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Exendin 4 Conjugation and Sequence Modification to Treat Type 2 Diabetes and Obesity

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Abstract

This thesis addresses three primary questions based on the pharmacodynamics (PD) or pharmacokinetics (PK) of the diabetes drug exendin 4 (Ex-4), and vitamin B₁₂ (B₁₂) bioconjugates, thereof.

Q1. (Chapter 2) What effect does B₁₂ conjugation to Ex-4 have on agonism of the glucagon-like peptide-1 receptor (GLP-1R) in vitro and on PD/PK (including brain uptake and function) in vivo?
   
   Goal: To remove side-effects (nausea, weight loss) of Ex-4 without loss of glucoregulation.

Q2. (Chapter 3) Can B₁₂ dietary uptake proteins such as gastric intrinsic factor offer protection to B₁₂ conjugated peptides or proteins (focusing on Ex-4), with a view to improving in vivo PK?
   
   Goal: To demonstrate (in vivo) that IF binding of B₁₂-Ex-4 confers protection against gastric proteolysis as a road-map to oral peptide delivery.

Q3. (Chapter 4) Can a dual agonist of the GLP-1R and neuropeptide Y2 receptor, based on the Ex-4 primary amino acid sequence, be designed and validated in vitro and in vivo.
   
   Goal: To create a new therapeutic to simultaneously treat diabetes and obesity.
Exendin 4 Conjugation and Sequence Modification to Treat Type 2 Diabetes and Obesity

By

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B.S. Chemistry, Rochester Institute of Technology 2012

DISSERTATION

Submitted in partial fulfillment for the degree of

Doctor of Philosophy in Chemistry

Syracuse University

December 2016
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Abbreviations

1. Cyanocobalamin
2. B₁₂-CA; B₁₂-carboxylic acid
3. B₁₂-propargyl amine
4. B₁₂-amino butyne
5. B₁₂-amino pentyne
6. B₁₂-amino hexyne
7. B₁₂(3)-Ex-4
8. B₁₂(4)-Ex-4; B₁₂-Ex-4
9. B₁₂(5)-Ex-4
10. B₁₂(6)-Ex-4
11. RLB001; HEGFTFTSDLKQMEEAEAVRLFIELKNGGPSSTRQRY-NH₂
12. RLB002; HEGFTFTSDLKQMEEAEAVRLFIELRHYLNLVTRQRY-NH₂
13. RLB003; IKPAREDASPEEDENQAYKEFIAYLNVLVTRQRY-NH₂
14. RLB004; HEGFTFTSDLKQMEEAEAVRLFIELKNGGPSRHYLNLVTRQRY-NH₂
15. RLB005; HEGFTFTSDLKQMEEAEAVRLFIELKNGGPSSTRQ-NH₂
16. RLB006; HEGFTFTSDLK(azido)QMEEAEAVRLFIELKNGGPSSTRQRY-NH₂

B₁₂ Vitamin B₁₂

GIT Gastrointestinal tract

IF Intrinsic factor

DMB 5,6-dimethylbenzimidazol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>COA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
<td>HC</td>
<td>Haptocorrin</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistant protein 1</td>
</tr>
<tr>
<td>TCII</td>
<td>Transcobalamin II</td>
</tr>
<tr>
<td>CD320</td>
<td>Cluster of differentiation 320</td>
</tr>
<tr>
<td>Cubam</td>
<td>Cubilin-amnionless</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic polypeptide</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>GLP-1 receptor</td>
</tr>
<tr>
<td>GLP-2R</td>
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<td>GIP receptor</td>
</tr>
<tr>
<td>GCGR</td>
<td>Glucagon receptor</td>
</tr>
<tr>
<td>Ex-4</td>
<td>Exendin 4</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamics</td>
</tr>
</tbody>
</table>
PK  Pharmacokinetics
EC₅₀  Effective concentration 50
GLP-2  Glucagon-like peptide-2
GPCR  G protein-coupled receptor
cAMP  Cyclic adenosine monophosphate
PYY  Peptide Tyrosine Tyrosine
NPY1R  Neuropeptide Y1 receptor
NPY2R  Neuropeptide Y2 receptor
IBX  2-iodoxybenzoic acid
HYP  2-hydroxypyridine
MALDI-ToF-MS  Matrix-assisted laser desorption-ionization time of flight mass spectrometry
EDC  1-ethyl-3-(3-3-dimethylaminopropyl)carbodiimide
HOBt  Hydroxybenzotriazole
DMSO  Dimethyl sulfoxide
DMF  Dimethyl formamide
RIP1  Rat insulin 1 promoter
CRE  cAMP response element
Luc  Luciferase
AKAR3  A kinase activity reporter
FRET  Fluorescence resonance energy transfer
A.C.  Adenylate cyclase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
</tr>
<tr>
<td>FHA</td>
<td>Forkhead associated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>AN</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>ESMS</td>
<td>Electrospray mass spectrometry</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Vitamin B\textsubscript{12} (B\textsubscript{12})

Vitamin B\textsubscript{12} (B\textsubscript{12}) is one of thirteen essential vitamins and the most recently discovered (see Figure 1).\textsuperscript{1} B\textsubscript{12} is a deep red color and has been referred to as “nature’s most beautiful cofactor”.\textsuperscript{2} B\textsubscript{12} is in a rare category as a naturally occurring organometallic and was the first such compound discovered.\textsuperscript{3,4} Humans cannot produce B\textsubscript{12}, as it is made only by certain prokaryotes such as Pseudomonas denitrificans, and so we must acquire it entirely through diet.\textsuperscript{5,6,7,8,9,10} B\textsubscript{12} is a water soluble molecule and readily degraded in the presence of acid.\textsuperscript{11} Humans employ a complex system of binding proteins and receptors in order to navigate the human gastrointestinal tract (GIT) and deliver B\textsubscript{12} into the blood stream, then proliferating cells, including those in the brain. A typical human takes in 1 -5 \(\mu\)g per day of B\textsubscript{12} but the body can store B\textsubscript{12} in the liver for up to five years and a deficiency can take years to discover.\textsuperscript{12} Deficiency is typically the result of a defect relating to the production of binding proteins such as intrinsic factor (IF). There are no known cases of overdosing from B\textsubscript{12} and no recommended upper limit in dosage.\textsuperscript{13}
Figure 1. B₁₂ (cobalamin) structure, shown as cyanocobalamin.

The core of B₁₂, also known as cobalamin, is comprised of a corrin ring chelating a cobalt(III) ion. This corrin ring, which is responsible for the red color of B₁₂, has two sides, α and β (see Figure 1). The corrin ring is similar to a porphyrin ring with key differences being the lower saturation in the corrin (3/4 vs 4/4) and the greater asymmetry of the corrin due to the loss of a methylene bridge between pyrrole rings C and D (also resulting in one less carbon (19) compared to the porphyrin (20)).¹⁴ On the α-side there is a 5,6-dimethylbenzimidazole (DMB) coordinated to the cobalt ion through a nitrogen atom. Off this DMB is a ribose moiety connected to the corrin ring through a phosphate and amide linker. Although there are several functional groups located on B₁₂, conjugations, made at the 5’-hydroxyl of the ribose moiety, maintain the most significant binding affinity by B₁₂ binding proteins.¹⁵,¹⁶

1.1.1 History of B₁₂: Discovery and Diseases

Symptoms of pernicious anemia, a disease characterized by pallor, sharp pains and excessive urination, were first described by physicians in the 1800s.¹⁷,¹⁸ Significant progress toward a cure for the disease, which was typically fatal at the time, did not come until the 1900s. The first major breakthrough was published in 1925 when Whipple, through research on anemia induced by blood loss in dogs, discovered that a diet composed of liver, particularly raw bovine liver, helped the dogs recover.¹⁹,²⁰ This led to the first idea of B₁₂, which was that there had to be something present in liver that was promoting recovery in pernicious anemia. Minot and Murphy then showed in 1926 that 45 patients with pernicious anemia given a daily meal consisting of bovine liver for several months, significantly improved.²¹ The discovery of effective pernicious anemia treatment won Minot, Murphy and Whipple the Nobel prize in chemistry in 1934.²²

Castle set out to address why liver was effective in treating pernicious anemia. His discoveries, through a set of experiments published in 1929 and 1930 using gastric juices from patients with and
without pernicious anemia, led him to believe that the meat had some factor that could only be utilized if those juices were present.\textsuperscript{23,24} What Castle realized was that, although the treatment to pernicious anemia was extrinsic factor (B\textsubscript{12}) and the cause was B\textsubscript{12} deficiency, the real problem was a lack of IF, which rendered the body unable to access B\textsubscript{12}.

The compound responsible for the Nobel prize in 1934 wasn’t isolated until over a decade later.\textsuperscript{25} In 1947, B\textsubscript{12} was simultaneously crystallized by Folks at Merck and Smith at Glaxo and published in Nature and Science, respectively.\textsuperscript{26,27} The second Nobel prize for B\textsubscript{12} was awarded in 1964 to Dorothy Hodgkin for determining the single-crystal X-ray structure of B\textsubscript{12} in 1955.\textsuperscript{28}

1.1.2 Metabolism of B\textsubscript{12}

B\textsubscript{12} acts as a cofactor in two enzymatic processes (see Figure 2). The first, with cytosolic methionine synthase, is the conversion of homocysteine to methionine, and the second, with mitochondrial methylmalonyl coenzyme A (COA) mutase, is the conversion of methylmalonyl-COA to succinyl-COA.

\textbf{Figure 2.} Representation of B\textsubscript{12} coenzyme function in methionine synthase and methylmalonyl-COA synthase.
As the coenzyme for methionine synthase, a methyl group is transferred to homocysteine from 5-methyl-tetrahydrofolate (5-methyl-THF) producing methionine and THF. During this transfer B12 plays a role in remethylating homocysteine and accepting a methyl group from 5-methyl-THF. In the absence of B12, 5-methyl-THF is not converted to THF, which is necessary for DNA synthesis, and homocysteine accumulates causing cellular stress. B12 is also essential for the conversion of methylmalonyl-CoA mutase to succinyl CoA. During this conversion adenosylcobalamin is the coenzyme to methylmalonyl-CoA mutase in the conversion of methylmalonyl-CoA to succinyl-CoA.

1.1.3 Dietary uptake of B12 in humans

Humans have a complicated uptake system for B12 requiring specific transport proteins to get B12 from dietary sources to proliferating cells. The uptake pathway of B12 is designed to both protect and transport. The first two proteins [haptocorrin (HC) and IF] transport B12 from the food into the blood and the last protein directs B12 to proliferating cells. Figure 3 shows the dietary uptake pathway of B12 and how each transport protein is utilized to bring B12 from the diet to the cell.
Figure 3. B$_{12}$ dietary uptake pathway. HC: Haptocorrin; MRP: Multidrug resistant protein 1; IF: Intrinsic factor; TCII: Transcobalamin II; CD320: Cluster of differentiation 320.

### 1.1.4 Transport proteins Involved in B$_{12}$ dietary uptake

There are three binding proteins for the dietary uptake of B$_{12}$. The binding proteins ensure that B$_{12}$ makes it from food to the cells. These proteins begin chaperoning B$_{12}$ as soon as the food starts to be broken down in the mouth. Protection is necessary since B$_{12}$ is unstable in the low pH environment of the stomach and, hence prone to degradation (much like a peptide would be).

#### 1.1.4.1 Haptocorrin (HC)

The first binding protein involved in B$_{12}$ trafficking is HC, alternative names for which are transcobalamin I or R-binder. HC is the least specific of B$_{12}$’s binding proteins and recognizes partially degraded fragments of B$_{12}$ along with intact B$_{12}$ with picomolar affinity. HC is a heavily glycosylated ~65 kDa protein and found in both the saliva and blood stream. Figure 3 shows the crystal structure of holo-HC. The HC protein encapsulates B$_{12}$ protecting it from degradation in the stomach. B$_{12}$ is then carried into the duodenum, where the HC is degraded by pancreatic proteases and the B$_{12}$ is picked up by IF.
Figure 4. Structure of Holo-HC (pdb: 4KKI), showing the hetero-dimeric nature of the protein with the B$_{12}$ bound at the interface of the dimer.

1.1.4.2 Intrinsic Factor (IF)

The second carrier protein responsible for transporting B$_{12}$ through the intestinal tract and into the blood is IF, a 50 kDa glycoprotein. The crystal structure of IF, shown in Figure 5 as an IF-B$_{12}$ complex, reveals that it is structurally very similar to HC, being composed of two separate domains and binding B$_{12}$ between these domains.$^{35}$ IF, unlike HC, is resistant to pancreatic proteases and binds B$_{12}$ in the duodenum with picomolar affinity.$^{34}$ IF-B$_{12}$ travels through the intestines and is bound by the cubilin-amnionless (cubam) receptor in the terminal ileum and internalized into the ileal enterocyte.$^{36}$ Cathepsin L is responsible for the breakdown of IF in the lysosome and subsequent release of B$_{12}$.37

A lack of IF causes most B$_{12}$ deficiencies and is particularly known as the cause of pernicious anemia.$^{37}$ Pernicious anemia is an autoimmune disorder that results in the loss of the gastric parietal cells responsible for producing IF. This disease is progressive and was considered fatal in the 1800s, since it led to an eventual clinical deficiency of B$_{12}$. Research into a cure for this disease drove the discovery of B$_{12}$.18
Figure 5. Structure of Holo-IF (pdb: 2PMV), showing the hetero-dimeric nature of the protein with the $\text{B}_{12}$ bound at the interface of the dimer.

