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A Synthetic Heterobivalent Ligand Composed of Glucagon-like Peptide 1 and Yohimbine Specifically Targets β -cells within the Pancreas

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Abstract

Purpose— β -cell specificity for a heterobivalent ligand composed of GLP-1 linked to yohimbine (GLP-1/Yhb) was evaluated to determine its utility as a noninvasive imaging agent.

Procedures—Competition binding assays were performed on β TC3 cells and isolated rat islets. Immunostaining for insulin was used to co-localized intravenously injected Cy5-labeled GLP-1/Yhb in β -cells of Sprague-Dawley rats. Rats were intravenously injected with ¹¹¹In-labeled GLP-1/Yhb to determine clearance rates and tissue biodistribution. Tissue specific binding was confirmed by competition with pre-administration of unlabeled GLP-1/Yhb and in Streptozotocin-induced diabetic rats.

Results—In β TC3 cells high affinity binding of GLP-1/Yhb required interactions with both receptors because monovalent competition or receptor knockdown with RNAi lowered specificity and avidity of the heterobivalent ligand. Binding specificity for isolated islets was 2.6 fold greater than that of acinar tissue or islets pre-incubated with excess unlabeled GLP-1/Yhb. Immunofluorescent localization of Cy5-labeled GLP-1/Yhb was restricted to pancreatic islets. Within 30 minutes, ~90% of the ¹¹¹In-labeled GLP-1/Yhb was cleared from blood. Tissue specific accumulation of radiolabeled ligand was apparent in the pancreas, but not other tissues within the abdominal imaging field. Pancreas specificity was lost in Streptozotocin-induced diabetic rats.

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Conflict of Interest

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contribution

L.V.S contributed to experimental design, data acquisition, analysis and interpretation and drafted the manuscript; K.A. contributed to experimental design, data collection and analysis; M.J.A, R.P, and A.C.K assisted with data collection and analysis; R.P and J.V contributed to experimental design and reagent and method development; R.M.L and S.W.L contributed to experimental design, data acquisition, analysis and interpretation. All authors have reviewed the manuscript and approved the final version.

Conclusions—The GLP-1/Yhb exhibits high specificity for β -cells, rapid blood clearance rates, and low non-specific uptake by other tissues within the abdominal imaging field. These characteristics of GLP-1/Yhb are desirable for application to β -cell imaging in vivo and provide a basis for developing additional multivalent β -cell specific targeting agents to aid in the management of Type 1 Diabetes.

Keywords

GLP-1; β -cell mass; imaging; adrenergic receptor; multivalency

Introduction

Loss of insulin secretion due to destruction of pancreatic β -cells is a distinguishing feature in patients with Type I Diabetes Mellitus. To evaluate the progression of β -cell loss prior to diabetes and to monitor the efficacy of therapeutic strategies to retard β -cell destruction in pre-diabetic patients, a non-invasive measure of β -cell mass is required. Imaging agents for β -cells have been identified and contrast agents linked to single ligands or antibodies have been used to evaluate β -cell mass [1–11]. For example, attempts to measure β -cell mass in vivo with agonists for the glucagon-like peptide 1 receptor (GLP-1R) have shown promise [9, 12, 13]. However, additional benefits exist for improved probe selectivity and avidity. These benefits include a greater dynamic range for detection. [3, 5].

We propose that β -cell imaging agents can be improved to meet the needed requirements by using the concept of multivalency. Multivalent ligands are composed of multiple binding domains tethered together by flexible linkers that allow each binding domain to simultaneously access their complementary receptors on a cell's surface. We previously demonstrated that multivalent ligands exhibit enhanced apparent affinity, to cells expressing the complementary receptor pair, when compared to their constituent monovalent molecules [14–16]. For example, a homomultivalent melanocyte stimulating hormone based ligand (two of the same moiety tethered together) bound with higher apparent affinity compared to the monovalent ligand [17, 18]. This enhanced affinity requires an ability of each binding domain within the ligand to access its receptors either simultaneously or within a limited time frame [19]. In this respect, a heteromultivalent ligand (two or more different moieties) exhibited a higher apparent binding affinity to cells expressing both receptors compared to cells that expressed only one of the complementary pair, demonstrating cell type specificity [16].

