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A Vitamin B12 Conjugate of Exendin-4 Improves Glucose Tolerance Without Associated Nausea or Hypophagia in Rodents

Elizabeth G. Mietlicki-Baase^{1,*}, Claudia G. Liberini^{1,*}, Jayme L. Worker^{2,*}, Ron L. Bonaccorso², Tito Borner³, David J. Reiner¹, Kieran Koch-Laskowski¹, Lauren E. McGrath¹, Rinzin Lhamo¹, Lauren M. Stein¹, Bart C. De Jonghe³, George G. Holz⁴, Christian L. Roth⁵, Robert P. Doyle^{2,4,&,#}, and Matthew R. Hayes^{1,3,&,#}

¹Department of Psychiatry, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

²Department of Chemistry, Syracuse University, Syracuse, NY 13244

³Department of Biobehavioral Health Sciences, School of Nursing, University of Pennsylvania, Philadelphia, PA 19104

⁴Department of Medicine, State University of New York, Upstate Medical University, Syracuse, New York 13210

⁵Center for Integrative Brain Research, Seattle Children's Research Institute, Division of Endocrinology, Department of Pediatrics, University of Washington, Seattle, WA

Abstract

Aims—While pharmacological glucagon-like peptide-1 receptor (GLP-1R) agonists are FDA-approved for treating type 2 diabetes mellitus (T2DM) and obesity, a major side effect is nausea/malaise. We recently developed a conjugate of vitamin B12 bound to the GLP-1R agonist exendin-4 (Ex4), which displays enhanced proteolytic stability and retention of GLP-1R agonism. Here, we evaluate whether the conjugate (B12-Ex4) can improve glucose tolerance without producing anorexia and malaise.

Materials and Methods—We evaluated the effects of systemic B12-Ex4 and unconjugated Ex4 on food intake and body weight change, oral glucose tolerance, and nausea/malaise in male rats, and on intraperitoneal glucose tolerance in mice. To evaluate whether differences in the profile of effects of B12-Ex4 versus unconjugated Ex4 are due to altered CNS penetrance, rats received systemic injections of fluorescein-Ex4 (Flex), Cy5-B12 or Cy5-B12-Ex4 and brain penetrance was

#Address correspondence to: Dr. Matthew R. Hayes, University of Pennsylvania, 125 South 31st St., Philadelphia, PA 19104, 215-573-6070, hayesmr@mail.med.upenn.edu; Dr. Robert P. Doyle, Syracuse University, 111 College Place, Syracuse, NY 13244, 315-443-3584, rpdoyle@syr.edu.

*These authors are considered to have co-first authorship

&These authors are considered to have co-senior authorship

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evaluated using confocal microscopy. Uptake of systemically administered Cy5-B12-Ex4 in insulin-containing pancreatic beta cells was also examined.

Results—B12-Ex4 conjugate improves glucose tolerance, but does not elicit the malaise and anorexia produced by unconjugated Ex4. While Flex robustly penetrates into the brain (dorsal vagal complex, paraventricular hypothalamus), Cy5-B12 and Cy5-B12-Ex4 fluorescence were not observed centrally, supporting a lack of CNS penetrance in line with observed reduction in CNS-associated Ex4 side effects. Cy5-B12-Ex4 colocalizes with insulin in the pancreas, suggesting direct pancreatic action as a potential mechanism underlying the hypoglycemic effects of B12-Ex4.

Conclusions—These novel findings highlight the potential clinical utility of B12-Ex4 conjugates as possible future T2DM therapeutics with reduced incidence of adverse effects.

Introduction

Multiple incretin-based therapeutics are approved for the treatment of type 2 diabetes mellitus (T2DM) due to their ability to elicit pancreatic insulin secretion and reduce blood glucose levels. These pharmacotherapies include compounds designed to increase endogenous concentrations of the incretin hormone glucagon-like peptide-1 (GLP-1) by inhibiting the endopeptidase DPP-IV, as well as synthetic peptide-based GLP-1 receptor (GLP-1R) agonists resistant to DPP-IV degradation¹. In addition to being the more potent class of GLP-1-based therapeutics for reducing glycemia, GLP-1R agonists significantly reduce food intake and body weight in both humans and animal models^{1–3}. This anorectic effect is attractive when considering the utilization of GLP-1R agonists as an on- or off-label treatment option for obesity, and indeed, the GLP-1R agonist liraglutide is FDA-approved for the treatment of obesity⁴. However, a sizeable percentage of individuals with T2DM do not have obesity or overweight^{5–7} and may want to avoid weight loss. Furthermore, it is important to note that the hypophagic effects of all GLP-1R agonists on the market are accompanied by pronounced incidence of nausea, vomiting, and malaise^{3,8,9}. In fact, ~20–50% of T2DM patients prescribed GLP-1-based medication experience nausea and/or vomiting, leading to discontinuation of drug treatment in ~6–10% and reduced dose tolerance in another ~15%^{10–14}. These adverse effects are surprisingly under-investigated, as they limit the widespread use, efficacy, and potential ubiquitous utility of GLP-1R agonists (e.g. liraglutide, exenatide) for the treatment of metabolic disease.