1.1.4.3 Transcobalamin (TCII)

TCII (shown in Figure 6), the final $\text{B}_{12}$ transport protein, is responsible for the delivery of $\text{B}_{12}$ to the cells. The TCII-$\text{B}_{12}$ complex is delivered to the cells through the CD320 receptor. Unsaturated TCII is found in blood at a concentration of 0.6-1.5 nM in humans, 2 nM in rats, and 20 nM in mice. It is important to consider that rat and mouse models differ in apo-TCII concentration. The TCII concentration is of particular importance when considering $\text{B}_{12}$ conjugation as a method to deliver peptides orally. This is because the concentration of $\text{B}_{12}$ will have to be over the TCII concentration, or the conjugate may be lost to $\text{B}_{12}$ uptake into proliferating cells.
1.1.5 Drug development using B_{12} and its dietary pathway

B_{12} and its dietary uptake pathway offer considerable potential in drug development.\textsuperscript{41} The first research avenue to be explored was oral delivery of peptides or proteins.\textsuperscript{42,43} The hypothesis was that the binding proteins that protect and transfer B_{12} could be manipulated to provide the same protection to peptides and proteins. Another area for research involves the brain uptake of B_{12}. The hydrophilic nature of B_{12} requires a receptor mediated transfer across the blood brain barrier (BBB). The transfer is done through the CD320 receptor and only transports B_{12} in the form of a TCII-B_{12}.

One concern involved with B_{12} conjugation work is the potential for B_{12} to simply act as B_{12}. Will a B_{12} compound act like B_{12} or the drug upon delivery? When B_{12} is in the blood TCII will bind it and deliver it to

**Figure 6.** Structure of Holo-TCII (pdb: 2BBS), showing the hetero-dimeric nature of the protein with the B_{12} bound at the interface of the dimer.
proliferating cells so administration over the TCII binding capacity should allow the B$_{12}$ conjugate to behave like the peptide. The second question is whether a B$_{12}$ conjugation will interfere with the function of the drug or molecule. If conjugation cannot be done in a way to maintain function the complex will not work. The obstacles are not unique to B$_{12}$ and can be mitigated with proper controls and dosing.

1.2 Diabetes Mellitus

Diabetes Mellitus is a disease that affects just under 30 million Americans. There are two types of diabetes mellitus, type 1 (T1DM) and type 2 (T2DM). T1DM is an autoimmune disease which destroys the insulin producing beta cells and is typically diagnosed before the age of 30. Since there are no beta cells and insulin cannot be produced the most effective form of treatment is insulin injections. T2DM is caused by a resistance to insulin. The β-cells are present and producing insulin in T2DM patients but the body isn’t properly utilizing it to clear the glucose from the blood into the cells.

Insulin and glucagon are used in the body to directly control glucose levels. A change in normal glucose levels can be very dangerous to the body so insulin and glucagon work to maintain an homeostasis. Insulin is responsible for glucose entering the cell and glucagon is responsible for the release of glucose stores into the blood. Both hypoglycemia and hyperglycemia can cause serious health issues and death if not properly treated.

Insulin is regulated through glucose stimulated insulin secretion (GSIS). An increase in blood glucose levels, causing hyperglycemia, triggers K$^+$ channels in β-cells to close depolarizing the cell. This causes an influx in Ca$^+$ and a release of insulin. The insulin then acts to remove the glucose from the blood and deliver it to the cells. In a fasting state, glucose levels are reduced causing hypoglycemia, which is countered through the release of glucose from glycogen stores in the liver. A drop in glucose levels also results in glucagon-induced gluconeogenesis, which helps to restore the glucose levels to normal.
1.2.1 Impact of T2DM

T2DM accounts for close to 95% of diabetes patients.\textsuperscript{55} Most cases go unnoticed and aren’t diagnosed for several years; it is estimated that up to a quarter of Americans with T2DM are undiagnosed.\textsuperscript{44} The most common risk factors for T2DM are age and a body mass index (BMI) over 25 kg/m\textsuperscript{2}.\textsuperscript{56} In many cases for people with these risk factors a change in diet can greatly reduce the chances of a diagnosis.\textsuperscript{57}

T2DM also has a high cost associated with it. Not only do individuals have to suffer from the ailment of T2DM but they also have to suffer from medical bills and treatment expenditures. Individuals diagnosed with diabetes average medical expenses 2.3 times higher than an individual without diabetes. In 2012 it is estimated that diabetes costs nationwide approached $245 billion.\textsuperscript{58} These costs reflect on the total impact of diabetes and just compound to the physical aspects of the disease.\textsuperscript{58}

1.2.2 Treatments options for T2DM

Since T2DM patients are able to make insulin, the treatment is different from a T1DM patient. A person with mild T2DM would typically be put on metformin initially. Metformin works to increase insulin sensitivity so that the insulin already produced in the body can work properly.\textsuperscript{59} After metformin treatment loses efficacy, typically after the first couple years of treatment, sulfonylureas are added to the treatment regimen.\textsuperscript{60} Sulfonylureas act by directly stimulating insulin secretion. This direct action makes sulfonylureas very potent but also presents the possibility of hypoglycemia in the case of an overdose. An alternative to sulfonylureas are dipeptidyl peptidase IV (DPP-IV) inhibitors.\textsuperscript{61} DPP-IV inhibitors prevent the degradation of incretin hormones which increase insulin levels. This inhibition lets the body release more insulin in a glucose dependent manner.\textsuperscript{62} Metformins, sulfonylureas and DPP-IV inhibitors are all available as oral medications, which is why these treatment options are considered first and second line therapies. The third line and fourth line treatments are injectable medications. The third line drugs are incretin mimetics, which act similar to an incretin hormone in the body and increase insulin levels in a glucose
The final treatment option is insulin injection, which is only used after no other treatments work.

### 1.2.3 Incretin Hormones

A spike in blood glucose levels after ingestion of nutrients causes an increase in insulin levels. This glucose dependent control of insulin is known as the incretin effect. The incretin effect is a response to peptides, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), released in the intestines. Activation of the incretin receptor on pancreatic β-cells results in insulin release dependent on glucose levels.

In T2DM patients the incretin effect is greatly reduced. This is due in large part to the insulinotropic effects of GIP and GLP-1 being either greatly reduced or not present. The insulinotropic effects of GIP are no longer present and GIP shows no effect on insulin secretion when administered even at pharmacological levels. GLP-1, however, has been shown to maintain some of its insulinotropic effects albeit at a significantly reduced rate compared to non-T2DM people. The greater effectiveness of GLP-1 to stimulate an insulin release in the presence of T2DM, compared to GIP, has led to the treatments utilizing GLP-1R agonism rather than GIPR agonism.

Of the treatment options for T2DM, only GLP-1 receptor agonist and DPP-IV inhibitors take advantage of the incretin effect. Incretin mimetic therapies are able to promote insulin release in a way that is glucose dependent which means the body is able to regulate how much insulin is released. This is an advantage that minimizes the risk of hypoglycemia. Along with minimized hypoglycemia, incretin therapies have been shown to induce weight loss, promote β-cell regeneration and improve cardiovascular health.
1.2.3.1 Glucose-dependent insulinotropic peptide

Glucose-dependent insulinotropic peptide (GIP) was the first incretin hormone found. It was initially thought to be only responsible for inhibiting gastric acid secretion but it was later discovered to be responsible for insulin secretion. GIP is released from the intestinal K cells post prandially and activates the GIP receptor (GIPR), a G-protein coupled receptor (GPCR), located in the pancreatic β-cells resulting in an increase in insulin secretion. The half-life of GIP is 2-5 min as a result of degradation from DPP-IV. GIP based T2DM therapies have not had much success because of their relatively weak physiological effects seen especially when compared to GLP-1. There are no current drugs marketed as a GIP agonist, but there is research into the effectiveness of GLP-1 and GIP dual agonism.

1.2.3.2 Glucagon-like peptide-1 (GLP-1)

GLP-1 is an incretin hormone in humans which is primarily responsible for controlling insulin levels in response to a rise in glucose levels in the blood. GLP-1 is produced in the intestinal L cells and is typically released post prandially. DPP-IV quickly degrades GLP-1 in the intestines resulting in rapid clearance ($t_{1/2} = \sim 2$ minutes). Most humans have a typical fasting plasma level of GLP-1 of 5-10 pmol/L, which can increase upon glucose addition. The GLP-1 receptor (GLP-1R) is located in the pancreatic β-cells as well as the brain, stomach and adipose tissue. Although GLP-1 and GIP are both incretin hormones and promote the release of insulin there are some key differences. GLP-1, unlike GIP, has been shown to inhibit gastric emptying, glucagon secretion and food intake.

1.2.3.3 Exendin 4 (Ex-4)

Ex-4 is an incretin mimetic, sharing 53% amino acid sequence similarity with 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 DPP-IV, which rapidly cleaves and inactivates GLP-1 in vivo. 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. Such resistance to DPP-IV does not, however, translate to other proteases, and exenatide (synthetic Ex-4) therefore must be administered subcutaneously.

Figure 7. One of the 36 accepted structures of the Ex-4 molecule, pdb1JRJ.87

1.2.3.3.1 Discovery of Ex-4

During the late 1980s and early 1990s John Eng, a researcher working for the Veteran Affairs Medical center in the Bronx, was searching for peptides in the glucagon superfamily. His search focused on peptides found in lizards particularly lizards of the Heloderma family. A narrow search for peptides found only in Heloderma suspectum containing the glucagon superfamily characteristic amino-terminal histidyl structure (His³) resulted in the discovery of exendin 3, a GLP-1R agonist, in 1990.88 This exciting result prompted an expansion on the search to include other species within the Heloderma family. Heloderma horridum was found to contain Ex-4, a potent version of exendin 3, with characteristics similar to those found in GLP-1 in 1992.89
1.2.3.4 Glucagon Receptor Family

The glucagon receptor superfamily includes the glucagon receptor (GCGR), GLP-1R, glucagon-like peptide 2 (GLP-2R) and GIPR. These receptors are also part of the larger family of G protein-coupled receptors, and as such contain a specific binding domain. Although the glucagon receptor family and corresponding peptide agonists share many similarities, each receptor maintains a high affinity for only one peptide. However, with careful engineering agonism can be achieved at multiple receptors creating dual and triple agonists of the glucagon receptor superfamily.  

There are three classes of GPCRs, class A, B, and C, and members of the glucagon receptor superfamily are part of class B. Class B GPCRs are part of the secretin family and are activated by endocrine hormones. These receptors contain a 7-transmembrane helical bundle with an N-terminal extracellular domain of 120 amino acids. Upon agonist binding a GPCR undergoes a conformational change resulting in the active state. The G protein acts as a signal transducer upon activation and begins a signal cascade depending on the subunit and pathway. There are three G protein subunits, α, β, and γ and each subunit can be tied to a number of different pathways with the most well know being Gs, Gq, and Gi. Upon GPCR signaling, β-arrestin proteins bind to the GPCR and desensitize the GPCR, ending the signal cascade.

Members of the glucagon receptor superfamily activate the Gs pathway resulting in an increase in cyclic adenosine monophosphate (cAMP). The increase in cAMP production activates the protein kinase A (PKA) in the cell. The increase in cAMP and active PKA can be used to follow agonism in receptor assays.

1.2.3.4.1 Pharmaceutical applications of Ex-4

Exenatide, a synthetic Ex-4, was approved as a pharmaceutical as the first incretin mimetic under the name Byetta in 2005. The role of exenatide is to increase insulin production and reduce the need for insulin injections. Typical treatment with exenatide is 5 µg injections twice daily for the first month then
10 µg injections twice daily.\textsuperscript{102} The half-life of exenatide is 2.1 hours and it is removed from the blood by glomerular filtration and is subsequently degraded by proteases.\textsuperscript{103}

1.2.4 Obesity

Obesity and diabetes are comorbidities and approximately 80-95% of patients with T2DM are obese.\textsuperscript{104} However, obesity alone affects significantly more people with estimates reporting over a third of Americans as obese.\textsuperscript{105} The recent rise in obesity and the projections of increased prevalence through 2050 along with the risk factors associated with the comorbidity of T2DM mean an effective drug to treat both is essential.\textsuperscript{106,107} The most effective treatment of obesity to date is bariatric surgery, which is an expensive and invasive to the patient.\textsuperscript{108} The invasive nature, severity and cost of bariatric surgery limit the procedure’s reach.\textsuperscript{109} However the alternative drugs currently on the market are only capable of 5-10% weight reduction.\textsuperscript{110} The effectiveness of weight-loss treatment, especially in single drug treatments, is often mitigated by counter-regulatory compensation effects.\textsuperscript{111}

1.2.4.1 Peptide Tyrosine Tyrosine (PYY)

Peptide tyrosine tyrosine (PYY) is a peptide is an appetite regulating peptide found in the neuropeptide Y hormone family.\textsuperscript{112} It is secreted from the enteroendocrine L-cells of the intestines postprandially. There are two active forms of PYY, PYY(1-36) and PYY(3-36). The initial and short lived PYY is PYY(1-36), which has a half-life only a couple of minutes in blood. The short half-life is due to dipeptidyl-peptidase IV (DPP-IV) which cleaves PYY(1-36) after the second N-terminal amino acid forming PYY(3-36), which has a half-life of just under 16 minutes.\textsuperscript{113} PYY(1-36) is an appetite stimulant which upon truncation becomes PYY(3-36), an appetite suppressant, and the major circulating form of PYY.

1.2.4.1.1 PYY(3-36) function

PYY(1-36) is cleaved by DPP-IV, which produces PYY(3-36) and eliminates any function at the neuropeptide Y1 receptor (NPY1R). PYY(3-36) acts only at the neuropeptide Y2 receptor (NPY2R) which is
responsible for appetite suppressing effects. Administration of PYY(3-36) peripherally has been shown to reduce food intake in both healthy and obese rodents.\textsuperscript{114} These results have laid a promising foundation for research into PYY(3-36) as an anti-obesity therapy.