In this study, we describe the synthesis and testing of a multivalent ligand composed of GLP-1 and a α_2 adrenergic receptor antagonist, yohimbine (Yhb). Glucagon-like peptide-1 receptor is abundantly expressed in rodent and human pancreatic β -cells making it a suitable target for noninvasive monitoring of β -cell mass [20–24]. In vivo and in vitro imaging approaches for GLP-1R agonists demonstrate it has a β -cell targeting capabilities [2, 9, 25, 26]. However, there also was accumulation in the kidney, liver, and lung, which lower resolution during image analysis [2, 25–27]. Therefore, approaches to enhance GLP-1 avidity and specificity would be advantageous. Alpha-2 adrenergic receptors (Adra2) also are present on human and rodent β -cells and provide a class of G-protein coupled receptors

with several well characterized and approved pharmaceutical agents [22, 28–30]. The aim of this study was to evaluate the ability of the GLP-1/Yhb construct to target specifically to β -cells, and thereby evaluate its potential as a noninvasive imaging agent.

Materials and Methods

Ligand synthesis

Heterobivalent DTPA-GLP-1/Yhb and Cy5-GLP-1/Yhb were synthesized using standard Fmoc/*t*Bu chemistry (summarized in Figure 1) on Rink Amide Tentagel resin (0.23mmol/g) [16, 31]. The resulting compounds were fully de-protected and cleaved from the resin by treatment with 91% trifluoroacetic acid (TFA) (3% water, 3% 1, 2-ethanedithiol, and 3% thioanisole). After ether extraction of scavengers, compounds were purified by high-performance liquid chromatography (HPLC) and/or size-exclusion chromatography (Sephadex G-25, 0.1 M acetic acid) to >95% purity. All compounds were analyzed for purity by analytical HPLC and mass spectrometry (MS).

Ligand radiolabeling

The DTPA-GLP-1/Yhb ligand was purified with preparative HPLC and lyophilized. The dry ligand was dissolved in 20% acetonitrile/0.5M ammonium acetate (1mg/mL) and 1.5 equivalent of ^{111}In chloride (specific activity 1.765 GBq/nmole; Perkin-Elmer, Boston MA, USA) was added. The mixture was stirred overnight at room temperature. The labeled ligand was desalted with Discovery DSC-8 50mg SPE column (Sigma-Aldrich, St. Louis, MO, USA) and eluted with 50% and 90% acetonitrile. The methods were previously verified by analytical HPLC and MALDI-MS with ^{111}In for chelation (99% purity, calculated mass: 5363.8, observed mass: 5362.9).

Cell Culture and shRNA transfection

SureSilencingTM shRNA plasmids containing GFP were purchased for GLP-1R and Adra2A (Qiagen Inc., Valencia, CA, USA). Control and gene specific shRNA plasmids for each receptor were amplified in Mach1 E.Coli cells (Invitrogen Life Technology, Grand Island, NY, USA) and purified with a Qiagen Plasmid Midi Kit. The control plasmid expresses a shRNA sequence that does not match anything in the relevant genome. βTC3 cells were cultured under standard conditions (humidified incubator at 37°C with 5%CO₂) in RPMI media supplemented with 10% FBS and 1% Penicillin/Streptomycin. Cells were plated in 6-well plates and cultured for 24 hours prior to transfection. βTC3 cells were transfected using Lipofectamine 2000 and shRNA plasmid (4 μg /well) complexes according to manufacturer's instructions. Transfection efficiency was determined by fluorescence detection of GFP. The duration post transfection was optimized to achieve maximum receptor knockdown of protein concentrations.

Western blotting

Polyacrylamide gel electrophoresis and immunoblotting was performed on 50 μg of βTC3 protein as described previously [32]. The primary antibodies for immunoblotting were raised in rabbit against GLP-1R, Adra2A and β -tubulin (1:500; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and detected with Super Signal West Pico chemiluminescent reagent

(Pierce, Rockford, IL). The membranes were exposed to x-ray film and densitometry measured with ImageJ image analysis software (National Institute of Mental Health, Bethesda, Maryland, USA).

β TC3 Receptor Binding Specificity

β TC3 Cells were incubated with designated concentrations of Cy5-labeled GLP-1/Yhb with and without unlabeled competitors (1 μ M GLP-1 or 1 μ M Yhb) for 2 minutes, then the media was replaced with ligand-free buffer. All images for Cy5-labeled GLP-1/Yhb binding were acquired 3 minutes after buffer replacement. This time point was chosen because previous experiments indicated that binding was complete, but removal from at or near the cell surface was minimum. On average, groups of 10–20 cells were imaged for each coverslip. A region of interest (ROI) was drawn at the cell membrane to obtain the average pixel intensity within that region using ImageJ software. Average intensities ($\pm$ SEM) are presented for three or more independent experiments.