A wealth of literature provides convincing evidence that a significant portion of the increase in glucose-stimulated insulin secretion following exogenous GLP-1R ligand administration is mediated by direct activation of GLP-1R expressed on pancreatic β -cells [see^{1–3,15} for review], mimicking the paracrine effects of pancreatic-derived GLP-1 that may not occur with endogenous L-cell-derived GLP-1^{16–18}. Importantly, activation of GLP-1R expressed on vagal afferent fibers and/or in discrete nuclei in the central nervous system (CNS) also contributes to exogenous GLP-1R agonist-mediated improvements in glycemic control^{1–3,15,19–21}. Interestingly, these dual sites of action (i.e. vagal and direct CNS activation) also mediate the intake- and body weight-suppressive effects of exogenous systemic GLP-1R agonist administration^{21–23}. Both liraglutide and exenatide can penetrate into the CNS and activate central GLP-1R-expressing nuclei leading to hypophagia and

weight loss^{22,24}. Remarkably, however, GLP-1R agonist-induced illness behaviors (e.g. nausea, conditioned taste avoidance, hypophagia, emesis) are mediated by GLP-1Rs expressed within the CNS and not by vagal afferent GLP-1Rs when these compounds are delivered systemically⁸. Although other T2DM medications (e.g., DPP-IV inhibitors) may improve glycemic control with minimal effects on energy balance^{25,26}, long-lasting GLP-1R agonists provide several advantages such as improved glycemic control and less frequent administration^{27–29}. Thus, from a therapeutic standpoint, designing a novel GLP-1R agonist that is resistant to DPP-IV degradation, does not penetrate readily into the CNS, but retains the enhanced pharmacokinetic and pharmacodynamic profile of such agonists on pancreatic β -cells would theoretically provide a new, improved pharmacological tool for glycemic control in T2DM patients without eliciting unwanted hypophagia and nausea.

Recently, we demonstrated that covalent conjugation of the GLP-1R agonist exendin-4 (Ex4) to vitamin B12 (Cyanocobalamin; B12) between the vitamin 5'-OH group and the K12 position of Ex4 retains picomolar agonism (68 pM)³⁰ of the GLP-1R, either as the free conjugate (B12-Ex4 - used in this study; see also Figure 1) or bound to Intrinsic Factor (IF; 126 pM)³⁰, a B12 transport protein critical for B12 absorption in humans. This work also confirmed that IF bound B12-Ex4 with low nanomolar affinity (as occurs with cyanocobalamin)³⁰.

Interestingly, while Ex4 readily penetrates the CNS^{22,31}, little is known about the penetrance of B12 in the brain. Uptake of B12 into the brain is putatively a receptor-mediated process with megalin (a receptor capable of TCII-B12 uptake in the kidney, for example) being expressed in the choroid plexus^{32,33}. Additional evidence points to the importance of the CD320 receptor, as genetic ablation in mice results in severe cobalamin deficiency in the mouse brain^{34,35}, as well as the transmembrane protein amnionless, natural mutations of which result in Imerslund-Gräsbeck syndrome and congenital cobalamin malabsorption³⁶. Collectively, this information points to a receptor-mediated process of B12 blood-brain barrier penetrance, but little is known of where B12 is transported in the brain, and to what extent³⁷, relative to total concentrations. It is evident that CNS uptake is considerably lower compared to other organs, especially the liver and kidney^{38,39}, with one recent study, using a B12-⁸⁹Zr PET probe, revealing less than 0.1% injected dose per gram (ID/g) in brain in rat models with over 5% ID/g observed in pancreas⁴⁰. We therefore hypothesized that a B12-Ex4 conjugate would retain the incretin profile of a GLP-1R agonist to improve glucose tolerance, but prevent development of nausea/malaise through reduced, or redirected, CNS/hypothalamic penetration of the agonist.

The rat is a unique animal model for pre-clinical testing of the B12-Ex4 conjugate. Rats, unlike humans and mice, demonstrate an unexpected hyperglycemic response to systemic Ex4 delivery. This hyperglycemic effect is unique to the Ex4 molecule (among approved GLP-1R agonists) in the rat and is due in part to CNS-mediated sympathetic activation⁴¹. Further, rats show well-documented hypophagic effects to GLP-1R ligands mediated partly by accompanying acute effects on nausea/malaise, similar to humans, but not to mice^{8,9}. Rats were therefore used as the primary model to evaluate the effects of B12-Ex4 on glycemic control, energy balance, and nausea/malaise, and these effects were compared with the response profile after peripheral administration of unconjugated Ex4. Given that Ex4

produces hypoglycemic effects in mice^{42,43}, similar to the effect observed in humans^{44,45}, blood glucose levels in mice were also assessed in a glucose tolerance test (GTT) upon B12-Ex4 or Ex4 administration. The data presented here provide evidence for second-generation “cobalaminylated” GLP-1R agonists for the treatment of T2DM, with a pronounced profile of effects that include glucoregulation without anorexia or body weight loss, and most critically an absence of nausea/malaise.

Materials and Methods

Animals

Adult male Sprague Dawley rats (Charles River) were singly housed in hanging wire mesh cages. Four-month old C57BL/6J mice (Jackson Laboratory) were singly housed in plastic cages. All animals were housed under a 12h:12h light/dark cycle in a temperature- and humidity-controlled environment. Standard rodent chow (Purina 5001) and tap water were available *ad libitum* except where noted. Procedures were approved by the Institutional Care and Use Committee of the University of Pennsylvania.