1.2.4.1.2 Neuropeptide Y receptors (NPYR)

The neuropeptide Y (NPY) receptor family is composed of four receptors (Y1, Y2, Y4 and Y5). Y1 and Y5 receptors are appetite stimulating and Y2 and Y4 are appetite suppressing.\textsuperscript{115} The NPY receptors are activated by the peptides NPY, pancreatic polypeptide, PYY(1-36), and PYY(3-36). The NPY receptors are class A GPCRs, which generally activate either G\textsubscript{i} or G\textsubscript{q} pathways. The G\textsubscript{q} protein activates a cascade which raises Ca\textsuperscript{2+} levels which can be followed through in vitro assays using Fura-2, a Ca\textsuperscript{2+} binding dye.\textsuperscript{116} The G\textsubscript{i} pathway causes a decrease in cAMP levels, which can be difficult to detect so a promiscuous G\textsubscript{q} protein is often introduced to produce a positive response.\textsuperscript{117}

1.2.4.1.3 PYY(3-36) pharmaceutical applications

PYY(3-36) has potential as a pharmacological peptide for appetite control and obesity. The first research to highlight the role of PYY(3-36) was published by Batterham in 2002, which showed food intake reductions of up to 30%.\textsuperscript{112} Since that paper there has been no shortage of research invested in finding a drug involving PYY(3-36), however no drugs have come to market. This lack of clinical translation is due to two characteristics of PYY(3-36): the first is the nausea associated with administration of the peptide and the second and most important factor is that the body is able to overcome its the appetite suppressing abilities after a couple of days of treatment.\textsuperscript{118,119}

Therapy involving PYY(3-36) has to be approached from more than one direction to compensate for the body’s ability to overcome the PYY(3-36) signaling. This can be approached through a combination therapy involving multiple peptides or through a single peptide targeting multiple targets with the ability to suppress appetite.
1.2.4.2 Combination therapy for diabetes and obesity treatment

Bariatric surgery has been around for decades and is established as a very effective way to treat obesity. Recently it has been realized that bariatric surgery is also an effective way to treat T2DM. Although the exact mechanisms which make bariatric surgery so effective are still relatively unknown, it is known that both GLP-1 and PYY(3-36) levels are considerably higher after surgery. Replication of the effect caused on hormones after bariatric surgery through medication would provide a great tool for treating both obesity and diabetes.

1.2.4.3 Summary

Diabetes is a disease that effects almost a tenth of all Americans, and though it is mostly controlled with proper medical care there is still room for improvement. Many treatment options for more severe cases of diabetes come with unpleasant side effects or an invasive method of administration. Changes in administration from injection to oral would do a great deal to improve patient compliance with third line medications. Changes in hydrophobicity could alter brain uptake and minimize unwanted side effects. The nature of B12 makes it a suitable candidate to alter both of these characteristics. This gives a B12-Ex-4 conjugate the potential to be an oral diabetes medication with limited side effects.

The comorbidity of obesity and diabetes is particularly concerning and although there are methods to control diabetes there are far fewer options to control obesity. Although Ex-4 isn’t approved for weight loss it is one of the side effects to the treatment. Enhancements to the appetite suppressing ability of Ex-4 without diminishing its ability to control glucose levels could lead to a diabetes/obesity treatment combined in one drug. PYY is a natural peptide designed to suppress appetite in humans, however the short half-life of PYY makes it unsuitable as a pharmaceutical. Tailoring Ex-4 to activate both the GLP-1R and NPY2R by constructing a hybrid Ex-4/PYY peptide could lead to a single peptide with dual agonism at both GLP-1R and NPY2R.
Modifications to Ex-4 either through B$_{12}$ conjugation or sequence manipulation could provide important improvements to the pharmacokinetics and pharmacodynamics of Ex-4. Success in this work could open the doors to both B$_{12}$ chemistry and Ex-4 chemistry. B$_{12}$ modification to Ex-4 could provide a method to mitigate side effects and be used as a tool in pharmaceutical peptide chemistry to alter brain uptake. A coagonist of GLP-1R and NPY2R drug would provide a method for treating both diabetes and obesity, simultaneously.

1.3 References


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Chapter 2: Synthesis, characterization, in vitro and in vivo analysis of B$_{12}$ conjugated to Exendin 4

The work reported in this chapter will be submitted to *Diabetes* with the title “Vitamin B12 conjugation of exendin-4 abrogates CNS induced nausea and food intake reduction and reverses hyperglycemic response in the rat” and coauthors: Ron L. Bonaccorso, Clinton L. Elfers, Elizabeth G Mietlicki-Baase, George G. Holz, Matthew Hayes, Christian L. Roth and Robert P. Doyle

The first trials of the in vivo glucose tolerance tests and food intake studies reported in this chapter were completed by me while visiting Seattle Children’s Research Institute (photo shown above).

2.1 Introduction

Controlling glucose levels is the goal of diabetes treatment. First and second line drugs, such as metformin and sulfonylureas, are effective, safe, and noninvasive.\(^1\) Third line drugs, though effective and very safe, are administered only through subcutaneous injection, leading to low patient compliance, and often come with unpleasant side effects.\(^2\) Nausea is reported in 50% of the patients taking exenatide, synthetic Ex-4, for example.\(^3\) This side effect is hypothesized to be a result of Ex-4 agonism of GLP-1R in the brain at the arcuate nucleus (AC) of the hypothalamus.\(^4\) GLP-1Rs in the brain are also responsible for the weight reduction associated with GLP-1 mimetics, but the pancreatic receptors are used to control
glucose levels and insulin release.\textsuperscript{5,6} If brain uptake can be shut down or reduced, both nausea and weight loss will be decreased.

Ex-4 is able to rapidly cross the blood brain barrier (BBB), due to its lipophilic properties.\textsuperscript{7} Ex-4 brain uptake studies have shown that almost 90\% of Ex-4 reaches the brain.\textsuperscript{8} To prevent BBB passage the lipophilicity of Ex-4 must be altered. One way to do this is through conjugation with a lipophobic molecule. B\textsubscript{12} transport across membranes is receptor mediated and not capable of passive diffusion which make it a good candidate for conjugation and subsequently preventing or at least inhibiting brain uptake.\textsuperscript{9} Only TCII bound B\textsubscript{12} and not HC bound B\textsubscript{12}, which composes almost 25\% of the total B\textsubscript{12} binding protein in blood, is able to pass through the blood brain barrier and only a third of serum B\textsubscript{12} enters the brain.\textsuperscript{10,11}

2.2 Design and syntheses of B\textsubscript{12}-Ex-4 conjugates (7-10)

Previously in the Doyle lab, B\textsubscript{12} had been functionalized with a carboxylic acid through modification of the 5’ hydroxyl group of the ribose moiety.\textsuperscript{12} Oxidation at this position through use of 2-iodoxybenzoic acid (IBX) allows for further modification and higher yielding peptide conjugations (over 90 percent yield).

2.2.1 Synthesis and characterization of B\textsubscript{12}-Ex-4 conjugates (7-10)

B\textsubscript{12}, as cyanocobalamin (1), had to be modified in a way that would allow high yielding conjugation between it and Ex-4. This was achieved by modifying the ribose hydroxyl group. B\textsubscript{12} was initially functionalized to a carboxylic acid.\textsuperscript{12} From the carboxylic acid a carbon linker and alkyne functional group could be formed.

2.2.1.1 B\textsubscript{12} modification of the 5’ hydroxyl to a carboxylic acid

This is done by converting the 5’ hydroxyl to a carboxylic acid as shown in Figure 1. Modifications are done at this position so that binding to the transfer proteins remains unhampered. This reaction is done using IBX and 2-hydroxypyridine (HYP). The primary alcohol is successfully converted to a carboxylic acid producing B\textsubscript{12}-CA (2) with a 30\% yield.
Figure 1. Oxidation of the 5’ hydroxyl group of 1 to a carboxylic acid.

2 was then purified using FPLC and characterized by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-ToF-MS). Figure 2 shows a m/z of 2 of 1343.6 which is consistent with the expected m/z of [M-CN].
Figure 2. MALDI-ToF MS of 2 expected [M-CN] 1344 m/z, observed 1343.6 m/z.

2.2.1.2 Functionalizing B$_{12}$ through addition of an alkyne via the carboxylic acid

Following the synthesis of 2, a series of B$_{12}$ molecules containing a terminal alkyne were synthesized. B$_{12}$ was primed for click chemistry by coupling an alkyne-amine to the carboxylic acid of 2. Four B$_{12}$-alkyne compounds (3-6) were synthesized with an alkyl chain containing a terminal alkyne varying from three to six carbons off of the carboxylic acid. Figure 3 shows the scheme used to synthesize the B$_{12}$-alkyne compounds (3-6) utilizing 1-ethyl-3-(3-3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazole (HOBt). Successful conversion yielded compounds 2-5 with a yield of >90%.
Figure 3. Synthesis of B$_{12}$-alkyne precursors for B$_{12}$-Ex-4. (i) EDCI, HOBr, propargyl amine, rt, DMSO, 16 h; (ii) EDCI, HOBr, 1-amino-3-butyno, rt, DMSO, 16 h; (iii) EDCI, HOBr, 1-amino-4-pentyne, rt, DMSO, 16 h; (iv) EDCI, HOBr, 1-amino-5-hexyne, rt, DMSO, 16 h; (v).

3-6 were successfully made and characterized through MALDI-ToF-MS as shown in Figure 4. 3 had an observed mass of [M-CN] 1381.7 m/z with an expected mass of 1382 m/z. 4 had an observed mass of [M-CN] 1393.6 m/z with an expected mass of 1394 m/z. 5 had an observed mass of [M-CN] 1407.7 m/z with an expected mass of 1408 m/z. 6 had an observed mass of [M-CN] 1421.9 m/z with an expected mass of 1422 m/z.
Figure 4A. B$_{12}$-propyne (3) MALDI-ToF MS expected [M-CN] 1380 m/z observed 1381.7 m/z.

Figure 4B. B$_{12}$-butyne (4) MALDI-ToF MS expected [M-CN] 1394 m/z observed 1393.6 m/z.
Figure 4C. B₁₂-pentyne (5) MALDI-ToF MS expected [M-CN] 1408 m/z observed 1407.7 m/z.

Figure 4D. B₁₂-hexyne (6) MALDI-ToF MS expected [M-CN] 1422 m/z observed 1421.9 m/z.
2.2.1.3 B₁₂-Ex-4 conjugates (7-10) synthesized via CuAAC

Compounds 2-5 were used to make a series of B₁₂-Ex-4 conjugates with various linker lengths between B₁₂ and Ex-4. B₁₂ was conjugated to Ex-4 through Sharpless/Huisgen copper-catalyzed azide-alkyne cycloaddition (CuAAC), click chemistry, utilizing copper(II) sulfate (CuSO₄) and sodium ascorbate.¹⁴,¹⁵,¹⁶ Figure 5 shows the schematic for the synthesis of compounds 6-9.

![Synthesis of B₁₂-conjugates 6-9.](image)

**Figure 5.** Synthesis of B₁₂-conjugates 6-9. (v) 3, CuSO₄, sodium ascorbate, Ex-4-K12-azido, Water/DMF 4:1, 1 h; (vi) 4, CuSO₄, sodium ascorbate, Ex-4-K12-azido, Water/DMF 4:1, 1 h; (vii) 4, CuSO₄, sodium ascorbate, Ex-4-K12-azido, Water/DMF, 4:1, 1 h; (viii) 6, CuSO₄, sodium ascorbate, Ex-4-K12-azido, Water/DMF; 1 h. >90% yield.

2.3 In vitro optimization of B₁₂-Ex-4 agonism at the GLP-1R

The B₁₂-Ex-4 series was first tested for function at the human GLP-1R in human embryonic kidney (HEK) cells, which had been stably transfected with the receptor. Activation of GLP-1R initiates the Gₛ cascade of the GPCR raising cAMP and PKA levels in the cell. This activity was monitored through a couple
of different assays using either a bioluminescence assay or FRET to follow the cascade resulting from activation of the GLP-1R.

The first method used to follow GLP-1R agonism involved monitoring a FRET ratio change to follow PKA activity. To follow PKA levels HEK-GLP-1R cells are treated with adenovirus incorporating an A kinase activity reporter (AKAR3). AKAR3 is activated and a FRET change is seen upon phosphorylation by PKA (see Figure 2). This change in FRET is dose dependent and can be used to determine an EC$_{50}$ for GLP-1R agonists.

**Figure 6.** GLP-1R agonist induced GPCR $G_s$ cascade resulting in AKAR3 activation by PKA. GLP-1R: glucagon-like peptide-1 receptor; A.C.: adenylate cyclase; HEK-293: human embryonic kidney cells; ATP: Adenosine
triphosphate; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; YFP: yellow fluorescent protein; CFP: cyan fluorescent protein; FHA: forkhead associated; FRET: fluorescence resonance energy transfer.

B_{12}-Ex-4 activates the G_{s} pathway of the GPCR upon binding to GLP-1R. The G_{s} pathway activates adenylate cyclase (A.C.) which converts ATP to cAMP. As the cAMP level rises the enzyme PKA is activated and can then phosphorylate the threonine amino acid of AKAR3. This phosphorylation of AKAR3 results in a conformational change producing a decrease of 485/535 nm emission FRET ratio. This decrease in FRET ratio is dose dependent and can be followed to show EC_{50} values in vitro.

Using AKAR3 to follow FRET ratio change allows for real time monitoring of changes. Figure 8 shows the raw data in real time for a dose response series of 8. At t=100 s the injection of either saline or 8 at a concentration between 10 pM and 10,000 pM was administered almost immediately resulting in an increase in PKA levels and change in FRET.