Cells were plated on 25mm no.1 glass coverslips in 6-well plates for competition binding and receptor knockdown experiments. Cells were imaged by placing a coverslip containing cells into a chamber held at 37°C on the stage of an Olympus IX70 inverted microscope equipped with a 40x 1.4 NA ultrafluor objective. The chamber was filled with Hanks Buffered Saline (HBSS pH 7.3; 5mM KCl, 0.3mM KH_2PO_4 , 138mM NaCl, 0.2mM NaHCO_3 , 0.3mM Na_2HPO_4 , 20mM HEPES, 1.3mM CaCl_2 , 0.4mM MgSO_4 , and 5.6mM glucose). Receptor binding was measured with the Cy5-labeled GLP-1/Yhb ligand. Cy5 fluorescence was excited using a 100 W Hg lamp as the excitation source with the white light passed through a 60nm band pass filter centered at 620nm. The emitted light was appropriately filtered at 700nm using a 75nm band pass prior to focusing the cell image onto a Photometrics Cascade 512B camera. Cy5 was chosen because background auto-fluorescence at these wavelengths was minimal, and therefore optimal for observing binding at low fluorophore/ligand concentrations.

For the knockdown experiments, β -TC3 cells were imaged first for GFP fluorescence to distinguish transfected (GFP positive) from non-transfected (GFP negative) cells then the filter was switched to the Cy5 filter set. Image acquisition and analysis for Cy5-labeled GLP-1/Yhb binding was identical to that described above.

Binding specificity for isolated rat islets

Islets were isolated using the collagenase (Sigma Type V) technique as described previously [33]. Eight isolations were performed in total with islets pooled from 2 rats per isolation. Rat islets were hand-picked under a microscope and cultured overnight in CMRL supplemented media (10% FBS, 1X Pen-Strep, and 11mM glucose) in an oxygen chamber supplemented with 95% O_2 and 5% CO_2 in a humidified incubator at 37°C. Partially digested acinar clusters were also cultured overnight under the same conditions. Eight binding experiments were carried out with 50 islets or acinar clusters (100 μ L CMRL media) in 100 μ L of HBSS media supplemented with 0.5% BSA (n=2 replicates/tissue type for each isolation/experiment). Half of the islet and acinar replicates were pre-incubated for 5 minutes with unlabeled GLP-1/Yhb ligand (2540 nmoles/L) to determine specific binding of the ^{111}In -

labeled GLP-1/Yhb ligand. The ^{111}In -labeled GLP-1/Yhb ligand (10.98 ± 1.47 nmoles/L in 50uL of saline with 0.5% BSA) was added to all replicates and incubated at 37°C for 5 minutes. Media were removed and tissues were washed in ice cold, serum-free DMEM media five times. Tissues were homogenized in RIPA buffer (50mM Tris, pH 7.4, 150mM NaCl, 2mM EDTA, pH 8.0, 50mM NaF, 1% Triton X-100, 1% Deoxycholic Acid, 0.1% SDS, 1mM DTT). Homogenized samples were used to determine radioactivity and protein concentrations (Pierce Microplate BCA Protein Assay Kit from Thermo Scientific, Rockford, IL, USA). Results are presented as disintegrations per minute (DPM) per protein content (DPM/ug of protein).

In vivo specificity of Cy5-labeled GLP-1/Yhb in the rat

Six anesthetized rats were injected with 5 nmoles of Cy5-labeled GLP-1/Yhb ligand via the saphenous vein to achieve an estimated blood concentration of 250 nmoles/L. Optimal estimated blood concentrations were previously determined with a dose response study (data not shown). Thirty minutes after ligand administration, rats were euthanized and pancreas was collected. Pancreas was also collected from control (non-injected) rats to identify background intensity. Pancreas tissue was fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and embedded in optimal cutting temperature (OCT) compound (Tissue Tek manufactured for Sakura Finetek Inc, Torrance, CA, USA). Pancreatic sections (6 μm) were immunostained for insulin (guinea pig anti-porcine insulin; 1:500, Dako, Carpinteria, CA, USA) and detected with affinity-purified secondary antiserum conjugated to aminomethylcoumarin acetate (AMCA, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) as previously described [34]. Independent images for each fluorophore were acquired using a Leica DM5500 Microscope System equipped with an A4 (insulin, Excitation Filter BP360/40, Dichromatic mirror 400, and Suppression Filter BP 470/40) and Y5 (Cy5, Excitation Filter BP 620/60, Dichromatic mirror 660, and Suppression Filter BP 700/75) filter cubes and a Leica EL600 compact light source with a HXP-R120W/45C VIS lamp as an excitation source. Images were digitally captured with 208 msec exposure for insulin and 10353 msec exposure for Cy5 using a Spot Pursuit 4 Megapixel CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Intensities for five islets and acinar tissue images were measured on 20 pancreas sections from each rat using Image Pro Plus 6.3 software (Media Cybernetics, Silver Spring, MD, USA), and the ratio of islet to acinar tissue intensities were calculated for each section then averaged for all sections.