Compounds

B12-conjugated exendin-4 (B12-Ex4) was produced, characterized and screened for agonism at the GLP-1R (EC₅₀ of 68 pM, relative to 28 pM for Ex4 in the same assay), as previously described³⁰. One addition to the characterization was the measure of TCII binding of B12-Ex4 (Figure 1D), which was conducted at the Department of Clinical Medicine-Clinical Biochemistry, University of Aarhus, Denmark as described⁴⁶. B12-Ex4, Ex4 (Bachem), and lithium chloride (LiCl; Sigma Aldrich) were dissolved in sterile 0.9% NaCl for peripheral injections. Injections were separated by at least 48h. For most *in vivo* experiments, injections were administered using a within-subjects, Latin square design. The exception was the conditioned taste avoidance (CTA) study, which used a between-subjects design.

Effects of B12-Ex4 on energy balance

Shortly before the onset of the dark phase, rats (n=12) were given subcutaneous (SC) injection of B12-Ex4 (1, 5, or 20µg/kg) or vehicle (1ml/kg sterile saline). Chow intake was measured at 1, 3, 6, and 24h post-injection. Food spillage was accounted for in all intake measurements. Body weight was also measured at 0 and 24h.

Effects of B12-Ex4 on glycemic control during an oral glucose tolerance test (OGTT)

Rats (n=12) were deprived of food overnight before testing. On the morning of testing, just after the onset of the dark phase, water was also removed from the cage. A small drop of blood was collected from the tail tip and analyzed for blood glucose (BG) level using a standard glucometer (AccuCheck). Immediately after this baseline BG reading (t=-30min), each rat received SC injection of B12-Ex4 (5 or 20µg/kg) or vehicle (1ml/kg sterile saline); doses of drug were selected based on the results of the feeding study. Thirty minutes later (t=0min), BG was measured and each rat received an oral gavage of glucose (2g/kg). Subsequent BG readings were taken at 20, 40, 60, and 120min after glucose gavage. After the final BG reading, food and water were returned.

Effects of systemic Ex4 on glycemic control and energy balance

The effects of unconjugated Ex4 were evaluated in an OGTT, using methods similar to those described above, with two major differences: SC injections were Ex4 (5 or 20 μ g/kg) or vehicle (1ml/kg sterile saline), and food intake and body weight change after the completion of the OGTT were monitored. Pre-weighed food was returned to the rats after the OGTT, and chow intake was measured for ~21.5h (e.g., until 24h after the SC injections). Spillage was accounted for in food intake measurements. Body weight was recorded at 0 and 24h. For the OGTT, n=10 rats were tested; food and body weight data were collected from n=9 due to a technical error in food intake measurement.

Effects of B12-Ex4 on expression of a conditioned taste avoidance (CTA)

Rats (n=8-10 per drug) were evaluated for expression of a CTA to a flavor paired with B12-Ex4 (5 μ g/kg, IP). Ex4 (5 μ g/kg, IP) and LiCl (0.15M) were used as positive controls. A two-bottle test was used so each rat had access to a flavor that had been paired previously with vehicle (1 ml/kg saline, IP) and a flavor that had been paired previously with drug (B12-Ex4, Ex4, or LiCl). See Supplemental Methods for more details.

Effect of B12-Ex4 on glycemic control in mice during an intraperitoneal glucose tolerance test (IPGTT)

The experimental procedure for IPGTT in mice was similar to that used for OGTT in rats. Briefly, mice (n=13; 8 females, 5 males) were food and water deprived for 4h before and during the IPGTT. Testing was completed mid-light phase. Blood was collected from the tail tip and analyzed for BG. Immediately after this baseline reading (t=-30min), each mouse received IP injection of Ex4 (5 μ g/kg), B12-Ex4 (equimolar dose to Ex4), or saline (10 μ l/g). Thirty minutes later (t=0min), BG was measured and each mouse received an IP injection of glucose (2g/kg). Subsequent BG readings were taken at 20, 40, 60, and 120min after glucose injection. After the final BG reading, food and water were returned. Area under the curve (AUC) was calculated from 0-120 min (e.g., beginning at the time of glucose administration) using the trapezoidal method.

B12-Exendin-4-Cyanine-5 (Cy5-B12-Ex4) synthesis

B12-Ex4 was synthesized as previously described³⁰. B12-Ex4 (0.5mg, 0.0001mmol) was dissolved in PBS buffer pH 7.6 (450 μ L) and sulfo-cyanine5-NHS-ester (1mg, 0.001mmol) (Lumiprobe) was added (in 50 μ L DMSO). The resulting solution was allowed to mix for 2h at room temperature, protected from light, and then purified through RP-HPLC on a Shimadzu Prominence HPLC using a C18 column (Eclipse XDB-C18 5 μ m, 4.6 $\times$ 150mm). Solvents: A: 0.1% TFA water and B: Acetonitrile. Method: B%: 1-70% over 15min. t_R : 12.1min. Yield: 98%. Emission and excitation were 648 and 670nm, respectively using a Varian Cary UV Spectrophotometer and Agilent Cary Eclipse Fluorescence Spectrophotometer, solvent H₂O/MeCN. LC-MS analysis (Shimadzu LCMS-8040, Method: 0.1% formic acid and 35% methanol water at 0.2mL/min, DL temp: 150°C, heat block temp: 400°C.): expected m/z: 6923 [B12-Ex4-(Cy5)₂], observed: 1383 [M+5H]⁺, 1728 [M+4H]⁺. See Figure 1 for more information.