![Figure 8](image_url)

**Figure 7.** Dose response of PKA phosphorylation of AKAR3 in real time after administration of either saline or 8 of concentrations ranging from 10-10,000 pM.
7-10 were tested for agonism at the GLP-1R. The optimal conjugated Ex-4 would be used as the lead conjugate for further testing. Figure 9 shows the dose response results of each conjugate. The most potent conjugate, 8, had an EC\textsubscript{50} of 68 pM followed by 7, 9, and 10, which had EC\textsubscript{50}'s of 121, 246 and 405 pM, respectively compared to Ex-4 at 26 pM. Ex-4 has been shown to have an EC\textsubscript{50} of 33 pM by Thorens \textit{et al.} by following cAMP production so this method is consistent with previous literature reports.\textsuperscript{18}

![Graph showing dose response results of each conjugate.](image)

**Figure 8.** Comparison of Ex-4, 7, 8, 9 and 10 EC\textsubscript{50} curves with EC\textsubscript{50} values of 27, 121, 68, 246 and 405 pM, respectively.

8 was screened as the optimal conjugate and was used for all further testing. Conjugation of B\textsubscript{12} to Ex-4 resulted in a relatively small loss of function and still maintained picomolar function at the GLP-1R. Linker length played a small role in receptor agonism potency as EC\textsubscript{50} of all of the conjugates were within one log unit, indicating that compounds 7-9 had essentially the same potency, of Ex-4 except for 10.
2.3.1.1 $\text{B}_{12}$-Ex-4 (8) affinity for $\text{B}_{12}$ binding proteins

After synthesis and optimization of the $\text{B}_{12}$-Ex-4 conjugates and analysis of function at the GLP-1R, binding affinities for the $\text{B}_{12}$ binding proteins had to be confirmed. For $\text{B}_{12}$ to be a viable oral delivery method modifications and conjugations have to be done in a way that won’t interfere with the ability of IF to recognize and bind the conjugate. TCII affinity is also important since it is responsible for delivery of $\text{B}_{12}$ to cells. Binding of the conjugate to TCII could result in delivery to the CD320 receptor rather than the GLP-1R.

IF and TCII binding of 8 was confirmed by radiometric chase assay using $^{57}$Co-labelled $\text{B}_{12}$ and compared to free $\text{B}_{12}$, as cyanocobalamin.$^{19}$ IF and TCII binding of 8 (Kd values of 6.8 nM and 0.75 nM, respectively) was maintained, albeit reduced from unmodified $\text{B}_{12}$ (0.12 nM and 0.098 nM respectively) see Figure 9.
Figure 9. TCII (A) and IF (B) binding of 8 compared to B_{12}.

After establishing binding to the B_{12} binding proteins 8 was tested for function at the GLP-1R while complexed to IF (see Figure 10). Since everywhere B_{12} is in the body it is bound to a binding protein, it is important to confirm that the conjugate could work while complexed to these B_{12} binding proteins. Picomolar agonism of GLP-1R is maintained even after IF complexation indication that 8 could work in the presence of these proteins.
The second method used for assaying GLP-1R agonism followed cAMP levels in the cell. HEK-293 cells stably expressing GLP-1R were first transfected with a rat insulin 1 promoter (RIP1) cAMP response element (CRE) encoding for the enzyme luciferase (Luc) (see Figure 11). The production of the enzyme luciferase is dependent on the cAMP levels after incubation with the GLP-1R agonist. Luciferin is then added to the cells and the amount catalyzed by luciferase can be determined through a bioluminescence readout. B₁₂-Ex-4 and B₁₂-Ex-4 complexed to the binding proteins IF and HC were tested for function and compared to Ex-4. Following cAMP through this RIP-CRE-Luc assay yielded an EC₅₀ of 85 pM for Ex-4, 362 pM for B₁₂-Ex-4, 434 for IF-B₁₂-Ex-4 and 426 for HC-B₁₂-Ex-4. Both IF and HC showed similar effect on agonism at GLP-1R.
2.4 In vivo testing of B₁₂-Ex-4 (8) to demonstrate reduced brain uptake compared to Ex-4

2.4.1 Glucose tolerance tests after B₁₂-Ex-4 and Ex-4 administration

Following the body’s response to a bolus of glucose is one of the oldest and most widely used tests to determine if a person is diabetic. A person with a normal response will show a blood glucose level below 140 mg/dL whereas a person with T2DM will reach a blood glucose level over 200 mg/dL. There are two different ways to test for glucose tolerance, either with an oral bolus of glucose or an intraperitoneal bolus of glucose followed by an A1C1 reading of the blood at various time intervals. Though in both instances the blood glucose levels rise, only through an oral bolus does the incretin effect occur. The body requires glucose to pass through the intestines for the appropriate receptors to be activated. This difference allows for determination of whether an incretin effect and intestinal stimulation is needed for our drug to elicit an effect.
2.4.1.1 Oral glucose tolerance test of 8 compared to Ex-4 and saline

An oral glucose tolerance test (OGTT) was performed on Sprague Dawley rats (n=12) (see Figure 10) to compare the glucose controlling effects of 8 and Ex-4. For testing brain uptake of 8 and Ex-4 the rat model is a particularly effective since activation of the rat’s sympathetic nervous system (SNS) causes a characteristic rise in glucose level unlike the sharp decrease seen in mice and humans. Elimination of this glucose spike and an actual decrease in glucose points to a reduction of brain uptake with 8 versus Ex-4, which showed the normal increase in glucose.

![Graph showing blood glucose levels over time for different treatments.](image)

Figure 12. Percent change in blood glucose (mg/dL) in Sprague Dawley rats (n=12) from baseline (t=-30) for vehicle, 0.9 nmol/kg Ex-4, 3.5 nmol/kg Ex-4, 0.9 nmol/kg 8, and 3.5 nmol/kg 8.

2.4.2 Food Intake and Body Weight change with 8 administration

A side effect associated with Ex-4 is weight loss, and though generally regarded as a positive it is unknown if the weight loss is a result of the nausea caused by Ex-4 or through some other process in the body. What is known though, is if GLP-1Rs in the brain are not activated the nausea and food intake reduction are not seen. If food intake reduction is mitigated, then brain activation of GLP-1Rs on the
AN are not activated. Figure 13 shows that administration of 8 at 0.2, 0.9 or 3.5 nmol/kg doses are ineffective at reducing food intake in rats (n=12).

![Graph showing cumulative food intake and BW change over 24 h for different doses of 8 and Ex-4.]

**Figure 13.** (Left) Food intake and (Right) body weight change over 24 h for either vehicle, 0.2 nmol/kg, 0.9 nmol/kg, or 3.5 nmol/kg of 8 (vehicle, 0.2 nmol/kg, 0.9 nmol/kg, and 3.5 nmol/kg).

Only at the two highest concentrations of 8 was there any change in food intake or body weight. Although this change was not significant the food intake and body weight study was repeated and compared to Ex-4 at a concentration of 0.9 nmol/kg (Figure 14 and Figure 15). Food intake reduction was significant for Ex-4 at 0.9 nmol/kg and significant for 8 but only at a concentration of 3.5 nmol/kg and with almost half the food intake reduction seen for Ex-4. Along with the reduction in food intake was a reduction in body weight. The largest average body weight reduction was from the Ex-4 treatment followed by 8 at 3.5 nmol/kg and then 8 at 0.9 nmol/kg.
Figure 14. 24 h food intake comparison of Ex-4 at 0.9 nmol/kg to 8 at 0.9 nmol/kg and 3.5 nmol/kg. p=0.00012 for Ex-4 and p=0.023 for 8 at 3.5 nmol/kg when compared to baseline.

Figure 15. 24 h body weight comparison of Ex-4 at 0.9 nmol/kg to 8 at 0.9 nmol/kg and 3.5 nmol/kg. p≤0.05
2.4.3 PICA studies establishing nausea levels for 8 vs. Ex-4

Measuring nausea in rats as a response to Ex-4 can be done by looking at the way the rats behave. Rats are particularly good model to study nausea on since they are unable to vomit and behave in a predictable way when experiencing nausea. There are a couple of tests that can be used to measure nausea. The first test is conditioned taste aversion and the second is pica, which is when the rat consumes a nonnutritive food such as kaolin.\textsuperscript{27,28,29}

A pica test was used to follow the level of nausea in the rats by following kaolin consumption. Kaolin intake was followed over 24 hours after administration of 0.9 nmol/kg of Ex-4, 0.9 nmol/kg of 8, or 3.5 nmol/kg of 8 (see Figure 14). Ex-4 treatment was the only instance of significant increase in kaolin intake when compared to baseline. Concentrations of 8 up to 4x that of Ex-4 still trended to lower nausea as indicated by the reduced kaolin intake. This study is still on-going to increase statistical significance prior to publication (To be submitted to the journal ‘Diabetes (IF 12)’ with this author as ‘first author’).

![Figure 16. 24 h kaolin intake after Ex-4 at 0.9 nmol/kg and 8 at 0.9 nmol/kg and 3.5 nmol/kg administration (n=6).](image-url)
2.5 Outcomes and conclusions

The in vitro assays show that 8 and the complex IF-8 maintain picomolar agonism at the GLP-1R and are both within one log order of the Ex-4 EC\textsubscript{50}, whether following cAMP directly or indirectly through PKA levels. The modifications to Ex-4 do not significantly change the agonism at the receptor, and the conjugation still allows for binding to the B\textsubscript{12} binding proteins. In vivo results reveal that the changes to the hydrophilicity have changed the pharmacodynamics of Ex-4. The conjugation to B\textsubscript{12} reverses the hyperglycemia seen in rats and works to lower the glucose levels. The most significant finding of this chapter is that B\textsubscript{12} conjugation to Ex-4 shows a smaller decrease of food intake and reduction of nausea. This suggests a blocking of central GLP-1R activation, presumably in the brain, while allowing peripheral GLP-1R activation to produce glucoregulation responses from the pancreas to continue. This work has considerable potential to be a new form of the Ex-4 pharmaceutical without the side-effect of chronic nausea. The goal of this chapter, to remove side-effects of Ex-4 without loss of glucoregulation, was achieved in a rat model.

2.6 Reference


Chapter 3: IF protection of 8 from systemic and pancreatic proteases


3.1 Introduction

The future of pharmaceutics and medicinal chemistry is in peptide therapeutics. Peptides in general offer several advantages over small molecule drugs, which have historically dominated pharmaceutical research and therapeutics, including fewer side effects, greater selectivity, and higher potency.1 Because of the high selectivity possible with peptides, new therapeutics can be used to target difficult to treat diseases.2 Peptide drug research is still relatively new, but it is a quickly growing field of pharmaceutical research.3

Despite the many benefits of peptide therapeutics, there are several major hurdles facing peptide therapeutics, including rapid degradation through proteolysis, short half-lives and rapid clearance, and low oral bioavailability.4 One of the many benefits of peptides as therapeutics is increased potency. Unfortunately, without an increase in half-life these peptides offer limited therapeutic value even with their higher potency. One such example of this is GLP-1, which, despite its potency, offers no therapeutic value due to its short half-life.5 In vivo half-life has been increased through use of albumin and pegylation.6,7 To increase the pharmaceutical potential of peptide research an effective and easily employable method of increasing oral bioavailability and protease resistance is essential.8

Extensive research into increasing oral bioavailability and peptide stability has led to promising results and several drugs using these new methodologies are in clinical trials.9 There is still no “go to” technique to provide oral delivery and protease resistance.10 Many of these techniques alter the potency and inactivate the drug. This underlies the reason to have multiple methodologies and techniques to use for peptide delivery and proteolysis resistance.
Most approved peptide medications are taken through injection.\textsuperscript{11} The low oral bioavailability of these peptides is due to their potential for degradation by enzymes and proteases.\textsuperscript{12} Ex-4, although resistant to degradation in the blood and currently available as a therapeutic, is an example of an injectable peptide prone to degradation by pancreatic proteases.\textsuperscript{13} In attempts to prevent degradation, Ex-4 can be used as a probe to detect proteolysis by following GLP-1R agonism after digestion. The level of maintained agonism at the receptor should correlate inversely with the amount of peptide digested.

In chapter 2 I demonstrated that IF binding of B\textsubscript{12}, upon conjugation to Ex-4, was maintained. It was also demonstrated that functional agonism of GLP-1R was maintained with low picomolar agonism. The question targeted herein is whether IF binding of 8 to a dietary uptake protein can offer protection to the conjugated peptide as well as the B\textsubscript{12} molecule.\textsuperscript{14} B\textsubscript{12} is a particularly attractive candidate for peptide conjugation since it possesses no inherent negative side effect.\textsuperscript{15} The \textbf{hypothesis} is that upon complexation IF should shield 8 from proteases thereby protecting it from degradation. IF’s natural resistance to proteolysis and encapsulation of B\textsubscript{12} make it an ideal shield for degradation of conjugates as well.\textsuperscript{16,17} To test this hypothesis 8 and IF-8 need to be incubated in the presence of proteases and then tested for agonism at the GLP-1R. Stability of Ex-4, 8 and IF-8 against the abundant intestinal endopeptidases, which represent both gastric and systemic proteases (see Table 1), trypsin, chymotrypsin, and meprin \( \beta \) were compared by measuring relative function at the GLP-1R after digestion.\textsuperscript{18,19,20} This work will test the value of IF in protecting B\textsubscript{12} from proteases. This work will show the potential of IF and B\textsubscript{12} conjugation and possibly provide evidence for oral delivery of therapeutics through the B\textsubscript{12} dietary pathway.
Table 1. Proteases and where they cut

<table>
<thead>
<tr>
<th></th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Meprin β</th>
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<tbody>
<tr>
<td><strong>Designation</strong></td>
<td>Basic</td>
<td>Aromatic</td>
<td>Acidic</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td>Gastric</td>
<td>Gastric</td>
<td>Systemic</td>
</tr>
<tr>
<td><strong>Digestion Locations</strong></td>
<td>Lysine and Arginine</td>
<td>Tyrosine, Tryptophan and Phenylalanine</td>
<td>Aspartic acid, Glutamic acid</td>
</tr>
</tbody>
</table>

3.2 In vitro testing of IF protection of 8

Ex-4, 8 and IF-8 were analyzed for stability against proteases by measuring remaining function at the receptor compared to undigested controls. Ex-4, 8 and IF-8 were tested for function at [100 nM], a concentration at which each had comparable percent change in FRET ratio (see Table 1). After establishing a reference point for 100% function each sample could be tested for agonism relative to 100% function after proteolysis.