Experimental Animals

Male Sprague-Dawley rats weighing 300 g at 8–9 weeks of age were purchased from Harlan (Livermore, CA, USA). Four rats received one intraperitoneal injection of Streptozotocin (60mg/kg; Sigma-Aldrich, St. Louis, MO, USA) reconstituted using 0.1M sodium citrate buffer (pH 4.5). Blood glucose concentrations were measured daily using a True Track Glucose Monitoring System (NIPRO Diagnostics, Fort Lauderdale, FL, USA). Rats were considered diabetic when their blood glucose concentrations exceeded 200 mg/dL. All rats exhibited blood glucose concentrations >200 mg/dL for three consecutive days prior to the start of the experiment. All experiments were performed with the approval of and in

accordance with guidelines established by the Institutional Animal Care and Use Committee at The University of Arizona.

Blood clearance rate and biodistribution

Rats were anesthetized with Ketamine and Xylazine and intravenously injected via the saphenous vein with the ^{111}In -labeled GLP-1/Yhb ligand (0.53 ± 0.06 nmoles in 1mL of sterile saline). Four rats received 20 nmoles of unlabeled GLP-1/Yhb ligand in 0.5mL of saline 10 minutes prior to administration of radiolabeled ligand and were compared to seven rats that received radiolabeled ligand only. Blood samples were collected from the tail vein at 0, 1, 3, 5, 10, 20, 30, and 45 minutes following ^{111}In -labeled GLP-1/Yhb administration. After the 45 minute blood sample was collected, the rats were euthanized, dissected, and organs were collected. The organs and tissues were rinsed in saline three times, blotted dry, and weighed. Five samples ($\sim 2 \text{ mm}^3$) were collected from each organ or tissue and weighed. Radioactivity for tissue samples and injectate was measured with an automatic gamma counter (Wizard² 2470, Perkin Elmer, Waltham, MA, USA). Results are presented as a percent of injected activity per gram of organ weight (%IA / g of tissue).

Analysis of pancreatic β -cell mass in Streptozotocin induced diabetic rats

Following the experiment, rats were euthanized and pancreas was collected. Pancreas tissue was fixed in 4% PFA in PBS and embedded in OCT. Pancreatic sections ($6 \mu\text{m}$) were immunostained for insulin, somatostatin (rabbit anti-bovine somatostatin; 1:500, DakoCarpinteria, CA, USA), and glucagon (mouse anti-porcine glucagon; 1:250, Sigma-Aldrich, St. Louis, MO, USA) and detected with affinity-purified secondary antiserum conjugated to AMCA, Dylight 594, and Alexa 488 respectively (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) as previously described [34]. Beta cell mass for each animal was obtained as described previously [34–36].

Statistical analysis

Fluorescence intensities for competitive binding experiments were analyzed within a concentration by a least squares analysis of variance with the use of the GLM procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA; v9.3, 2011). A two tailed unpaired t-test was performed to observe significant ($P < 0.05$) differences in βTC3 cells transfected with shRNA plasmids. Differences for the tissue biodistribution, Cy5-labeled GLP-1/Yhb, and in vitro specificity experiments were determined by least squares analysis of variance with the use of the GLM procedure of SAS. Blood clearance rate was determined using the GraphPad Prism Software (GraphPad Software Inc., La Jolla, CA, USA, V6, 2012). A significance level of $P < 0.05$ was used for all statistical tests. Results are presented as mean $\pm$ SEM.

Results

Binding Specificity of GLP-1/Yhb Requires Bivalent Interaction with Complementary Receptors

Saturation binding of Cy5-labeled GLP-1/Yhb to βTC3 cells was monitored in the presence of competitors against a single receptor to evaluate monovalent binding of the individual

domains (Figure 2). High affinity binding (at 1 and 5 nmoles/L) was observed only when both ligands within the GLP-1/Yhb have access to their receptors; i.e., significant binding of the GLP-1/Yhb was not observed below 50 nM in the presence of either one of the cognate monomers. In the presence of unlabeled Yhb (1 μ M), Cy5-labeled GLP-1/Yhb binding to the GLP-1R was observed at concentrations of 50 nmoles/L and above. Specific binding to Ad α 2A (in the presence of cold GLP-1) also was observed at 50 nmoles/L and 100 nmoles/L Cy5-labeled GLP-1/Yhb (in the presence of saturating monomeric GLP-1). Previously, we reported that on average 250,000 – 300,000 GLP-1R are expressed per β TC3 cell (passage 46–47), using a high throughput screening assay [14]. This is at least 2 fold higher than the number of Ad α 2A receptors expressed on these β TC3 cells. Therefore the magnitude of bivalent binding (below 50 nmoles/L) is limited by the number of Ad α 2A. Conversely, the high level of binding of the GLP-1 element within the GLP-1/Yhb, at 50 nmoles/L and above (in the presence of cold Yhb), is due to binding to available ‘spare’ GLP-1R [14].

