their effect versus the specific protease. The pH sensitivity of the assay prevented the use of actual intestinal fluids when testing the compounds.

Digestion was conducted in a standard extracellular solution containing trypsin at 11, 22, or 50 μg/mL, chymotrypsin at 1.25, 3, or 6.25 μg/mL, or meprin β at 1 or 5 μg/mL (see Figures 4 and S2).

At the lowest concentrations of trypsin (11 μg/mL) and chymotrypsin (1.25 μg/mL) there is up to 50% greater function for IF-**4** relative to **4** alone with the highest concentration of trypsin (50 μg/mL) and chymotrypsin (6.25 μg/mL) assayed showing complete lack of function for all systems. The digestion was monitored by measuring agonism of the drugs at the GLP-1R, initially over the course of 3 h, although it was quickly noted that there was no change after 1.5 h indicating that the digestion had stopped by this time point (data not shown). Subsequent triplicate runs were then performed on digestions of 1.5 h.

Meprin β digestion revealed a 2-fold increase in function with B_{12} conjugation and a 3-fold increase in function when prebound to IF (see Figures 5 and S3). No function was seen for **1** at concentrations greater than 3 μg/mL. The protection provided from B_{12} conjugation and subsequent binding to IF show that key residues are being protected. Results of the AKAR3 assays show maintenance of function where otherwise none was observed or improvement of function when **4** is first bound to IF.

■ CONCLUSIONS

The conservation or improved relative function demonstrated herein for Ex-4 when conjugated to B_{12}, and more significantly when bound by IF, is an important first-step in addressing the use and putative role of IF in protecting an administered peptide (orally or by injected means). Protection against pancreatic protease-catalyzed hydrolytic digestion of **4** was maximal at a trypsin concentration of 22 μg/mL and 3 μg/mL of chymotrypsin when **4** was prebound to IF, providing a 4-fold and 5-fold positive increase in function, respectively, as measured by GLP-1R agonism (utilizing the AKAR3 screening assay). The digestion with metalloendoprotease meprin β showed the most significant protection when comparing **1** and IF-**4**. No reduction in function was seen at the highest concentration of meprin β tested (5 μg/mL), while **1** showed no function at concentrations greater than 1 μg/mL of meprin β. B_{12} provided some protection against trypsin relative to the native peptide. The effect is seen at 11 μg/mL of trypsin with a relative 4-fold increase and at 1.25 and 3 μg/mL of chymotrypsin with a relative increase of 3- and 5-fold. The fact that the IF bound form IF-**4** still maintained significant function at the GLP-1R is also highly significant since many routes to protect against intestinal degradation involve encapsulation, which prevents possible luminal function or absorption when in place. The use of IF to improve the protease stability of a peptide offers significant scope for exploitation. Even a small improvement in oral function, for example, may be sufficient to achieve the desired effect. Combining this approach with a highly potent peptide with known gut receptors that can produce a vagal afferent response (such as, but not limited to, GLP-1/Ex-4 or PYY3-36), for example, may allow for a positive clinical outcome to be achieved orally, without need even for systemic delivery. Finally, as demonstrated by the stability against meprin β, there is no suggestion that this approach is limited to oral use against

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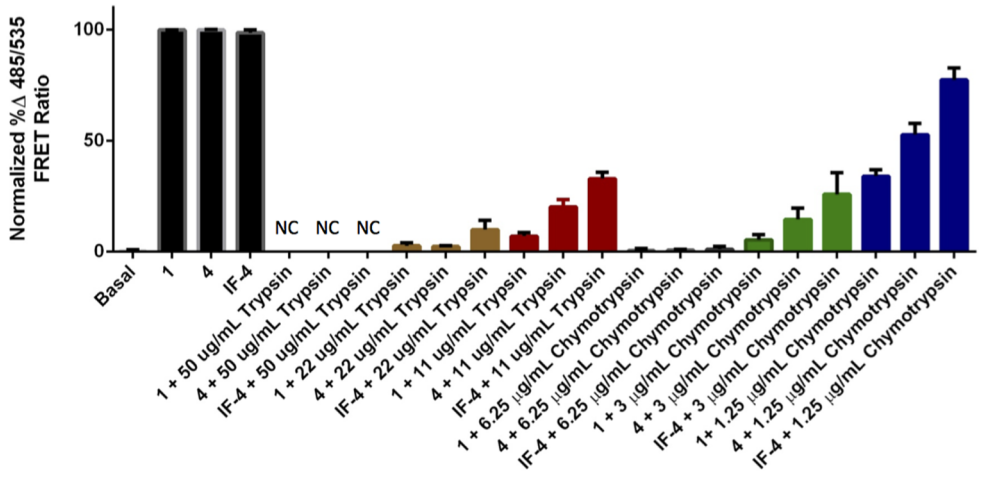

Figure 4. Digestion for 1.5 h of 100 nM **1**, **4**, and IF-**4** with 50, 22, or 11 μg/mL of trypsin or 1.25, 3, or 6.25 μg/mL of chymotrypsin using AKAR3 to measure function. The data shows the maximum expression normalized to 100% of the conjugates done in triplicate (mean ± SEM). Basal control contained trypsin at 50 μg/mL of trypsin. (N.C. = no change). A scatterplot analysis is provided in the Supporting Information (Figure S2).

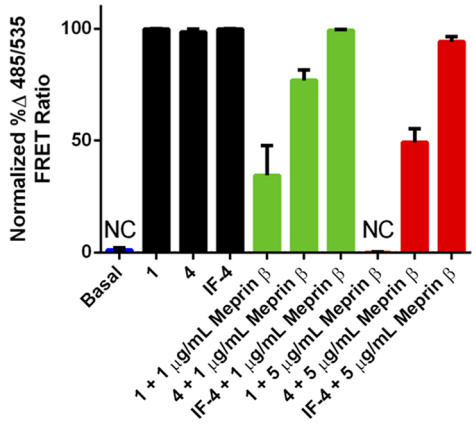

Figure 5. Thirty minute meprin β digestion of 100 nM **1**, **4** and IF-**4** with 1 and 5 μg/mL of meprin β. The data shows the maximum expression normalized to 100% of the conjugates done in triplicate (mean ± SEM). Basal control contained 2 μg/mL of meprin β. Recombinant human meprin β was produced in insect cells and purified and activated as described previously.[29] A scatterplot analysis is provided in the Supporting Information (Figure S3).

gastric proteases, but could also be expanded into serum (through subcutaneous or intravenous injection of IF bound B_{12}-peptide conjugates, for instance), facilitating greatly improved pharmacokinetics (especially when combined with prior results showing B_{12} conjugation already improved sc absorption of a PYY3-36 conjugate[8]), making this a possible platform technology for peptide drug development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00390.

NMR data and scatterplot analyses (PDF)

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Notes

The authors declare no competing financial interest.

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