B12-Cyanine-5 (Cy5-B12) synthesis

Cy5-B12 was synthesized through Huisgen/Sharpless 'Click' Chemistry^{47,48}. Cu(I) (1mg, 0.005mmol) and Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (3.5mg, 0.006mmol) were dissolved in 0.5mL DMF/H₂O (4:1 v/v). Once color change occurred, the previously synthesized B12-Azide (3mg, 0.002mmol)⁴⁹ and Cyanine-5 alkyne (0.5mg, 0.0007mmol) (Lumiprobe) was dissolved in the solution and allowed to stir at room temperature overnight protected from light. This was purified through RP-HPLC on a Shimadzu Prominence HPLC using a C18 column (Eclipse XDB-C18 5 μ m, 4.6 $\times$ 150mm). Solvents: A: 0.1% TFA water and B: Acetonitrile. Method: B%: 20-72% over 18min. t_R : 4.7min. Yield: 94%. LC-MS analysis (Shimadzu LCMS-8040, Method: 0.1% Formic acid and 35% methanol water at 0.2mL/min, DL temp: 150°C, heat block temp: 400°C.): expected m/z: 2059 observed: 1031 [M+2H]⁺, 1042 [M+Na+2H]⁺, and 1050 [M+K+2H]⁺. Emission and excitation were 645 and 682nm, respectively using a Varian Cary UV Spectrophotometer and Agilent Cary Eclipse Fluorescence Spectrophotometer, solvent H₂O. See Figure 1 for more information.

GLP-1 assay for Cy5-B12-Ex4

Agonism at the GLP-1 receptor was monitored using HEK-293 cells stably transfected with the GLP-1 receptor cultured in DMEM with 10% FBS, 1% pen/strep, and 250 μ g/mL geneticin/g-418. Cells were plated on a rat-tail-collagen-coated 96-well plate at 60,000 cell/well and allowed to adhere overnight. The cells were infected with an adenovirus to express the H188 FRET reporter using a 25 MOI for 16-20h in 75 μ L of DMEM-1% FBS. After viral incubation, the cells were placed in 200 μ L standard extracellular matrix with glucose and 0.1% BSA. Conjugates were added to each well at 5 $\times$ the required concentration. Agonism was determined through an increase in 485/553nm FRET ratio indicative of an increase in cAMP level through cAMP binding to an EPAC (exchange protein directly activated by cAMP)⁵⁰.

Immunohistochemical procedures and confocal imaging

Rats (n=4/group) were given IP injection of fluorophore-labeled Ex-4 (Flex; 5 μ g/kg; 0.0001nM; AnaSpec⁵¹), Cy5-B12-Ex4 (5 μ g/kg; 0.03nM), Cy5-B12 (5 μ g/kg), or Cy5-B12-Ex4 delivered at an equimolar dose to Flex (0.0001nM). Rats were transcardially perfused 3h after injection, using 0.1M PBS followed by 4% paraformaldehyde (PFA). Brains were collected and sections from the area postrema and hypothalamus were processed via immunohistochemistry for NeuN and GFAP, mounted, and coverslipped with DAPI mounting medium. Sections were visualized via confocal microscopy. See Supplemental Methods for more detail.

To evaluate the penetrance of B12-Ex4 in the pancreas, rats (n=2) were given IP injection of Cy5-B12-Ex4 (5 μ g/kg) and transcardially perfused 3h later with 4% paraformaldehyde in PBS. Pancreases were collected and sagittally sectioned, processed via immunohistochemistry for insulin, and coverslipped with DAPI mounting medium. Sections were visualized with confocal microscopy and three-dimensional rotational animations were rendered from the collected z-stack images using Imaris 8.1.2 (Bitplane). See Supplemental Methods for more information.

Statistical analyses

See Supplemental Methods.

Results

B12-Ex4 has potent beneficial effects on glycemic control, but minimal impact on feeding and body weight in rats

Ex4 and other GLP-1R agonists reduce blood glucose levels, and are used clinically to treat T2DM⁵². In addition, the food intake- and body weight-suppressive effects of GLP-1R agonists have highlighted the utility of these pharmacotherapies for the treatment of obesity⁵³. To evaluate whether the metabolic effects of B12-Ex4 are similar to those of other GLP-1R agonists such as unconjugated Ex4, the effects of SC injection of B12-Ex4 on energy balance and glycemic control were evaluated. To confirm TCII binding of B12 in its Ex4 conjugated form (i.e. B12-Ex4), a radio chase assay using⁵⁷ Co-labelled B12 was conducted⁴⁶ and confirmed low nanomolar binding (~ 75nM) was maintained (Figure 1D).