The requirement of both complementary receptors for binding below 50 nmoles/L was evaluated by reducing receptor expression with shRNA. GLP-1R or Ad α 2A protein concentrations were decreased with transient transfections of shRNA plasmids in β TC3 cells (Figure 3). GLP-1R was reduced by 82–96% and Ad α 2A was reduced by 93–97% at 72 hours post transfection.

Binding of Cy5-labeled GLP-1/Yhb to cells transiently transfected with receptor specific shRNA plasmid was reduced (Figure 4). On addition of 25nM Cy5-GLP-1/Yhb, less binding was seen on cells with knockdown of either GLP-1R (Figure 4C) or α 2AR (Figure 4D) compared to substantial binding (~2-fold greater intensity) in non-transfected cells within the same field of view. The average fluorescent intensity of β TC3 cells that bound Cy5-labeled GLP-1/Yhb and were transfected with GLP-1R or Ad α 2A shRNA plasmids was 43.1% or 45.3% less, respectively, than the non-transfected cells expressing both receptors. Together, these data demonstrate that at low concentrations (< 50 nmoles/L), GLP-1/Yhb binds preferentially to cells when both of its cognate receptors are accessible for binding relative to cells where only one receptor is accessible on the cell surface.

Isolated islet sequestration of ^{111}In -labeled GLP-1/Yhb

In isolated islets incubated with ^{111}In -labeled GLP-1/Yhb, accumulation was 2.6 fold greater ($P < 0.05$) than that of islets incubated with ^{111}In -labeled GLP-1/Yhb in the presence of 230 fold greater unlabeled GLP-1/Yhb (Figure 6). In contrast, specific binding was not observed in acinar tissue because unlabeled competitor was ineffective. Furthermore, islets incubated with only ^{111}In -labeled GLP-1/Yhb had accumulation greater than ($P < 0.05$) both acinar tissue treatment groups.

Pancreatic cellular specificity of Cy5-labeled GLP-1/Yhb

After an intravenous injection, Cy5-labeled GLP-1/Yhb co-localized with β -cells that were immunostained with insulin (Figure 7). Fluorescence intensities for Cy5 were compared as ratios for insulin positive areas to surrounding pancreas tissue (Figure 7). The average intensity ratio for sections acquired from non-injected rats was not different than 1,

indicating that background in the Cy5 filter was homogeneous for β -cell and acinar tissues. The β -cell to acinar fluorescence intensity ratio was 1.8 fold in rats injected with Cy5-labeled GLP-1/Yhb, which was greater ($P < 0.01$) than non-injected rats.

Biodistribution of ^{111}In -labeled GLP-1/Yhb

In circulation maximum blood concentrations of ^{111}In -labeled GLP-1/Yhb were observed 1 minute (338.1 ± 3.4 nmoles/L) post intravenous injection (Figure 5). The rate of clearance of ^{111}In -labeled GLP-1/Yhb from the blood was 50% of maximal at 5 minutes following administration and ~90% of maximal after 30 minutes. The clearance from circulation was best fit by the equation $y = 3.2 \times 10^8 \exp(-0.26x) + 4.74 \times 10^7$, where x is time in minutes and y is radioactivity in DPMs. Less than 13% (44.89 ± 6.78 nmol/L) of the ^{111}In -labeled GLP-1/Yhb was present in circulation 45 minutes after injection.

Organs were collected 45 minutes following injection, a time after which blood activity was 0.70 ± 0.53 %IA / g of tissue. Tissue distribution analysis for the ^{111}In -labeled GLP-1/Yhb showed that the kidneys accumulated the greatest proportion of the injected activity (16.24 ± 1.45 %IA / g of tissue; Table 1). The lungs acquired the second highest proportion of ^{111}In activity at 1.38 ± 0.43 %IA / g of tissue, while the pancreas proportion of ^{111}In activity was 0.18 ± 0.07 %IA / g of tissue, with all other tissues retaining less than 1.0 %IA / g of tissue 45 minutes after injection.