Table 2. % Change in FRET at 100 nM for Ex-4, 8 and IF-8.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Change in FRET at 100 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex-4</td>
<td>-12± 0.01</td>
</tr>
<tr>
<td>8</td>
<td>-13± 0.02</td>
</tr>
<tr>
<td>IF-8</td>
<td>-12± 0.01</td>
</tr>
</tbody>
</table>

Digestion was conducted in a standard extracellular solution containing either trypsin at 11, 22, or 50 μg/mL, chymotrypsin at 1.25, 3, or 6.25 μg/mL or meprin β at 2 or 10 μg/mL see Figure 1 and Figure 2. Three different concentrations of the pancreatic proteases were used to track the relative differences between 8 and IF-8 (see Figure 1). At the lowest concentration of trypsin (11 μg/mL) and chymotrypsin (1.25 μg/mL) there is up to 50% greater function for IF-8 relative to 8 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.

Figure 1. Top: column graph and Bottom: scatter plot of 1.5 h trypsin and chymotrypsin digestion of 100
nM Ex-4, 8 and IF-8 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).

Meprin β digestion revealed a 3.3 fold increase in function with B₁₂ conjugation and a 4.5 fold increase in function when pre-bound to IF (see Figure 5). No function was seen for Ex-4 at concentrations greater than 3 μg/mL. The protection provided from B₁₂ 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. B₁₂ conjugation itself offered some protection at the low trypsin concentrations tested.

Figure 2. Left: column graph and Right: scatter plot of 30 min meprin β digestion of 100 nM Ex-4, 8 and IF-8 with 2 and 10 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.
3.3 Outcomes and conclusions

The conservation or improved relative function demonstrated herein for Ex-4 when conjugated to B\textsubscript{12} and more significantly, when bound by IF is an important first-step in addressing the use, and putative role, of IF in delivering and protecting an orally administered peptide. Protection against pancreatic protease-catalyzed hydrolytic digestion of \textit{8} was maximal at an optimal trypsin concentration of 22 μg/mL of trypsin and 3 μg/mL of chymotrypsin when \textit{8} was pre-bound to IF, providing a 4-fold and 7.5-fold positive increase in function respectively as measured by GLP-1R agonism (utilizing a new AKAR3 screening assay). The digestion with metalloendoprotease meprin β showed the most significant protection when comparing Ex-4 and IF-\textit{8}. No reduction in function was seen at the highest concentration of meprin β while 1 showed no function at concentrations greater than 2 μg/mL of meprin β. B\textsubscript{12} provided some protection against trypsin relative to the native peptide. The effect is seen at 11 μg/mL of trypsin with a relative increase of 3-fold and at 1.25 and 3 μg/mL of chymotrypsin with a relative increase of 3 and 1.6-fold. The fact that the IF bound form IF-\textit{8} 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 may be sufficient to achieve the targeted 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 PYY(3-36)) 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 use against gastric proteases, but could also be expanded into serum (through intravenous injection of IF bound peptide conjugates for instance), facilitating greatly improved pharmacokinetics, making this a possible platform technology for peptide drug delivery. The
goal of this chapter, to test that IF binding of 8 confers protection against gastric proteolysis as a road map to oral peptide delivery.

3.4 References

6. Sleep, D.; Cameron, J.; Evans, L. R., Albumin as a versatile platform for drug half-life extension. Biochimica et Biophysica Acta (BBA) - General Subjects 2013, 1830 (12), 5526-5534.


Chapter 4: Rationally designed GLP-1R/Y2R agonist peptide

The work in this chapter was done in collaboration with Professor George G. Holz and Dr. Christian L. Roth.

The work reported in this chapter will be submitted to PNAS with the title “Diabetes and weight loss therapy through hybrid peptides” and coauthors: Ron L. Bonaccorso, Clinton L. Elfers, George G. Holz, Christian L. Roth and Robert P. Doyle.

The work from this chapter resulted in a patent pending: SU100846_156P471

4.1 Introduction

Weight loss research has been given a lot of attention in recent years due to the growing epidemic of obesity.\(^1\) Despite all of the research and therapeutic options, effective weight loss treatments have been difficult to find.\(^2\) There are only five FDA approved drugs marketed for treatment of obesity but the effectiveness of these drugs are variable and not consistent from patient to patient.\(^3\) Several diabetes medications have been shown to cause weight loss and recently liraglutide has even been approved as a weight loss medication.\(^4\) Most of these drugs show limited effectiveness, and many potential drugs either lose effectiveness over time or result in significant weight gain after treatment has ended.\(^5\) The body is able to invoke a counter-regulatory response and ignore the signals suppressing appetite deeming the drug ineffective.\(^6\)

Studies have shown that combination therapy can be effective at treating obesity.\(^7\) Drug cocktails however are difficult and expensive to test since there are multiple PK and PD pathways to test and side effects to consider. A single peptide that targets multiple receptors is an ideal candidate to treat obesity.\(^8\) Combination treatments consisting of GLP-1R agonists and NPY2R agonists have shown to be particularly effective at treating both diabetes and obesity.\(^8\) Coagonism of GLP-1R and either the glucagon receptor (GCGR) or GIPR have shown to have enhanced glucose and weight loss control as well.\(^9,10\) Current co-
agonists have shown to be effective in vitro but have limited efficacy in vivo. T2DM and obesity management through combination therapies have potential to be effective and are expected to be the future of weight control therapeutics.\textsuperscript{11}

### 4.2 Design of hybrid proteins

To overcome the compensatory effects of PYY(3-36), a hybrid peptide, made from fragments of Ex-4 and PYY(3-36), should be able to target both GLP-1R and NPY2R without the PD effects of two different drugs. A series of peptides were designed with the goal of combining fragments of Ex-4, which were vital for GLP-1R agonism, and fragments of PYY(3-36), which are necessary for maintaining agonism to the NPY2R.\textsuperscript{12} The similarity between glucagon receptor superfamily peptides opens the possibility of receptor agonism at GIPR and GCCR.\textsuperscript{13,14} An initial series of three sequences were designed with the first two peptides composed primarily of Ex-4 sequence and the third as a modified version of PYY(3-36). Table 1 shows the comparison of each peptide in relation to Ex-4 and PYY. An initial series of 3 peptides, RLB001 (\textsuperscript{11}), RLB002 (\textsuperscript{12}) and RLB003 (\textsuperscript{13}), was designed to screen for dual agonism. After the results of these three peptides an additional three, RLB004 (\textsuperscript{14}), RLB005 (\textsuperscript{15}) and RLB006 (\textsuperscript{16}), were designed. The last three peptides were designed to try and improve upon the initial series of \textsuperscript{11}, \textsuperscript{12} and \textsuperscript{13} to produce an effective dual or even triagonist.\textsuperscript{15}
Table 1. Hybrid peptide design and comparison. Sequences corresponding to 11-16 were designed with elements of PYY(3-36) and Ex-4. Amino acid sequence taken from PYY(3-36) is shown in blue and amino acid sequence taken from Ex-4 is shown in black.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYY(3-36)</td>
<td>IKPEAPREDASPEELNRYASYARHYLNLVTRQY-NH2</td>
</tr>
<tr>
<td>Ex-4</td>
<td>HEGTFTSDLKQMEEEAVRLFIELWLNKGGPSGRPPSS-NH2</td>
</tr>
<tr>
<td>(11)RLB001</td>
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<td>(12)RLB002</td>
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<td>(15)RLB005</td>
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</tr>
<tr>
<td>(16)RLB006</td>
<td>HEGTFTSDLKazido)QMEEEAVRLFIELWLNKGGPSSTRQY-NH2</td>
</tr>
</tbody>
</table>

4.3 In vitro screening and testing

The RLB series peptides were tested for function at receptors corresponding to the glucagon super family of receptors and NPY receptors. These receptors were transfected into either HEK293 cells or CHO cells. HEK 293 cells stably transfected with either GLP-1R or GIPR followed by an infection of adenovirus incorporating AKAR3 to indirectly follow cAMP production. CHO cells were transfected with NPY2R and a promiscuous g protein using FURA2 to follow calcium release.

4.3.1 Screening the RLB series for GLP-1R agonism and dose response

Function was first established at the GLP-1R by screening the initial conjugates in the HEK-GLP-1R cells. These cells were treated with adenovirus containing AKAR3 and a agonism was determined by a decrease in FRET ratio. The initial screen of compounds is found in Figure 1 and shows that 11, 12 and 14 have agonism at the GLP-1R. 13 showed no function at the GLP-1R at 300 nM making ineffective as a GLP-1R and NPY2R coagonist.
Figure 1. A screen of RLB series peptides at the GLP-1R at 300 nM.

The initial screen showed that modifications to Ex-4 don’t completely hinder function at the GLP-1R. The peptides showing function at the GLP-1R were then analyzed further to establish an EC$_{50}$. Figure 2 shows that 11 and 14 are the most effective with EC$_{50}$ values of 50 ± 3 pM and 128 ± 30 pM, respectively and followed by 12 at 253 ± 50 pM.

Figure 2. Dose response comparison of 11, 12 and 14 to Ex-4. 12 has a p-value of 0.0032 compared to Ex-4 and 11.
11, 12 and 14 all retained picomolar agonism at GLP-1R warranting further analysis at NPY2R and other glucagon superfamily receptors. 13 was discarded as a potential dual agonist due to its loss of function.

4.3.2 Screening the RLB series for NPY2R agonism and dose response

Following a screen at the GLP-1R, a screen for agonism at NPY2R was conducted. Figure 4 shows full function was only achieved with 12 and 14. Positive results show that a successful in vitro dual agonist of GLP-1R and NPY2R was achieved.

![Graph showing FURA2 Response vs. NPY2R agonists at 300 nM concentration levels](image)

**Figure 3.** NPY2R screen with RLB series of peptides at [300 nM].

12 and 14 were followed up with a full dose response curve to establish an EC50 for each peptide (see Figure 4). PYY(3-36) was used as the positive control to show that each peptide shows comparable agonism at the NPY2R through in vitro trials. 14 had an EC50 of 107 ± 9 nM and 12 had an EC50 of 59 nM.
4.3.3 Screening the RLB series for GIPR and GCGR agonism and dose response

After establishing agonism at the GLP-1R, the RLB series peptides were tested against similar receptors. Receptors in the glucagon receptor superfamily were tested based on the similarities of native peptide agonists. Shown in Figure 5 are the results of the GIPR screen, where only 11 and 15 showed function at the GIPR and Ex-4, PYY, 12 and 14 showed no function under 1 µM.

Figure 4. NPY2R EC_{50} of 12, 59 nM, and 14, 107 nM.
Figure 5. GIPR dose response and EC<sub>50</sub> values of 11 (275 nM) and 15 (173 nM). Note: Ex-4, 12, 14 and PYY were all tested with no agonism up to 1 µM.

Peptides 11 and 14 were screened for GCGR agonism compared to Ex-4. Each of these peptides, including Ex-4, showed slight agonism at the receptor at high concentrations (>300 nM). Only peptides 11, 12 and 14 were confirmed to have function at more than one receptor. 11 is an effective in vitro dual agonist of GLP-1R and GIPR and 12 and 14 are effective agonists of GLP-1R and NPY2R.

4.4 In vivo testing of 11 compared to Ex-4 and PYY(3-36)

After the initial screening of the RLB series peptides an in vivo study was planned to compare the dual agonists 11 and 14 to Ex-4 and PYY(3-36). Since 12 was significantly less potent at the GLP-1R it was not selected for in vivo testing. A one-day food intake screen of 11 and 14 showed that 11 had a reduction in food intake but 14 actually resulted in the opposite effect and caused an increased food intake from the rats (see Figure 6). Given the initial results only 11 was pursued.
**Figure 6.** Initial screen of food intake for compounds 11 and 14 at 2.4 nmol/kg over 16 h.

11 was isolated as the lead candidate for both blood glucose control and weight loss. A dose response study in was then completed in rats to determine the optimal concentration of 11. A two day food intake study was conducted to verify that 11 behaved uniquely when compared to Ex-4 and co-administration of Ex-4 and PYY(3-36). Figure 7 shows the results of a two day food intake study comparing 11, Ex-4 and a mixture of Ex-4 and PYY(3-36) all at 2.4 nmol/kg per day. The total reduction of food intake for all three samples is shown in Figure 8. 11 shows a significant improvement over Ex-4 alone and an average lower food intake when compared to Ex-4 and PYY(3-36) co-administration. This establishes that 11 is not Ex-4 but rather a new and different drug.
Figure 7. Inhibition of food intake of 11 compared to Ex-4 and Ex-4 and PYY(3-36) combined each at 2.4 nmol/kg/d.

Figure 8. Normalized food intake against baseline levels for 11, Ex-4 and Ex-4 and PYY(3-36) shown over 12 and 23 h.
Food intake was studied further in diet induced obese (DIO) rats over two days comparing 11’s treatment at 2.4 nmol/kg to an equimolar mixture of Ex-4 and PYY(3-36) at 2.4 nmol/kg (shown in Figure 9). In each category 11 is out performing Ex-4 and PYY(3-36) combination treatments.