First, to test whether B12-Ex4 has similar intake- and body weight-suppressive effects as Ex4, rats were given SC injection of B12-Ex4 (0, 1, 5, or 20µg/kg in 1ml/kg sterile saline), and subsequent food intake (1, 3, 6, 24h) and body weight gain were measured. The highest dose of B12-Ex4, 20µg/kg, significantly suppressed food intake at 3h and 6h post-injection (Figure 2A; drug × time interaction, $F_{9,99}=3.69$, $p<0.001$; 0µg/kg versus 20µg/kg, $p<0.05$ at 3h and 6h). No other significant effects on food intake were observed at other times or by other doses of drug (all other $p>0.05$). There was also no significant effect of B12-Ex4 on 24h body weight change (Figure 2B; $F_{3,33}=0.50$, $p=0.69$), which is consistent with the fact that cumulative 24h energy intake was similar among the treatment conditions.

Next, the glycemic effects of B12-Ex4 (5 or 20µg/kg) or vehicle (1ml/kg sterile saline, SC) were evaluated via OGTT. B12-Ex4 significantly reduced blood glucose levels in the OGTT (Figure 2C; main effect of drug, $F_{2,22}=4.01$, $p<0.04$; drug × time interaction, $F_{10,110}=17.29$, $p<0.000001$). Posthoc analyses showed that both doses of B12-Ex4 significantly suppressed BG at 20 and 40min after glucose gavage (versus vehicle, all $p<0.05$). A dose-responsive effect is also suggested by the finding that 20µg/kg B12-Ex4 had more potent BG-suppressive effects than 5µg/kg at 20min after glucose gavage ($p<0.05$). Interestingly, BG levels were increased by both doses of B12-Ex4 at 60min and by the higher dose at 120min (all $p<0.05$). Importantly, injection of B12-Ex4 had no effect on blood glucose levels on its own ($t=0$, all $p>0.05$).

Systemic injection of Ex4 produces hyperglycemia, hypophagia, and weight loss

The rat is a particularly interesting model to test the effects of an Ex4-based drug on glycemic and energy balance control, because rats exhibit a hyperglycemic response to acute peripheral administration of Ex4 due to sympathetic activation⁴¹, but also show pronounced reductions in feeding and body weight gain^{54–56}. To evaluate the effects of SC administration of unconjugated Ex4 on these measures, and to be able to more directly compare the effects of B12-Ex4 to those of Ex4, an OGTT was administered to rats after SC injection of Ex4 (5 or 20µg/kg) or vehicle (1ml/kg), and subsequent chow intake and body

weight were monitored after the OGTT. Similar to previous findings⁴¹, systemic Ex4 produced a pronounced hyperglycemic response in the rats (Figure 3A, main effect of drug, $F_{2,18}=8.84$, $p<0.01$; drug $\times$ time interaction, $F_{10,90}=11.89$, $p<0.000001$). Injection of either dose of Ex4 increased BG on its own (e.g., before administration of glucose; at $t=0$ min, vehicle versus 5 or 20 μ g/kg, $p<0.05$). BG levels remained significantly elevated in Ex4-treated rats at 40, 60, and 120min after the glucose gavage (vehicle versus 5 or 20 μ g/kg, all $p<0.05$).

When food was returned after the last BG reading, Ex4-treated rats ate significantly less than did vehicle-treated controls in the subsequent 21.5h (Figure 3B, $F_{2,16}=43.74$, $p<0.000001$; vehicle versus 5 or 20 μ g/kg, $p<0.05$) and gained less body weight (Figure 3C, $F_{2,16}=8.31$, $p<0.01$; vehicle versus 20 μ g/kg, $p<0.05$). These results demonstrate the unique constellation of effects produced by peripheral Ex4 administration in the rat, and more importantly, highlight the distinct differences between Ex4 and B12-Ex4 for glycemic and energy balance control.

Ex4 elicits expression of a robust CTA that is not observed with B12-Ex4

GLP-1R agonists such as Ex4 have undesired side effects including nausea/malaise^{8,52}. To evaluate whether B12-Ex4 produces nausea/malaise, rats were evaluated for expression of a conditioned taste avoidance (CTA) to B12-Ex4 (5 μ g/kg, IP). Additional groups of rats were evaluated in this experiment for CTA to Ex4 (5 μ g/kg, IP) or to LiCl (0.15M, IP), which is well known to produce nausea and CTA in rodents^{8,57,58}. As shown in Figure 3D, acceptance of the drug-paired flavor was significantly higher in the B12-Ex4-treated group compared to either LiCl or Ex4 ($F_{2,24}=5.29$, $p<0.01$; B12-Ex4 versus LiCl or Ex4, $p<0.05$; LiCl versus Ex4, $p>0.05$), suggesting that B12-Ex4 does not produce the same nausea/malaise as Ex4.