Specific binding of ^{111}In -labeled GLP-1/Yhb within each tissue was determined by pre-injection of saturating unlabeled GLP-1/Yhb (Table 1). Tissue specific accumulation for the radiolabeled ligand was apparent only in the pancreas ($P < 0.05$) and lung ($P < 0.05$). No differences were observed in other tissues between ^{111}In -labeled GLP-1/Yhb and unlabeled GLP-1/Yhb.

Biodistribution of ^{111}In -labeled GLP-1/Yhb in Streptozotocin-induced diabetic rats

Streptozotocin-induced diabetic rats were used to evaluate pancreas specificity for ^{111}In -labeled GLP-1/Yhb. Blood clearance kinetics in the β -cell depleted rats was similar to those non-diabetic rats (Figure 5). Biodistribution also was similar in control and diabetic rats, except that the specific binding in the pancreas was lost (Table 1).

^{111}In -labeled GLP-1/Yhb in the pancreas was similar to that observed for pancreata obtained from rats injected with unlabeled GLP-1/Yhb prior to receiving the ^{111}In -labeled GLP-1/Yhb (Table 1). Both groups of rats have lower ($P < 0.05$) pancreas binding compared to control rats injected only with ^{111}In -labeled GLP-1/Yhb. Streptozotocin-induced β -cell demise in diabetic rats was confirmed by immuno-fluorescent analysis for insulin positive area. Streptozotocin-induced diabetic rats had $0.17 \pm 0.01\%$ β -cell positive area, which was less ($P < 0.01$) than the $1.93 \pm 0.01\%$ β -cell positive area found in normal, control rat pancreas. The pancreata from Streptozotocin-induced diabetic rats had similar α -cell and δ -cell areas as controls.

Discussion

Prerequisite for non-invasive β -cell imaging agents requires that probes have high specificity and avidity for β -cells and be readily cleared from circulation to reduce background in adjacent tissues due to the relatively small size of β -cell mass. In this study, a newly synthesized heterobivalent ligand composed of GLP-1 and yohimbine was evaluated, and for the first time we demonstrate the capability of a multivalent synthetic molecule to bind to an insulinoma cell line and untransformed β -cells. Binding experiments with the β TC3 cells demonstrate that access to both cognate receptors is required for high affinity GLP-1/Yhb. Moreover, in vivo experiments demonstrate that, within the pancreas and abdominal tissues, GLP-1/Yhb binding was specific for β -cells, which was further confirmed by loss of specific binding in pancreata of Streptozotocin-induced diabetic rats and in isolated rat islets.

An important characteristic of these multivalent ligands is their relatively small molecular weight which should provide kinetic properties required of an imaging agent. The washout to steady state of ^{111}In -labeled GLP-1/Yhb occurred within 30 minutes of injection confirming this advantage. In order to evaluate the level of binding of ^{111}In -labeled GLP-1/Yhb to islets, an estimate of β -cell mass was required, which was determined to be approximately 2% of the pancreas mass in control rats, using the approach described in the methods. This measurement of β -cell mass was determined using sibling cohort of rats (age and weight matched) from the same delivery as the rats used for the ^{111}In -labeled in vivo studies. To determine islet specific binding within the pancreas, %IA / g of tissue for Streptozotocin-induced diabetic rats was subtracted from the % IA / g of tissue for the ^{111}In -labeled GLP-1/Yhb injected rats. The Streptozotocin-induced diabetic rats lack β -cells and therefore any measurement would be background (non-specific binding). The same results are found if %IA / g of tissue for rats injected with unlabeled GLP-1/Yhb plus ^{111}In -labeled GLP-1/Yhb is subtracted from the %IA / g of tissue for ^{111}In -labeled GLP-1/Yhb injected rats. Rats injected with ^{111}In -labeled GLP-1/Yhb had an activity of 7.45 ± 1.15 %IA / g of tissue, which was greater ($P < 0.05$) than the level found in rats injected with unlabeled GLP-1/Yhb plus ^{111}In -labeled GLP-1/Yhb (0.68 ± 0.10 %IA / g of tissue) and Streptozotocin-induced diabetic rats (0.70 ± 0.06 %IA / g of tissue). These findings demonstrate the utility for multivalent constructs with low retention times in circulation to bind with high specificity to native pancreatic β -cells. Furthermore, the flexibility in synthesis of the multivalent construct suggests potential to further refine β -cell targeting agents by using either additional β -cell ligands or other novel ligand combinations.