**Figure 9.** On the left is shown food intake over a two day study with DIO rats administered either 2.4 nmol/kg of 11 or an equimolar mixture of Ex-4 and PYY(3-36). On the right is shown the change in body weight gain of the 11 treatment vs the equimolar Ex-4 and PYY(3-36).

**Figure 10.** Food intake comparison for 11 and Ex-4 at 10 nmol/kg/d over two days normalized to pretreatment baseline in young 9 week old rats (3 males and 2 females per group).
Direct comparison of 11 and Ex-4 at 10 nmol/kg dosing is seen in Figure 10. 11 is able to provide an average decrease in food intake lower than Ex-4 at both 12 h and 23 h time points.

4.5 Glucoregulatory effects of 11 versus Ex-4

11 was compared directly to Ex-4 to determine efficacy in blood glucose control. An intraperitoneal glucose tolerance test was performed on rats to treated with 11, Ex-4 and saline (see Figure 11). Both Ex-4 and 11 showed a similar prolonged increase in glucose levels characteristic of Ex-4 in rats. This indicates that the mechanism of action for 11 to control glucose is the same as Ex-4.

![Blood Glucose Levels](image1)

**Figure 11.** (Left) Blood glucose levels after administration of saline, 11 and Ex-4 following a 10 day treatment (10 nmol/kg/d, 2 males and 2 females per group). (Right) Fasting glucose level reduction after a two day treatment with 10 nmol/kg/d 11 in 31-wk old male rats.

4.6 Outcomes and conclusions

A summary of the results from the in vitro agonism of each receptor is highlighted in table 2. In vitro results were able to confirm dual agonist function with 11 at the GLP-1R and GIPR and dual agonist function with peptides 12 and 14 at the GLP-1R and NPY2R. 11 and 14 were the most potent GLP-1R agonist in vitro and were initially chosen for in vivo trials. No peptide showed any significant function at the NPY1R or the glucagon receptor.
Table 2. Summary of receptor agonism results. (NT= Not tested, *11 and 14 saw no change in EC50 after 3 months in solution, **B12-16 was tested in place of 16.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EC50 at Receptors Tested</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>NPY1</td>
</tr>
<tr>
<td>PYY(3-36)</td>
<td>&gt;300 nM</td>
</tr>
<tr>
<td>Ex-4</td>
<td>&gt; 1 µM</td>
</tr>
<tr>
<td>*(11)</td>
<td>&gt; 1 µM</td>
</tr>
<tr>
<td>(12)</td>
<td>&gt; 1 µM</td>
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<tr>
<td>(13)</td>
<td>&gt; 1 µM</td>
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<tr>
<td>*(14)</td>
<td>&gt; 1 µM</td>
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<tr>
<td>(15)</td>
<td>NT</td>
</tr>
<tr>
<td>**(16)</td>
<td>NT</td>
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The in vivo results showed that 14 had the reverse effect on appetite and weight loss and caused an increase in both. 11 despite no NPY2R agonism in vitro showed improved efficacy at reducing weight gain and suppressing appetite. 11 even showed improved function over combination therapy of Ex-4 and PYY(3-36) at equimolar dosing at controlling food intake and weight reduction. 11 and Ex-4 showed similar profiles in their glucoregulatory abilities though. 11, although similar in glucose control, possesses an enhanced ability to control appetite and promote weight loss. There is a need for effective treatment of obesity and T2DM and the initial testing of 11 shows that it there is great potential for it as a lead drug candidate. The goal of this chapter was to create a new lead therapeutic that simultaneously treats diabetes and obesity and this was accomplished.
4.7 References


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Chapter 5: Experimental

5.1 Materials and Methods

The chemicals and solvents were purchased from Sigma-Aldrich, VWR, or Thermo Fisher Scientific and used without further purification. Cyanocobalamin (B₁₂), 2-iodoxybenzoic acid (IBX), 2-hydroxypyridine (HYP), dimethyl sulfoxide (DMSO), propargyl amine, 1-amino-3-butynle, 1-amino-4-pentyne, 1-amino-5-hexyne, 1,1′-carbonyldi(1,2,4-triazole), trimethylamine (TEA), carbodiimide, hydroxybenzotriazole (HOBt), dimethyl formamide (DMF), copper(II) sulfate (CuSO₄) sodium ascorbate, and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma Aldrich. Black costar plates with a clear bottom were purchased from Thermo Fisher Scientific. PYY(3-36) K₄-azido, PYY(1-36), Ex-4 K12-azido, 11, 12 and 13 were purchased from C.S. Bio Lab (Cambridge, MA). 14, 15, 16 and 14 azido were all purchased from NeoBiolab (Cambridge, MA). H₂O was distilled and deionized using an 18.2 mΩ Barnstead Nano Diamond ultra-purification system. Fetal bovine serum (FBS), PenStrep, Dulbecco’s modified eagle media (DMEM), F-12K, Pluronic F-127 and Fura-2 acetoxyethyl ester (Fura-2AM) were purchased from Invitrogen Life Technologies, Carlsbad, CA. Acetonitrile, methanol and tetraflouro acetic acid (TFA) were purchased from VWR. In vitro plate assays were completed using a Molecular Devices FlexStation III running SoftMaxPro software. Xeragenix supplied the human intrinsic factor, which was expressed in Arabidopsis thaliana.

RP-HPLC was performed using either an Agilent 1100 system or a Shimadzu Prominance with an Agilent Eclipse C₁₈ XBD analytical column (5 µm x 4.6mm x 150 mm). Fast protein liquid chromatography (FPLC) was performed using an AKTAPrime plus (GE Healthcare) liquid chromatograph with an automated fraction collector and 5 mL diethyleaminoethyl (DEAE) anion-exchange (AE) column.

MALDI-ToF MS was performed on a Bruker Autoflex III Smartbeam with a laser intensity between 10-20% for samples smaller than 2,000 Da (B₁₂ precursor compounds) and between 40-50% for samples larger than 2,000 Da (peptides and bioconjugates). A CHCA matrix was used for all samples and was prepared by
dissolving 10 mg of CHCA to a 50:50 mixture of water and acetonitrile containing 0.1% TFA. Samples were then analyzed by MALDI-ToF-MS using a 50:50 mixture of sample to matrix.

5.2 B_{12}-Ex-4 Synthesis

5.2.1 Synthesis of B_{12}-Carboxylic Acid

1 (1 equivalent), IBX (2.5 equivalents) and HYP (5 equivalents) were dissolved in DMSO at 60 °C for 3 h. The reaction was then precipitated out using 15:1 diethyl ether and acetone. The crude reaction was then dissolved in H_{2}O and purified by FPLC using 100% water to bind 2 to the DEAE AE column and 2% NaCl in water to elute pure 2.

5.2.2 Synthesis of B_{12}-Alkyne Compounds (3-6)

2 was combined with 10 equivalents of EDCI and 20 equivalents of HOBt in dry DMSO under argon and stirred for 20 minutes. To this reaction 10 equivalents of 1-amino-3-butyne was added. This reaction proceeded for 16 h and was then precipitated out with the addition of acetone and ether at a 4:9 ratio. This reaction was separated by HPLC with a flow rate of 1 mL/min and a 1 mL/min gradient of 0-13% acetonitrile over 13 minutes with a retention time of 5 min.

5.2.3 Synthesis of B_{12}-Ex-4 compounds (7-10)

Ex-4 was then conjugated to 2 using copper-catalyzed alkyne-azide cycloaddition. This conjugation was achieved by dissolving Ex-4-K12-azido with either 3, 4, 5, or 6 in a 1:3 ratio, respectively with copper(II) sulfate (CuSO_{4}) and sodium ascorbate in water/DMF (4:1) and stirring for 1 h. The reaction yields were greater than 90% with isolated purity greater than 95% for 6-9 as indicated by HPLC in all cases.

6-9 were separated from Ex-4 and unreacted 1 on a C18 column (see Figure 3) monitored at both 280 and 360nm. A mobile phase of 0.1% TFA water was used with a flow rate of 1 mL/min and a gradient from 20-42.5% acetonitrile for 3 min then 42.5-47% acetonitrile for 12 min. Isolated conjugates 6-9 were
confirmed by electrospray mass spectrometry. The bioconjugates were separated as two peaks due to the aggregation of Ex-4.

5.3 In vitro work following agonism at GLP-1R, GiPR, NPY2R and NPY1R

5.3.1 GLP-1R agonism

HEK-293 cells stably transfected with human GLP-1R (HEK-GLP-1R) were used to test for agonism of each peptide and peptide conjugate. The HEK-GLP-1R cells were grown in DMEM with 10% fetal bovine serum (FBS), 1% pen-strep and 0.1% geneticin all produced by Gibco. Cells were incubated at 37 °C in a humidified incubator with 5% CO₂. Cells were plated at 60,000 cells per well on a 96 well plate coated with rat tail collagen and incubated for 24 h. To follow protein kinase A activity media was removed from the cells and DMEM containing 1% FBS and adenovirus incorporating the A-kinase activity reporter 3 (AKAR3) with a multiplicity of infection per cell of 25 was added to each well. After 20 h of incubation the media was removed and 200 µL of standard extracellular solution (SES) was added to each well. Another 50 µL of SES containing conjugate was added to each well and immediately measured at 485 and 535 nm upon excitation at 440 nm. To follow cAMP production media was removed from the cells and DMEM containing RIP-CRE-Luc (200 ng/well), Lipofectamine, and Plus reagent was added to each well. Cells were incubated for 3 h and then an additional 200 µL of DMEM with 10% fetal bovine serum and 1% penstrep was added. After 36 of incubation the media was removed and 100 µL of DMEM with 0.1% bovine serum albumin containing the conjugate to be tested was added to each well and incubated for an additional 4 h. Cells were then lysed from the plate and tested for luciferase activity by measuring photoemissions.

5.3.2 GiPR agonism

HEK-293 cells stably transfected with human GiPR (HEK-GiPR) were used to test for agonism of 11, 12, 14, and 15. The HEK-GiPR cells were grown in DMEM with 10% fetal bovine serum (FBS), 1% pen-strep and 0.1% geneticin all produced by Gibco. Cells were incubated at 37 °C in a humidified incubator with
5% CO$_2$. Cells were plated at 60,000 cells per well on a 96 well plate coated with rat tail collagen and incubated for 24 h. To follow protein kinase A activity media was removed from the cells and DMEM containing 1% FBS and adenovirus incorporating the A-kinase activity reporter 3 (AKAR3) with a multiplicity of infection per cell of 25 was added to each well. After 20 h of incubation the media was removed and 200 µL of standard extracellular solution (SES) was added to each well. Another 50 µL of SES containing conjugate was added to each well and immediately measured at 485 and 535 nm upon excitation at 440 nm.

5.3.3 NPY1R and NPY2R agonism

CHO cells were used to test for agonism of each peptide and peptide conjugate. The CHO cells were grown in F12K media with 10% fetal bovine serum (FBS) and 1% pen-strep. Cells were incubated at 37 °C in a humidified incubator with 5% CO$_2$. Cells were plated at 20,000 cells per well on a 96 well plate coated with rat tail collagen and incubated for 24 h. To follow NPY2R or NPY1R activation, media was removed from the cells and F-12k containing either NPY2R or NPY1R (20 ng/well), Lipofectamine, and Plus reagent was added to each well. Cells were incubated for 3 h and an additional 200 µL of DMEM with 10% fetal bovine serum and 1% pen-strep was added. After 36 of incubation the media was removed and 100 µL of SES containing Fura-2 AM and pluronic acid F-127 were added to the cells to follow Ca$^{2+}$ production and incubated for 30 min. The SES mixture was removed and 150 µL of SES was added to each well. Another 50 µL of SES containing conjugate was added to each well and excited at both 355/9 nm and 375/9 nm. Upon Fura-2 coordination with Ca$^{2+}$ 505 nm light was emitted.
5.4 In vivo studies following glucoregulation and food intake after 8 and 11 administrations in Sprague Dawley rats

In vivo studies on 8 were done in collaboration with the Hayes lab at UPenn, and in vivo studies on 11 were done in collaboration with the Roth lab at Seattle Children’s Research Institute. In vivo studies are still ongoing for both 8 and 11.

5.5 Glucoregulation Studies

Either saline, Ex-4 or 8 was administered at t=-30 min followed by an oral bolus of glucose at t=0 min. Blood glucose levels were monitored at t=-30, 0, 30, 60, and 120 min. Either saline, Ex-4 or 11 was administered at t=-30 min followed by an oral bolus of glucose at t=0 min. Blood glucose levels were monitored at t=-30, 0, 15, 30, 60, and 120 min.

5.6 Food intake and body weight

Food intake was monitored using a metabolic cage. Food was removed from the rats during the last hour of the light cycle while the rats were weighed.

5.7 Pica study upon treatment with saline, Ex-4 or 8

Sprague Dawley rats were administered treatment and left alone in cages with both kaolin and chow present. Total kaolin consumption was measured after 24 h. A high consumption of kaolin is indicative of nausea.

5.8 References

6 Future Work

6.1 Retest pica and perform conditioned taste aversion testing on rats administered 8

The pica study on rats after administration of 8 will be expended on to include a larger sample size and more accurate error bars. The rats will be administered either saline, Ex-4 or 8 and then exposed to kaolin again. Consumption of kaolin will be calculated and compared to baseline to determine relative levels of nausea caused by each treatment.