In mice, B12-Ex4 and Ex4 suppress blood glucose levels in a glucose tolerance test

To confirm the ability of B12-Ex4 to improve glycemic control in species that do not exhibit Ex4-induced stress-mediated hyperglycemic responses, the glycemic effects of equimolar doses of B12-Ex4 and Ex4 were tested via IPGTT in mice. In contrast to rats, and more in line with human data^{44,45}, Ex4 administration strongly attenuated the increase in blood glucose levels after IP glucose administration. Similarly, B12-Ex4 reduced blood glucose levels in the IPGTT (Figure 4A; main effect of drug, $F_{2,24}=67.13$, $p<0.0001$; drug $\times$ time interaction, $F_{10,120}=15.07$, $p<0.0001$). Posthoc analyses showed that both compounds significantly suppressed BG at 20, 40, 60, and 120min after glucose injection (all $p<0.05$). Interestingly, injection of B12-Ex4 or Ex4 also reduced BG levels prior to IP glucose injection ($t=0$, all $p<0.05$). Although Ex4 had a more potent effect on BG at 20min compared to B12-Ex4 ($p<0.05$), area under the curve analyses revealed that both compounds had hypoglycemic effects post-glucose load compared to saline (Figure 4B; $F_{2,24}=62.74$, $p<0.0001$; vehicle versus B12-Ex4 or versus Ex4, $p<0.05$).

Unlike Ex4, B12-Ex4 does not readily penetrate into the CNS

Previous work shows that Ex4 crosses the blood-brain barrier to exert effects on energy balance and illness/malaise^{22,31,51}. As B12-Ex4 treatment produces the glycemic benefits

associated with Ex4 without producing the centrally-mediated effects of hypophagia and nausea, this suggests that B12-Ex4 may be excluded from the CNS. To evaluate this possibility, rats were treated systemically with a fluorescent-tagged version of B12-Ex4 (Cy5-B12-Ex4), and penetrance into the brain was evaluated using confocal microscopy. The results were compared with CNS penetrance of a fluorescent-tagged version of Ex4 (Flex), which has been shown to penetrate into the CNS⁵¹, and fluorescent-tagged B12 (Cy5-B12). The presence of each of these fluorescent compounds was evaluated in the dorsal vagal complex (DVC; Figure 5) and paraventricular nucleus of the hypothalamus (PVN; Figure 6), due to the known importance of these areas in mediating the feeding effects of GLP-1R activation^{59,60} and the hyperglycemic response observed in rats after systemic Ex4⁴¹. Consistent with previous data⁵¹ Flex was observed in the DVC, and also was observed within the PVN. In contrast, Cy5-B12 and Cy5-B12-Ex4 were not detected in either nucleus, suggesting that exogenously-injected B12 does not readily penetrate into these regions of the CNS, and hence that conjugation of B12 to Ex4 greatly reduces or prevents Ex4 from entering the same areas.

B12-Ex4 is colocalized on insulin-producing pancreatic beta cells

The finding that peripherally administered B12-Ex4 is not detected in the DVC or PVN suggests that the glycemic effects of the compound are likely mediated via peripheral actions. The pancreas is a prime candidate for a peripheral site of action responsible for the glycemic-suppressive effects of B12-Ex4. Indeed, GLP-1R agonists can act directly on pancreatic beta cells to stimulate insulin release, thereby improving blood glucose levels^{61–63}. To assess whether B12-Ex4 is taken up by insulin-producing pancreatic beta cells, rats were given systemic injection of Cy5-B12-Ex4 (5µg/kg) and colocalization with insulin-expressing cells was analyzed in the pancreas with 3-dimensional confocal microscopy. Results show robust colocalization of Cy5-B12-Ex4 with insulin in pancreatic sections (Figure 7; Supplemental Materials, Videos 1 and 2), supporting the hypothesis that B12-Ex4 acts at the pancreas to improve glycemic control.

Discussion

GLP-1-based pharmacotherapies for T2DM have been revolutionary in providing largely safe and efficacious means to reduce chronic hyperglycemia [see^{1,3,15} for review]. However, due to side effects of current GLP-1-based compounds including anorexia, nausea, and vomiting, nearly one in four T2DM patients are not able to benefit from the full pharmaceutical advantages of these pharmacotherapies^{10–14}. There is clearly a critical need to develop a new generation of GLP-1 pharmacotherapies that provide hypoglycemic benefit without eliciting detrimental side effects. Although the hypophagic effects of GLP-1R agonists are often attractive to clinicians and to T2DM patients with overweight or obesity, much of the same CNS circuitry underlying GLP-1R ligand-mediated anorexia is also partially responsible for mediating nausea/malaise⁸. Moreover, weight loss may be undesirable for some T2DM patients, such as individuals with a normal BMI. As the hypophagia and illness-like effects of existing GLP-1R agonists require CNS penetrance and direct central action^{8,21–23}, we sought to create a GLP-1R agonist conjugate that minimizes anorexia and nausea by reducing CNS penetrance, but that retains potent pharmacodynamics

and pharmacokinetic profile on peripheral GLP-1R populations to exert glycemic benefits. This report shows for the first time the ability of B12-Ex4 [see³⁰ for previous biochemical GLP-1R agonism analyses] to improve glucose tolerance in rodents without producing hypophagia, body weight loss, or CTA. Supporting a recent finding showing that vitamin B12 is not actively transported into the adult brain⁴⁰, current immunohistochemical data suggest that the unique profile of glycemic effects without the same hypophagic / CTA-producing effects of Ex4 involves a direct effect of B12-Ex4 on pancreatic beta cells coupled with a virtual absence of CNS penetrance of the compound.

