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Quantitative Impact of Plasma Clearance and Down-regulation on GLP-1 Receptor Molecular Imaging

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Abstract

Purpose—Quantitative molecular imaging of beta cell mass (BCM) would enable early detection and treatment monitoring of type-1 diabetes. The glucagon like peptide-1 (GLP-1) receptor is an attractive target due to its beta cell specificity and cell surface location. We quantitatively investigated the impact of plasma clearance and receptor internalization on targeting efficiency in healthy B6 mice.

Procedures—Four exenatide-based probes were synthesized that varied in molecular weight, binding affinity, and plasma clearance. The GLP-1 receptor internalization rate and *in vivo* receptor expression were quantified.

Results—Receptor internalization (54,000 receptors/cell *in vivo*) decreased significantly within minutes, reducing the benefit of a slower clearing agent. The multimers and albumin binding probes had higher kidney and liver uptake, respectively.

Conclusions—Slow plasma clearance is beneficial for GLP-1 receptor peptide therapeutics. However, for exendin-based imaging of islets, downregulation of the GLP-1 receptor and non-specific background uptake result in a higher TBR for fast-clearing agents.

Keywords

exendin; glucagon like peptide 1 receptor; type 1 diabetes imaging

Introduction

Type-1 diabetes mellitus (T1DM) is the result of an autoimmune destruction of beta cells, leading to loss of endogenous insulin production and unregulated blood glucose levels. Although the pathophysiology of T1DM is under investigation, the ability to monitor and measure the pancreatic beta cell mass (BCM) is important for assessing the magnitude of autoimmune destruction[1–7]. Most patients display symptoms after losing the majority of their beta cell mass, complicating efforts to prevent further destruction using immunomodulatory therapy[8–9]. The ability to accurately measure beta cell mass would

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Conflict of Interest

The authors declare that they have no conflict of interest.

also be informative during clinical trials to test the efficacy of treatment [10]. Although the regional distribution of beta cells within islets of Langerhans differs across species, the BCM makes up less than 2% of the pancreatic mass in humans[11–12]. Due to this low fraction of beta cells in the pancreas, a robust, non-invasive technique for monitoring and measuring BCM to predict disease onset has proven elusive[10, 13]. Currently, stimulated insulin secretion to indirectly measure the BCM showed success with healthy animal models but failed to predict the development of diabetes because of its limited ability to monitor BCM functionality[6, 14–15]. Other studies were able to correlate hyperglycemia only with large reductions in BCM[16–17]. Imaging agents developed for targeting beta cells such as antibodies, sulfonylureas, and vesicular monoamine transporter (VMAT) receptor agonists have been used in the past but face problems due to low target-to-background ratios (TBR) during imaging, often from low target expression or poor specificity[18–23].

There is a strong demand in the diabetes community to establish an imaging technique to quantify and monitor the beta cell mass as the destruction of these cells is responsible for the onset of type-1 diabetes[24–25]. However, quantitative imaging is challenging because islets are 100–300 microns in diameter, much smaller than the resolution of PET and SPECT. Consequently, quantification of probe concentration will be diluted significantly by partial volume effects[26–31]. Additionally, the loss of beta cell mass requires measurement of a reduction in signal in the context of increased non-specific uptake due to inflammatory conditions during insulinitis[32]

Exendin-4, a 39-amino acid peptide originally isolated from *Heloderma suspectum*, is used for the treatment of type 2 diabetes, and the ligand-receptor interaction between exendin and glucagon-like peptide-1 receptor (GLP-1R) has been characterized for imaging beta cells in T1DM[24, 33–36]. Expression of GLP-1R in beta cells, high vascularization of tissue within the islets of Langerhans, rapid binding kinetics, and *in vivo* target specificity have led to the development of multiple radiolabeled exendin molecules to track the beta cell mass as well as detection of insulinomas[24, 33–34, 37–42]. Owing to its fast binding kinetics, rapid uptake in target tissue, and ability to be stabilized to resist protease degradation, exendin and related peptides have been investigated as molecular imaging agents for measuring beta cell mass *in vivo*[24, 36, 43–44]. However, current approaches lack the sensitivity required for clinical application[25, 41, 45], so more development is needed. Modifications such as PEGylation have shown prolonged therapeutic effects[46–47], which may benefit a slower-clearing imaging agent if rapid distribution and blood clearance result in low targeting. An imaging agent that clears from the blood slowly has more time to accumulate in the target tissue, and in the well-vascularized islets, endocytosis of GLP-1R agonists occurs rapidly[48], suggesting a slower clearing agent may theoretically result in a higher TBR in the pancreas. However, potential downregulation of GLP-1R expression may reduce beta cell internalization rates to values lower than non-target uptake at longer times, and TBR will be reduced due to high background signal from lack of clearance. The multiple competing factors require quantitative analysis of molecular probe properties to design efficient imaging agents. The paucity of data on several parameters, including the number of GLP-1 receptors per cell and transient changes in internalization rate and down-regulation prevented accurate simulations of optimal probe properties. To quantify the internalization rate of GLP-1R and determine the role of receptor recycling, downregulation, and plasma

clearance on imaging contrast, we synthesized a series of molecular imaging probes based on the exendin-4 peptide that vary in molecular weight and plasma clearance to determine the impact on TBR (Fig. 1). We also measured the time-dependent internalization rate using a quenchable probe and quantified the *in vivo* receptor expression. The data will help the design of improved imaging agents and predict scaling to the clinic.