Nausea will also be measured through conditioned taste aversion (CTA). The rats will first be placed under water depravation for one week and be allowed water for only a limited amount of time a day. After a week rats were given a flavored water solution during the period of water access. Right after the flavored water is taken away the rats are administered either vehicle or drug. This is done for a couple of days followed by resting period. Rats are then given a choice of flavored water and the position of the flavored waters is switched half way through the water period. A preference for the new flavor indicates CTA.1

6.2 Test for IF-8 protection against IF degrading proteases

IF-8 will be screen against more proteases. Cathepsin L is known to degrade IF so the question that will be answered is whether IF will continue to protect B₁₂ and 8 in the presence of cathepsin L.2 I have shown that IF protects 8 from degradation relative to unbound controls in an environment where IF is resistant to the only proteases present. Upon internalization into the ileum IF is degraded by lysosomal protease cathepsin L. Will this environment also damage 8 or will IF offer enough protection to allow a high enough percentage of 8 to enter the blood stream intact.

The first part of this experiment will involve running control to determine if 8 is digested by cathepsin L. Once the level of degradation is known, a protease cocktail will be used to degrade IF-8. The protease mixture will first contain proteases used in the previously published work such as trypsin, chymotrypsin
and meprin β. Additional proteases will need to be tested especially other cathepsins. After establishing the robustness of IF-8 protection other B₁₂-peptide conjugates will be tested for stability. Establishing IF binding as a way to improve protease resistance for a drug will open IF and B₁₂ conjugation to investigation as a potentially new oral delivery technique.

6.3 Continue in vitro and in vivo testing on 11

6.3.1 Screen for activity at NPY1R and NPY2R following the native Gᵢ pathway

An assay has been developed to follow the Gᵢ pathway in NPY2R agonism. The current assay however does not produce a strong enough response to report an accurate dose response curve. HEK-293 cells endogenously express the adenosine receptor, which upon agonism causes an increase in cAMP levels.³ A two injection system was used in preliminary data to first stimulate cAMP production through the Gᵢ pathway, with an adenosine injection, and the second injection was used to shut down cAMP production through the Gᵢ pathway using PYY(3-36). Figure 1 shows the ability of PYY(3-36) to lower cAMP levels and prevent FRET ratio from decreasing further.
**Figure 1.** Adenosine induced cAMP rise to show PYY(3-36)’s ability to work through the Gi pathway and lower cAMP levels.

The raw data is shown as a ratio of 485/535 emission. The total reduction in is calculated by taking the total average FRET ratio change between 360 s and 420 s to determine the relative effectiveness of raising and lowering the cAMP and PKA levels.
Although the current assay to show native G\textsubscript{i} pathway activation after NPY1R and NPY2R agonism does show a decrease in FRET ratio change the assay does not provide enough of a change to calculate a reliable dose response. This assay will have to be improved by either increasing cAMP levels or causing a greater difference in FRET ratio between the adenosine/SES and adenosine/PYY(3-36) samples. This can be done by modifying the assay in one or more of the following ways: optimizing receptor transfection, changing cell line, or changing the reporter.

6.3.2 Determine half-life of 11 in vivo

From chapter 4 I have shown that 11 has improved food intake reduction over Ex-4, but similar blood glucose control. One suspected reason for this is 11 has a longer half-life than Ex-4 causing appetite
suppressing effects to last longer. To test for this an ELISA will be used to test blood sample taken after administration of 11. These samples will be analyzed for total in vivo residency and be used to calculate a half-life for 11.

6.3.3 Modify 11 to improve upon the pharmacokinetic properties

Although 11 has improved weight loss capabilities when compared to Ex-4 it may be able to be further optimized. Additional sequence modifications to 11 could yield a more potent dual agonist or even triagonist. The in vivo effects of 11 are superior to Ex-4 and Ex-4/PYY(3-36) coadministration despite no evidence of NPY2R agonism and only slight agonism at the GIPR, when compared to native GIP. It is possible that GIPR agonism could be optimized through additional modifications. For this to be effective a binding study simulation between 11 and GIPR would need to be done.

Addition of PYY(3-36) amino acid sequence could alter 11 enough to add NPY2R agonism. 14 has a significantly greater portion of PYY(3-36) and work at nanomolar concentration in vitro although showed no food intake reduction in vivo. Glucagon receptor agonism may also be added through an amino acid modification of E3Q. 4

6.3.4 Test 11 for pica and CTA response in rats

11 is similar to Ex-4 in amino acid sequence and glucose control but more potent at appetite suppressing. If this enhanced appetite suppressing ability is due to an increase in brain uptake and increased receptor agonism of GLP-1R in the AN, an increase in nausea may play a role in weight loss. To test for an increase in nausea a pica and CTA test will be run on rats administered 11.
References


Education

**Syracuse University**
Ph.D. Chemistry  
Syracuse, NY  
Expected 2016

**Rochester Institute of Technology**
B.S. Chemistry  
Rochester, NY  
2012

I have been involved in research concerning many aspects of medicinal chemistry. I have been lead on projects that range from oral delivery of peptides to designing and optimizing novel peptides featuring dual agonism and/or biased agonism, primarily at G-coupled protein receptors. My research has focused on protein/peptide conjugation, with my interest lying in the use of the vitamin B12 dietary uptake pathway. My research has allowed me to pursue projects from concept to in vivo outcome (rodent models). My work has resulted in a paper in *Molecular Pharmaceutics* and two additional papers in the final in vivo stages of completion for publication in early 2017. Along with my advisor Professor Robert Doyle, I am co-author of a filed patent describing bi-agonists of both the Glucagon-like peptide-1 receptor and Neuropeptide Y2 receptor, aiming to both control glucose levels and facilitate food intake reduction concomitantly through a peptide pharmaceutical. My goal upon graduating in early summer 2016 is to continue to pursue research and development of in pharmaceuticals.

**Skills**
Designing mosaic peptides, GLP-1 and PYY assay design, G protein coupled receptor assay development for appetite and diabetes peptide drug development, fluorescent and bioluminescence plate assays, mammalian cell culture and cell transfection, plasmid isolation, site specific peptide/protein conjugation, click chemistry, organometallic synthesis, RP-HPLC, FPLC, (affinity, size-exclusion, IEC), MALDI-ToF-MS and ESI-MS, FTIR, 1- and 2-D NMR, western blot, fluorescence spectroscopy.

**Research Experience**

**Graduate Research Assistant:** Syracuse University  
Worked in peptide conjugation, including purification and characterization (Syracuse University; advisor: Prof. Robert P. Doyle) along with cell culture and in vitro testing (SUNY Upstate collaborator, Prof. George G. Holz) and in vivo testing (Seattle Children’s Research Institute; collaborator Prof. Christian L. Roth).

**Undergraduate Research Technician:** Rochester Institute of Technology  
Designing an indicator to be used as a quick field test to check for counterfeit malaria medication in high risk countries.

**Laboratory Technician:** University of Rochester Laboratory for Laser Energetics  
Synthesis and purification of ligands for inorganic dyes.
Awards
Syracuse University Travel Award Summer 2013 – ACS Indianapolis
Fall 2015 – AAPS Orlando

Publications and Patents


- **Bonaccorso R. L.**, Doyle R. P., Coagonists of Glucagon-Like Peptide 1 Receptor and Neuropeptide Y2 Receptor *Patent Pending* SU100846

- **Bonaccorso R. L.**, Clinton E., Chepurny O. G., Holz G. G., Doyle R. P., Intrinsic Factor Binding of a Vitamin B12 Conjugate of Exendin-4 Shows Improved Glycemic Control in Rats *In preparation*

- **Bonaccorso R. L.**, Chepurny O. G., Holz G. G., Doyle R. P., Dual Agonism of the GLP-1 Receptor and NPY2-Receptor Through rational design of a Peptide Coagonist *In preparation*

Presentations and Posters
- **246th ACS National Meeting and Expo**, Indianapolis September 2013; *Poster*
- **Upstate Medical University**, Syracuse, NY, October 3, 2014; *Presentation*
- **Pfizer**, Cambridge, MA, November 21, 2014; *Invited Presentation*
- **ACS 2015 Northeast Regional Meeting**, Ithaca, NY, June 12, 2015; *Presentation*
- **AAPS National Conference Orlando** October 26, 2015; *Poster*
- **NYAS GLP-1 Treatment for Diabetes and Beyond** December 8, 2015; *Poster*

Professional Affiliations
- American Chemical Society (ACS)
- American Association of Pharmaceutical Scientists (AAPS)
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Teaching Assistantships
Organic Chemistry I Lab, Fall 2012
Organic Chemistry II Lab, Spring 2013
General Chemistry II Recitation, Spring 2014
General Chemistry I Recitation, Fall 2014
General Chemistry II Lab, Spring 2015
General Chemistry I Recitation, Fall 2015
General Chemistry II Lab, Spring 2015
Enhanced Peptide Stability Against Protease Digestion Induced by Intrinsic Factor Binding of a Vitamin B₁₂ Conjugate of Exendin-4

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ABSTRACT: Peptide digestion from proteases is a significant limitation in peptide therapeutic development. It has been hypothesized that the dietary pathway of vitamin B₁₂ (B₁₂) may be exploited in this area, but an open question is whether B₁₂-peptide conjugates bound to the B₁₂ gastric uptake protein intrinsic factor (IF) can provide any stability against proteases. Herein, we describe a new conjugate of B₁₂ with the incretin peptide exendin 4 that demonstrates picomolar agonism of the glucagon-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 B₁₂ conjugate to IF, however, resulted in up to a 4-fold greater activity of the B₁₂-Ex-4 conjugate relative to Ex-4, when the IF-B₁₂-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 up to 5 μg/mL of meprin β.

KEYWORDS: vitamin B₁₂, exendin-4, intrinsic factor, trypsin, AKAR3

INTRODUCTION

The human vitamin B₁₂ (B₁₂) 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 primarily protects B₁₂ against acid digestion in the stomach and critical for oral uptake (the third, transcobalamin II (TCII), 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 B₁₂ to Ex-4 and subsequently adding IF, assuming the necessary maintenance of B₁₂ binding by IF and Ex-4 agonism are controlled. To test these hypotheses we

Prebinding the B₁₂ conjugate to IF, however, resulted in up to a 4-fold greater activity of the B₁₂-Ex-4 conjugate relative to Ex-4, when the IF-B₁₂-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 up to 5 μg/mL of meprin β.

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Mol. Pharmaceutics 2015, 12, 3502−3506
synthesized a B12-Ex-4 conjugate focusing on the lysine 12 (K12) position of Ex-4 and the ribose 5′-hydroxyl group of the B12 moiety as sites of conjugation since both sites on the respective moieties had published precedent for allowable modification. 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), B12-Ex-4 (4), and IF-B12-Ex-4 (IF-4). With such establishing parameters controlled for stability against the abundant intestinal endopeptidases, trypsin, chymotrypsin, and the kidney protease meprin β were compared for 1, 4, and IF-4.

## EXPERIMENTAL SECTION

(AzidoK12)-Ex-4 (1) was conjugated to B12 at the K12 position using Huisgens/Sharpless click chemistry, using Ex-4 modified at the lysine 12 ε-amine with an azido group during solid-phase synthesis. The 5′ hydroxyl group of B12 was also modified prior to coupling, being selectively oxidized to a carboxylic acid (2) using 2-iodoxybenzoic acid, as previously described by us. Subsequent coupling of 1-amino-3-butyne to 2 with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDCI) and 1-hydroxybenzo-triazole (HOBt) produced B12 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 B12-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.

## DISCUSSION

Initially, IF binding of 4 was confirmed by radiometric chase assay using 57Co-labeled B12 and compared to free B12, as cyanocobalamin (see Figure 2). Significant IF binding of 4 (6.8 nM) was maintained, albeit reduced from unmodified B12 (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). To this

**Scheme 1. Synthesis of B12-Ex-4 Conjugate 4**

![Scheme 1](image-url)

"Reagents and conditions: (i) EDCI, HOBt, 1-amino-3-butyne, rt, 16 h; (ii) CuSO4, 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."
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.\textsuperscript{26}–\textsuperscript{28} This is the first instance to our knowledge of a FRET assay for the GLP-1R using viral AKAR3 and offers a ready route to sensitive high-throughput screening of the GLP-1R. An EC\textsubscript{50} for \textit{I}, 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 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.\textsuperscript{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 nature of B\textsubscript{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 \textmu g/mL, chymotrypsin at 1.25, 3, or 6.25 \textmu g/mL, or meprin \beta at 1 or 5 \textmu g/mL (see Figures 4 and S2).

At the lowest concentrations of trypsin (11 \textmu g/mL) and chymotrypsin (1.25 \textmu g/mL) there is up to 50% greater function for IF-4 relative to 4 alone with the highest concentration of trypsin (50 \textmu g/mL) and chymotrypsin (6.25 \textmu g/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 \beta digestion revealed a 2-fold increase in function with B\textsubscript{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 \textmu g/mL. The protection provided from B\textsubscript{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\textsubscript{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 \textmu g/mL and 3 \textmu g/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 \beta showed the most significant protection when comparing 1 and IF-4. No reduction in function was seen at the highest concentration of meprin \beta tested (5 \textmu g/mL), while 1 showed no function at concentrations greater than 1 \textmu g/mL of meprin \beta. B\textsubscript{12} provided some protection against trypsin relative to the native peptide. The effect is seen at 11 \textmu g/mL of trypsin with a relative 4-fold increase and at 1.25 and 3 \textmu g/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 PYY\textsubscript{3-36}), 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 \beta, there is no suggestion that this approach is limited to oral use against

![Figure 2](image1.png)  
Figure 2. IF binding to B\textsubscript{12} (0.12 nM) and 4 (6.8 nM). IF used in these assays was produced in the plant \textit{Arabidopsis} in the apo-form and of high purity.\textsuperscript{24}

![Figure 3](image2.png)  
Figure 3. Dose–response analysis of 1, 4, and IF-4 yielded EC\textsubscript{50} values of 26, 68, and 132 pM, respectively, as determined by monitoring the 485/535 nm FRET emission ratio.