The rat provides a unique model for the proof-of-concept testing needed for the preclinical evaluation of B12-Ex4. Rats show an unexpected hyperglycemic response to Ex4, due in part to a CNS-mediated activation of the sympathetic nervous system⁴¹. In addition, like humans, rats show a pronounced profile of behavioral effects to systemic Ex4 including reduced food intake and body weight, as well as illness-like behaviors, again due to CNS action^{8,22,64}. B12-Ex4 did not produce the same suppression of food intake, reduction in body weight, and induction of CTA as did Ex4 in rats. The effect of B12-Ex4 on glycemic control was also evaluated in mice, a species in which Ex4 produces a hypoglycemic response similar to that observed in humans. In mice, B12-Ex4 and unconjugated Ex4 each elicited hypoglycemic responses in an IPGTT. Collectively, these data provide an ideal preclinical set of outcomes to support the therapeutic potential of this conjugate as a future antidiabetic drug for humans.

The *in vivo* behavioral data were supported by our immunohistochemical analyses showing a virtual absence of B12-Ex4 CNS penetrance in the DVC and PVN, two areas of the brain showing unconjugated Ex4 penetrance and believed to mediate in part the hyperglycemic, hypophagic, body weight suppressive, and malaise-producing effects of Ex4 in rats. Future studies are warranted to identify the mechanisms responsible for the minimal CNS uptake of B12 and the molecular mechanisms by which B12 conjugation reduces CNS Ex4 access. It will also be important to address whether higher doses of B12-Ex4 are able to more effectively penetrate the CNS. The 5µg/kg dose of Cy5-B12-Ex4 used for this study was selected because 5µg/kg B12-Ex4 had no effect on feeding or body weight in rats, but produced hypoglycemia in the OGTT, suggesting that a lower dose of B12-Ex4 elicits an optimal profile of glycemic and energy balance effects. In contrast, a higher dose of B12-Ex4 (20µg/kg) reduced blood glucose but also caused a small but significant transient suppression of feeding, suggesting that higher doses may have a slightly different pattern of effects. Nevertheless, these results clearly underscore the reduced CNS penetrance but retention of glycemic benefits by lower doses of B12-Ex4.

As B12-Ex4 is not extensively penetrating into the CNS, pancreatic GLP-1R represents the likely cellular substrate mediating the hypoglycemic effects of B12-Ex4. Further analyses supported this hypothesis, as immunohistochemical data showed colocalization of Cy5-B12-Ex4 with insulin in the pancreas. This suggests that B12-Ex4 may exert its glycemic effects via direct action at pancreatic beta cells, while CNS-mediated effects of GLP-1R activation such as hypophagia, nausea, and malaise are minimal or absent due to lack of penetrance of B12-Ex4 into the brain, consistent with previously reported radio-probe data⁴⁰.

The current data provide novel mechanistic evidence that B12 conjugation to a GLP-1R agonist can be used as a means to retain the hypoglycemic properties of GLP-1R agonists but greatly reduce the CNS-mediated anorexia and illness effects observed with all current approved GLP-1-based ligands. These studies are far from the complete set of *in vivo* glycemic analyses needed for B12-Ex4, but certainly justify the need for more comprehensive future analyses. Further investigations are warranted to examine the acute actions of B12-Ex4 in diabetic animal models, as well as to evaluate the metabolic effects of chronic B12-Ex4 administration. It will also be critical to evaluate whether, and to what extent, B12-Ex4 may localize within other CNS nuclei not examined here. Collectively, these data highlight the discovery that B12 conjugation to Ex4 results in a next-generation incretin therapeutic with the clinically desired hypoglycemic effects but not concomitant hypophagia, body weight loss and, most notably, illness-like behaviors, ideal for the future of T2DM treatment in humans. This method of conjugation may also be broadly beneficial to other therapeutics that would benefit from reduced CNS penetrance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Synthetic scheme for Cy5-B12, Cy5 alkyne was “clicked” onto a B12-azide conjugate. (B) RP-HPLC of Cy5-B12 showing purity 98% and LC-MS showing 1031[M+2H]⁺, 1042[M+Na+2H]⁺, and 1050 [M+K+2H]⁺, consistent with the conjugate. (C) Excitation and emission spectra of Cy5-B12 at 645 and 682nm, respectively. (D) Human recombinant TCII binding of B12-Ex4 and cyano-B12 with a K_d of 0.75 and 0.98nM, respectively. (E) Synthetic scheme for Cy5-B12-Ex4, Cy5-NHS ester was conjugated to Ex4’s lysine 26 and N-terminal. (F) RP-HPLC of Cy5-B12-Ex4 showing purity 91% and LC-MS showing m/z = 1728 [M+4H]⁺, consistent with conjugate containing two molecules of Cy5 per B12-Ex4 component. (G) Excitation and emission spectra of Cy5-B12-Ex4 at 648 and 670 nm, respectively. (H) Cy5-B12-Ex4 agonism at the GLP-1 receptor reported using the FRET reporter H188; EC₅₀ = 13nM. Note that B12-Ex4 agonism at the GLP-1 receptor was previously reported (68 pM)³⁰.

Figure 2.