A range of β -cell active agents were previously evaluated and shown to have sub-optimal binding specificity and sensitivity for in vivo imaging [5]. The β -cell specific monoclonal antibodies, K14D10 and IC2, were evaluated in vivo, but exhibited slow clearance rates from circulation and accumulated in the liver [3]. Although not exclusively expressed in the β -cell, ligands with high specificity for β -cells also have been tested, including glibenclamide derivatives (sulfonylurea receptor 1; SUR1) [37], dihydrotetrabenazine (vesicular monoamine transporter type 2; VMAT2) [38], and naphthylalanin derivatives (somatostatin receptor) [39]. These ligands demonstrated pancreatic binding, but accurate assessment or visualization of β -cell mass in vivo was limited by non-specific binding [5, 37, 39]. One family of ligands that show promise for β -cell targeting are modulators of the

GLP-1 receptor, which has been evaluated for noninvasive monitoring of β -cell mass and tumor detection, because of its robust expression in β -cells compared to surrounding pancreatic tissue [2, 9–13, 25, 40, 41]. Here, we expand on GLP-1 as a targeting agent, and show GLP-1/Yhb biodistribution is restricted to the lung and pancreas. Moreover, specificity for pancreatic β -cells is improved with bivalent binding of GLP-1/Yhb compared to monovalent GLP-1R (Figures 2 & 4).

The specificity and avidity of the GLP-1/Yhb construct is greater than the individual binding characteristics due to the requirement of simultaneous interaction of the two independent binding domains (GLP-1, Yhb) to their complementary receptors (Figures 2 and 4) [19]. Furthermore, the innate affinity of the GLP-1 binding domain within the construct for the GLP-1 receptor is predicted to be decreased (Figure 3) [14], due to steric hindrance caused by the linker and second binding domain [14, 15], which would subsequently lower binding to tissues expressing the GLP-1R alone. Regardless, the overall avidity of the ligand is improved by the second moiety, in this case Yhb, eliciting greater specificity. This finding is consistent with previous studies showing enhanced specificity of a melanocyte-stimulating hormone (MSH)/(CCK) bivalent construct for transformed cell lines expressing Human Melanocortin-4 (MSH) and Cholecystokinin-2 (CCK) receptors [15, 16, 42].

Improving current targeting agents will increase the prospects for noninvasive β -cell imaging for clinical evaluation of β -cell mass. Although some ligands for single receptors or molecules show promise, we demonstrate that synthetic heterobivalent ligands, such as GLP-1/Yhb, can be developed to increase cellular specificity and sensitivity while maintaining low retention times in circulation [14, 15]. These qualities meet necessary criteria for a successful imaging agent, low retention and increased β -cell sensitivity. Moreover, we show that GLP-1/Yhb exhibits high specificity for native β -cells and low non-specific uptake into other organs within the abdominal field. This experimental design provides a basis for developing additional β -cells specific imaging agents by using other ligand combinations or additional binding domains [17].

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Figure 1.
Synthesis of GLP-1/Yhb-DTPA ¹¹¹In labeling (a) or GLP-1/Yhb Cy5 labeling (b).

Figure 2. Bivalent receptor binding increases GLP-1/Yhb ligand specificity in β TC3 cells
 Average fluorescence intensities (a.u.f.; $\pm$ SEM) are shown for β TC3 cells incubated with varying concentrations of Cy5-labeled GLP-1/Yhb. Cells were incubated for two minutes with labeled ligand, the media was replaced with ligand free media and images were acquired after additional 3 minutes. Black bars represent Cy5-labeled GLP-1/Yhb binding without competition. White bars present average fluorescence intensity for Cy5-labeled GLP-1/Yhb in presence of saturated GLP-1R (unlabeled $1\mu\text{M}$ GLP-1), demonstrating monovalent Yhb binding. Gray bars represent average fluorescence intensity for Cy5-labeled GLP-1/Yhb in presence of saturated Adora2A (unlabeled $1\mu\text{M}$ Yhb), demonstrating monovalent GLP-1 binding. * = $P < 0.05$ for no competitor vs. either monovalent binding. # = $P < 0.05$ for monovalent GLP-1 binding vs. monovalent Yhb binding. No significant difference was observed between bivalent GLP-1/Yhb binding (black bars) and the estimated additive binding density for GLP-1/Yhb in the presence of the independent monomeric competitors, at 100 nM ligand.

Figure 3. GLP-1R or Adra2A knockdown in β TC3 cells

β TC3 cells were transiently transfected with either Adra2A (a) or GLP-1R (b) shRNA plasmids. Protein was isolated 72 hours after the transfection and representative Western blots are shown for Adra2A, GLP-1R, and β -tubulin (loading control). Two shRNA plasmids were evaluated for each gene and compared to a control plasmid. Duplicate lanes are shown for each transient transfection. Protein expression for shRNA plasmid 1 (light grey bars) and plasmid 2 (dark grey bars) for each gene is presented as a percentage of the control plasmid (black bars). Three independent transient transfections were used to determine receptor protein expression.