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

<table>
<thead>
<tr>
<th>compd</th>
<th>% change in FRET at 100 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−12 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>−13 ± 0.02</td>
</tr>
<tr>
<td>IF-4</td>
<td>−12 ± 0.01</td>
</tr>
</tbody>
</table>
gastric proteases, but could also be expanded into serum (through subcutaneous or intravenous injection of IF bound B12-peptide conjugates, for instance), facilitating greatly improved pharmacokinetics (especially when combined with prior results showing B12 conjugation already improved sc absorption of a PYY3-36 conjugate⁸), 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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Solution Structure and Constrained Molecular Dynamics Study of Vitamin B₁₂ Conjugates of the Anorectic Peptide PYY(3–36)


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.

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 enteroneuroendocrine 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,[15, 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) G₃₉-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 protein.[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 G₃₉ 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 proteins to lower levels of intracellular cyclic adenosine monophosphate (cAMP).[25] 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 K<sub>PYY</sub>, showing both conjugates are less active than K<sub>PYY</sub> and that 4 is more active than 3. Both conjugates are, however, within one-half log order of K<sub>PYY</sub>.

This difference in EC<sub>50</sub> values in comparing conjugates with K<sub>PYY</sub> 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 B<sub>12</sub>. 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 a 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 Hx 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.

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.
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. If the
The C-terminal pentapeptide plays a role in NPY to undergo remarkable structural changes within the second extracellular loop by hydrophobic contacts and extensive and susceptible interactions in NPY; a network that is critical for Y2 receptor binding and activation. 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 the control for all assays. Characterization of the alkyne precursor 1 and 2, including RP-HPLC, MALDI-ToF MS, and NMR can be found in the Supporting Information (Figure S13–16).

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. 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 with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazole (HOBr) 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 was implemented using a copper iodide (Cul) and tris[(1-benzyl-1H-1,2,3-triazol-4-ylimethyl)amine (TBTA) method, adapted from Gryko et al. Copper(I)-catalyzed alkyne–azole cycloaddition (CuAAC) synthesis of conjugates 3 and 4 via the alkyne precursors 1 and 2 and a Kp-azido PYY(3–36) (KpPYY) is described in Scheme 2 (spacer length n = 2, 3 for precursors 1 and 2). KpPYY was initially tested against PYY(3–36) amide (Sigma–Aldrich), and there was no observed difference in Y2-R agonism. Subsequently, KpPYY 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<sub>i</sub>: 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). 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 EC<sub>50</sub> 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 K<sub>4</sub>PYY 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

Scheme 1. Synthesis of B<sub>12</sub>–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 B<sub>12</sub>–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 B<sub>12</sub> and the triazole linkage to K<sub>4</sub>PYY. Reagents and conditions: Cu(1); TBTA; reactions were carried out for 16 h at RT. PYY(3–36) adapted from PDB ID: 2DF0.

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.

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)\[10\] software package. The NMR distance lists, ranges, and tabulated distances from the time-averaged MD structure for 3 are provided in the Supporting Information.

**Acknowledgements**

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**Keywords:** GPCR · molecular dynamics · NMR spectroscopy · PY(3–36) · vitamin B12


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BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

[0001] The present invention relates to peptides for treating weight loss and glucose levels and, more specifically, to a single peptide that can trigger weight loss while controlling glucose levels.

2. DESCRIPTION OF THE RELATED ART

[0002] The glucagon-like peptide 1 receptor (GLP-1R) is involved in stimulating the release of insulin in a glucose dependent manner. As a result, GLP-1R agonists have been the source of development for drugs that can be used to treat insulin deficiency diseases such as diabetes. The neuropeptide Y2 receptor (NPYR2) is involved in appetite signaling and suppression. Accordingly, there is a need in the art for a drug that could serve as an agonist of both receptors, thereby more fully addressing the problems associated with glucose regulation and food intake.

[0003] BRIEF SUMMARY OF THE INVENTION

[0004] The present invention comprises peptide sequences that can serve as agonists of both the glucagon-like peptide 1 receptor (GLP-1R) and the neuropeptide Y2 receptor (NPYR2). In particular, the invention comprises certain non-naturally occurring peptides that can successfully serve as agonists for both receptors. The present invention may also be used as a coagonist at other receptors, such as the NPYR1 or NPYR4 or NPYR5, and not necessarily exclusively GLP-1R and NPYR2.
BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

[0005] The present invention will be more fully understood and appreciated by reading the following Detailed Description in conjunction with the accompanying drawings, in which:

[0006] FIGS. 1A and 1B are graphs of the results of agonism screening for certain peptides according to the present invention;

[0007] FIGS. 2A and 2B are graphs of the results of agonism screening for certain peptides according to the present invention;

[0008] Fig. 3 is a chart of GLP-1R agonism screening for certain peptides at a concentration of 100nM;

[0009] Fig. 4 is a chart of NPYR2 agonism screening for certain peptides at a concentration of 100nM;

[0010] Fig. 5 is a graph of GLP-1R agonism of an exemplary peptide according to the present invention;

[0011] Fig. 6 is a graph of NPYR2 agonism of an exemplary peptide according to the present invention;

[0012] Fig. 7 shows the comparison of Ex-4 and PYY and highlights the critical components of each; and

[0013] Fig. 8 shows potential variations of RLB001 that may have comparable or better function at both the GLP-1R and NPYR2.

DETAILED DESCRIPTION OF THE INVENTION

[0014] Referring now to the drawings, wherein like reference numerals refer to like parts throughout, the present invention comprises peptide sequences that are coagonists of GLP-1R and NPYR2. The sequences of the present invention were designed by combining certain
aspects of the natural substrates or known agonists of GLP-1R and NPYR2., such as Peptide YY and exendin-4.

[0015] Following the approach of the present invention, the sequences

HGEGTFTSDLKQMEEEAVRLFIEWLKNGGPSSTRQRY-NH2 (SEQ. ID NO. 1), referred to herein as Peptide RLB001, HGEGTFTSDLKQMEEEAVRLFIEWLRHYLNVLVTRQRY-NH2 (SEQ. ID NO. 2), referred to herein as Peptide RLB002, and

IKPEAPREDASPEEENQAYKEFIAYLNVLVTRQRY-NH2 (SEQ. ID. NO. 3), referred to herein as Peptide RLB003 were designed and synthesized through solid phase peptide synthesis.

[0016] Referring to FIGS. 1A and 1B, the sequences were screened for agonism with respect to each of GLP-1R and NPYR2 by performing a either a Fluorescence Resonance Energy Transfer (FRET) assay to show GLP-1R agonism or following the fluorescence of FURA 2 upon calcium release to show NPYR2 agonism and plotting the change in either FRET or fluorescence over a predetermined time period. These assays have been used previously to show function at each receptor respectively. The peptides synthesized according to the present invention were compared against a basal measurement, exendin-4 (a known agonist of GLP-1R), Peptide YY (a known agonist of NPY2R), and ionomycin.

[0017] Referring to FIGS. 2A and 2B, Peptide RLB001 was screened for agonism with respect to each of GLP-1R and NPYR2, as well as the controls discussed above. Referring to FIG. 3, screening of the peptides for GLP-1R agonism revealed that RLB001 and RLB002 were effective agonists. Referring to FIG. 4, screening of the peptides for NPYR2 agonism revealed that RLB001 was even more effective that known agonists of NPY2R, such as PYY. As seen in FIG. 5, Peptide RLB001 done in triplicate has an EC_{50} of 50 pM at GLP-1R. As seen in FIG. 6, Peptide RLB001 done in duplicate has an EC_{50} of 81 nM at NPYR2. The relevant portions of
PYY and exendin-4 used as a basis for forming the sequences according to the present invention are seen in FIG. 7. Referring to FIG. 8, SEQ ID NO. 4 through SEQ ID NO. 12 represent variations of RLB001 that may have comparable or better function at both the GLP-1R and NPYR2 and may be readily tested as described above to confirm their efficacy. The present invention may also be used as a coagonist at other receptors, such as the NPYR1 or NPYR4 or NPYR5, and not necessarily exclusively GLP-1R and NPYR2.

These results demonstrate that the peptide RLB001 has comparable function at the GLP-1R and the NPYR2. A single peptide may thus be used to activate two receptors responsible for glucose control and appetite suppression. In addition, the unique profile of calcium release after activation at the NPYR2 may indicate biased agonism.

Sequences according to the present invention may be further modified according to known processes, such as glycosylation, to improve the use of the sequences as pharmaceuticals by assisting with the delivery of the protein to a subject. For example, N-linked glycosylation may be used to attach oligosaccharides to a nitrogen atom, such as the N4 of asparagine residues. Similarly, O-linked glycosylation may be used to attach glycans to serine and threonine and C-linked glycosylation used for the covalent attachment of a mannose residue to a tryptophan residue.

Sequences according to the present invention may be further modified by pegylation, which is the attachment of a therapeutic protein to poly(ethylene glycol) polymer chains (PEG). The attachment of poly(ethylene glycol) chains can prevent degradation by proteolytic enzymes, reduce rapid clearance of the sequences by the kidneys, and increase the circulating half-life. As is known in the art, PEG is linked to a protein sequence through reactive molecular groups on amino acid side chains such as lysine.
Sequences according to the present invention may be further modified by lipidation. The presence of a lipid group in peptides modulates their hydrophobicity, secondary structures and self-assembling propensities while retaining their abilities to bind to target receptors. Lipidation improves metabolic stability, membrane permeability, bioavailability, and changes pharmacokinetic and pharmacodynamic properties of peptides.

The sequences may also be encapsulated in a suitable vehicle to either aid in the delivery of the compound to target cells, to increase the stability of the composition, or to minimize potential toxicity of the composition. For example, nanoparticles, liposomes, microemulsions, micelles, dendrimers and other phospholipid-containing systems may be used as is known in the art.

Sequences according to the present invention may further be conjugated to known conjugation partners to assist in the use of the sequences as a pharmaceutical. For example, conjugation partners such as an organic drug molecule, an enzyme label, a toxin, a cytostatic agent, a label which can be photoactivated and which is suitable in photodynamic therapy, a pharmaceutically suitable radioactive label, a hapten, digoxigenin, biotin, a chemotherapeutic metal complex or metal, colloidal gold, or a moiety that extends the serum half-life may be used to assist with the delivery of the present invention.

The present invention includes pharmaceutical compositions comprising a preparation of the sequences of the invention. Such pharmaceutical compositions may be for administration for injection, or for oral, nasal, transdermal or other forms of administration, including, e.g., by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, intraocular, retrobulbar, intrapulmonary (e.g., aerosolized drugs) or subcutaneous injection (including depot administration for long term release); by sublingual, anal, vaginal, or
by surgical implantation. The treatment may consist of a single dose or a plurality of doses over a period of time. In general, pharmaceutical compositions comprising effective amounts of a sequence according to the invention, whether modified as described above or not, together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content, pH and ionic strength; additives such as detergents and solubilizing agents, anti-oxidants, preservatives and bulking substances. The pharmaceutical compositions optionally may include still other pharmaceutically acceptable liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. Implantable sustained release formulations are also contemplated, as are transdermal formulations.
WHAT IS CLAIMED IS:

1. A composition for simultaneously treating obesity and insulin deficiency, comprising a peptide sequence having a first portion that corresponds to a selected portion of the sequence of an agonist of the glucagon-like peptide 1 receptor (GLP-1R) and a second portion that corresponds to a selected portion of the sequence of an agonist of the neuropeptide Y2 receptor (NPYR2).

2. The composition of claim 1, wherein the sequence comprises HEGTFTSDLSKQMEEEAVRLFIEWLKNNGPSSTRQRY-NH2 (SEQ. ID NO. 1).

3. A method of simultaneously treating obesity and insulin deficiency, comprising the steps of administering a peptide sequence having a first portion that corresponds to a selected portion of the sequence of an agonist of the glucagon-like peptide 1 receptor (GLP-1R) and a second portion that corresponds to a selected portion of the sequence of an agonist of the neuropeptide Y2 receptor (NPYR2).

4. The method of claim 1, wherein the sequence comprises HEGTFTSDLSKQMEEEAVRLFIEWLKNNGPSSTRQRY-NH2 (SEQ. ID NO. 1).
ABSTRACT

Peptide sequences that can serve as agonists of both the glucagon-like peptide 1 receptor (GLP-1R) and the neuropeptide Y2 receptor (NPYR2). The peptide sequences include regions that correspond to certain aspect of natural substrates and known agonists of the glucagon-like peptide 1 receptor (GLP-1R) and the neuropeptide Y2 receptor (NPYR2) in a single sequence.
FIGS. 1A and 1B
FIGS. 2A and 2B
FIG. 3

GLP-1R Screen at 100 nM

%Δ A 485/535 FRET Ratio

Basal Ex-4 PYY RLB001 RLB002 RLB003
FIG. 4

NPY2R Screen at 300 nM

Δ FURA2 Response

Basal  PYY  Ex-4  Ionomycin  RLB001  RLB002  RLB003
Dose Response Curve of RLB001 at GLP-1R

FIG. 5
Dose Response Curve of RLB001 at NPYR2

FIG. 6
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<th>RLB001</th>
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**FIG. 7**
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<tr>
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<tr>
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</tbody>
</table>

Fig. 8
Vita

Ron Bonaccorso was born in Gainsville, FL in August of 1990. He grew up in Rochester, NY and then completed undergraduate work at Rochester Institute of Technology in 2012. In 2016 he completed his PhD in Chemistry at Syracuse University.