B12-Ex4 potently suppresses blood glucose in an oral glucose tolerance test but has minimal effects on energy balance control. Food intake and body weight change were measured after SC administration of B12-Ex4. Only the highest dose of drug, 20 $\mu\text{g/kg}$, produced any reduction in feeding (A). No changes in 24h body weight gain were observed as a result of B12-Ex4 administration (B). In a separate experiment, SC injection of B12-Ex4 (0 $\mu\text{g/kg}$ indicated by white circles, 5 $\mu\text{g/kg}$ by lighter blue circles, 20 $\mu\text{g/kg}$ by darker blue circles) reduced blood glucose in an oral glucose tolerance test from 20-40min after injection (C). *, significantly different from vehicle ($p<0.05$); &, significantly different from 5 $\mu\text{g/kg}$ ($p<0.05$). Key in (A) also applies to (B). Data are mean $\pm$ SEM.

Figure 3.

Systemic administration of Ex4 produces a different profile of metabolic effects than B12-Ex4. In contrast to the potent suppression of blood glucose produced by B12-Ex4, SC injection of Ex4 (0 $\mu\text{g/kg}$ indicated by white squares, 5 $\mu\text{g/kg}$ by lighter red squares, 20 $\mu\text{g/kg}$ by darker red squares) produced a robust hyperglycemic response (A). Food intake (B) and body weight gain (C) were suppressed by SC Ex4. To compare the induction of nausea/malaise by Ex4 with that potentially produced by B12-Ex4, rats were tested for expression of a conditioned taste avoidance (CTA) of a flavor paired with IP injection of B12-Ex4 (5 $\mu\text{g/kg}$), Ex4 (5 $\mu\text{g/kg}$), or LiCl as a positive control (0.15M). The percent acceptance of the drug-paired flavor is shown as a box-and-whiskers plot in (D). Both Ex4 (individual responses represented by black circles, overall group response represented in light gray box) and LiCl (individual responses represented by white circles, overall group response represented in white box) produce avoidance of the drug-paired flavor, as indicated by a reduced acceptance of the flavor. These effects are significantly different from acceptance of the drug-paired flavor in B12-Ex4-treated animals (individual responses represented by

white squares, overall group response represented in dark gray box). *, significantly different from vehicle ($p < 0.05$); &, significantly different from all other groups ($p < 0.05$). Key under (B) and (C) applies to both panels. Data in (A-C) are mean $\pm$ SEM.

Figure 4.

Systemic administration of B12-Ex4 or Ex4 suppresses blood glucose in mice. In an intraperitoneal glucose tolerance test, Ex4 (5 μ g/kg) and B12-Ex4 (dose equimolar to Ex4) suppressed blood glucose levels prior to (t=0min) and after (t=20, 40, 60, 120min) IP glucose administration (A); vehicle versus B12-Ex4: ** p<0.01, *** p<0.001; vehicle versus Ex4: ### p<0.001; B12-Ex4 versus Ex4: §§ p<0.01. Area under the curve analyses from 0-120min (i.e., post-glucose load) show that B12-Ex4 (blue) and Ex4 (red) reduce AUC compared to saline vehicle (white) (B); * p<0.05, *** p<0.001. Data are mean $\pm$ SEM.

Figure 5.

Systemically-delivered fluorescently labeled Ex-4 (Flex) highly penetrates within the DVC, whereas Cy5-B12 and Cy5-B12-Ex4 do not. Images were acquired at 10-20 $\times$ (A,C,E,G) or 63 $\times$ (with 2-3 $\times$ optical zoom) (B,D,F,H) magnifications. Brains were processed for immunohistochemistry to label Flex, Equimolar-Flex, Cy5-B12 and Cy5-B12-Ex4 (yellow), astrocytes (GFAP; green) and neurons (NeuN; red). Sections were counterstained using DAPI (blue) to visualize cell nuclei. (B) Flex and (D) equimolar-Flex immunoreactivity is readily visualized in neurons and astrocytes in the DVC. (F) Cy5-B12 and (H) Cy5-B12-Ex4

are not present either in neurons or in astrocytes within the DVC. AP, area postrema; CC, central canal; DVC, dorsal vagal complex; NTS, nucleus tractus solitarius.

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Figure 6.

Systemically-delivered fluorescently labeled Ex-4 (Flex) highly penetrates within the PVN, whereas Cy5-B12 and Cy5-B12-Ex4 do not. Images were acquired at 10-20× (A,C,E,G) or 63× (with 2-3× optical zoom) (B,D,F,H) magnifications. Brains were processed for immunohistochemistry to label Flex, Equimolar-Flex, Cy5-B12 and Cy5-B12-Ex4 (yellow), astrocytes (GFAP; green) and neurons (NeuN; red). Sections were counterstained using DAPI (blue) to visualize cell nuclei. (B) Flex and (D) equimolar-Flex immunoreactivity is readily visualized in neurons in the PVN. (F) Cy5-B12 and (H) Cy5-B12-Ex4 are not

present either in neurons or in astrocytes within the PVN. 3V, third ventricle; PVN paraventricular hypothalamic nucleus.

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Figure 7.

Systemically-delivered Cy5-B12-Ex4 is colocalized with insulin in the pancreas. The representative still images from three-dimensional rotational videos (Supplemental Materials) demonstrate that Cy5-B12-Ex4 (yellow) is colocalized with insulin (red) in pancreatic beta cells. Sections were counterstained with DAPI (blue). Videos and corresponding representative images were taken from a z-stack (2μm step size) at 40× (A; Video 1) and from a z-stack (1μm step size) at 40× with 4-5× optical zoom (B; Video 2).