Figure 4. Individual receptor knockdown decreases Cy5-labeled GLP-1/Yhb binding in β TC3 cells

Binding was evaluated after incubation with 25 nM Cy5-labeled GLP-1/Yhb in β TC cells transiently transfected with either GLP-1R (a, c) or Adra2A (b, d) shRNA plasmids. GFP expression is presented in (a, b) and distinguishes cells in which either GLP-1R (a) or Adra2A (b) expression is depressed. Cells in the field of view which were not transfected (i.e. do not express GFP), and therefore are presumed to have a full receptor complement are highlighted by dashed lines. Cy5-labeled GLP-1/Yhb binds preferentially non-transfected β TC3 cells.

Figure 5. Isolated islet sequestration of ^{111}In -labeled GLP-1/Yhb

Isolated rat islets and acinar tissue were incubated with ^{111}In -labeled GLP-1/Yhb (n=8) or co-incubated with unlabeled GLP-1/Yhb (2540 nmoles/L, n=8). Tissue type is indicated on the x-axis with total radioactivity per protein content of sample on the y-axis. *P < 0.05 indicates significant difference between ^{111}In -labeled GLP-1/Yhb and unlabeled GLP-1/Yhb and tissue type.

Figure 6. Pancreatic specificity of Cy5-labeled GLP-1/Yhb

a) Pancreata were collected from injected (right panel) and non-injected (left panel) rats. Representative images were captured for insulin and Cy5. The merged image for the injected panel shows the co-localization of GLP-1/Yhb binding and β -cells. b) Relative fluorescent intensity ratios for five islets/section (n=20 sections) from non-injected and Cy5-labeled GLP-1/Yhb (250 nmoles/L) injected rats are presented. *P < 0.05 indicates significant differences between treatments.

Figure 7. ^{111}In -labeled GLP-1/Yhb and clearance rates

Blood clearance rates of ^{111}In -labeled GLP-1/Yhb in normal (closed circle, $n=7$), Streptozotocin-induced (open circle, $n=4$), and ^{111}In -labeled GLP-1/Yhb + Unlabeled (triangle, $n=4$) injected rats following intravenous injections with time (min) on the x-axis and radioactivity (DPM) on the y-axis.

Table 1
¹¹¹In-labeled GLP-1/Yhb Biodistribution in Sprague-Dawley Rats

Rats were injected with only ¹¹¹In-labeled GLP-1/Yhb (n=7), ¹¹¹In-labeled GLP/Yhb blocked by pre-injection of 20 nmoles/L unlabeled GLP-1/Yhb (n=4), or in a Streptozotocin-induced diabetic model (n=4). Tissue samples collected are indicated in the first column and the average %IA / g of tissue or organ ± SEM is presented for each group.

Tissue	¹¹¹ In-Labeled GLP-1/Yhb	¹¹¹ In-Labeled GLP-1/Yhb + Unlabeled	Streptozotocin-Induced Diabetic Model
Blood	0.701 ± 0.528	0.128 ± 0.013	0.111 ± 0.009
Pancreas	0.184 ± 0.065 ^a	0.034 ± 0.005 ^b	0.035 ± 0.003 ^b
Liver	0.291 ± 0.047	0.418 ± 0.024	0.484 ± 0.017
Kidney	16.24 ± 1.448	17.31 ± 4.333	11.44 ± 1.202
Small Intestine	0.132 ± 0.031	0.069 ± 0.013	0.095 ± 0.029
Large Intestine	0.207 ± 0.045	0.087 ± 0.013	0.119 ± 0.018
Spleen	0.306 ± 0.088	0.098 ± 0.016	0.167 ± 0.011
Stomach	0.225 ± 0.066	0.067 ± 0.016	0.079 ± 0.014
Diaphragm	0.086 ± 0.023	0.037 ± 0.006	0.055 ± 0.016
Heart	0.128 ± 0.042	0.042 ± 0.003	0.039 ± 0.005
Lung	1.023 ± 0.309 ^a	0.121 ± 0.014 ^b	0.501 ± 0.051 ^a
Fat	0.051 ± 0.011	0.023 ± 0.004	0.027 ± 0.003
Muscle	0.048 ± 0.012	0.019 ± 0.003	0.019 ± 0.003

^{a,b} P < 0.05 indicates significant difference compared to ¹¹¹In-labeled GLP-1/Yhb blocked by pre-injection of unlabeled GLP-1/Yhb. A one-way ANOVA with an LSD test was used to identify significant difference between treatments.