Materials and Methods

All chemicals, unless specified, were purchased from Sigma-Aldrich (Milwaukee, WI) and were used as received. Fluorochromes AF647 alkyne and AF488 alkyne were purchased from Life Technologies (Carlsbad, CA); Cy7 was purchased from Lumiprobe (Hallandale Beach, FL); IRDye 800CW was purchased from Licor (Lincoln, NE). Mutant exendin-4 (HGEGTFTSDLSKQXEEEAVRLFIEWLKNGGPSSGAPPPS), where X is the non-natural amino acid azidohomoalanine, was purchased from Innoprep (San Diego, CA). Reversed phase high performance liquid chromatography (RP-HPLC) and size-exclusion chromatography were performed on a Shimadzu LC unit using Luna C18(2) and Yarra SEC-2000 columns, respectively (Torrance, CA). ESI-MS data were obtained using an Agilent Q-TOF 1200 series. MALDI-MS spectra were collected using a Bruker Autoflex mass spectrometer. NMR spectra were collected using a Varian MR400 spectrometer. All MALDI-TOF and ESI-Mass Spectrometry data were collected at the University of Michigan Department of Chemistry's Core Facility. Fluorescence microscopy was performed using an Olympus FV 1200 microscope and an Olympus-IX81 spinning disk confocal microscope.

Preparation of *N,N'*-di(prop-2-yn-1-yl)pentane-1,5-diamine (1) and 5-amino-*N,N,N'*-tri(prop-2-yn-1-yl)pentan-1-aminium (2)

N-Boc-cadaverine (800 μ mol) was added to potassium carbonate (2.4 mmol) in 2 mL acetonitrile (MeCN). Propargyl bromide in toluene was added dropwise (2.0 mmol) and the reaction mixture was stirred overnight at room temperature before being concentrated under reduced pressure. The reaction mixture was filtered and deprotected using 50% trifluoroacetic acid in dichloromethane, and purified using preparative RP-HPLC using a constant gradient of 5:95 of MeCN:H₂O to yield **1** and **2** (212 μ mol, 27%; 127 μ mol, 16%). **1**: ¹H NMR (400 MHz, CD₃OD): δ 4.22 [d, *J* = 2.4, 4 H], 3.37 [t, *J* = 2.8, 2 H], 3.32 [t, *J* = 7.6, 2 H], 2.94 [t, *J* = 7.6, 2 H], 1.82 – 1.68 [m, 4 H], 1.47 [quint, *J* = 7.6, 2 H]. HRMS: *m/z* calculated for C₁₁H₁₈N₂: 178.1470, found: 179.1543 [MH⁺]. **2**: ¹H NMR (400 MHz, CD₃OD): δ 4.50 [d, *J* = 2.4, 6 H], 3.64 – 3.59 [m, 5 H], 2.95 [t, *J* = 7.6, 2 H], 1.89 – 1.85 [m, 2 H], 1.75 [quint, *J* = 7.6, 2 H], 1.48 [quint, *J* = 7.6, 2 H]. HRMS: *m/z* calculated for C₁₄H₂₁N₂: 217.1705, found: 217.1699.

IRDye 800CW conjugation to (1) and (2)

IRDye 800CW NHS ester (1 μ mol in DMSO) was added to an aqueous solution containing either **1** or **2** (10 μ mol) buffered with 7.5% sodium bicarbonate. The reaction was stirred at room temperature for 30 min followed by purification on preparative RP-HPLC (12 mL/min; A: 0.1% trifluoroacetic acid in water, B: 0.1% trifluoroacetic acid in acetonitrile; 20–60% B 0.1–10 min) to give fluorescent di-alkyne and tri-alkyne **3** and **4**, respectively; **3**: *t_R* = 8.4

min; **4**: $t_R = 7.3$ min MALDI-TOF: **3**: m/z calculated: 1160.42, found: 1163.49; **4**: m/z calculated: 1198.47, found: 1201.49;

Preparation of Cy7 alkyne

Propargylamine (5 μmol) was added to 200 μL of 1:1 water:acetonitrile buffered with 7.5% sodium bicarbonate. Cy7 NHS ester (0.5 μmol in DMSO) was added and the reaction was stirred at room temperature for 30 min followed by purification on preparative RP-HPLC (Cy7 alkyne: 65% B 0.1–20 min: $t_R = 16.4$ min). HRMS: m/z calculated for $\text{C}_{14}\text{H}_{21}\text{N}_2$: 586.3797, found: 586.3795.

Preparation of fluorescent exendin monomers

AF488 alkyne (0.2 μmol), AF647 alkyne (0.2 μmol), IRDye 800CW alkyne (0.2 μmol), or Cy7 alkyne (0.2 μmol) was added to 200 μL of water followed by $\text{CuSO}_4\cdot\text{TBTA}$ (20 nmol) and sodium ascorbate (1 μmol). Lastly, mutant exendin (0.2 μmol) was added and the reaction mixture was stirred at room temperature for 12 h followed by purification using preparative RP-HPLC (AF488 monomer: 20–55% B 0.1–17 min: $t_R = 15.3$ min; AF647 monomer: 20–30% B 0.1–4 min, 30% B 4–7 min, 30–55% B 7–17 min: $t_R = 15.3$ min; IRDye 800CW monomer: 20–70% B 0.1–15 min: $t_R = 9.7$ min; Cy7 monomer: 30–90% B 0.1–22 min: $t_R = 11.1$ min). MALDI-TOF: AF488 monomer: m/z calculated: 4750, found: 4754; AF647 monomer: m/z calculated: MW of dye unpublished, found: 4859; IRDye 800CW monomer: m/z calculated: 5219, found: 5220; Cy7 monomer: m/z calculated: 4768, found: 4764.

Preparation of IRDye 800CW dimer and IRDye 800CW trimer

3 or **4** (0.2 μmol) was added to 200 μL of water followed by $\text{CuSO}_4\cdot\text{TBTA}$ (20 nmol) and sodium ascorbate (1 μmol). Lastly, mutant exendin (0.6 μmol for IRDye 800CW dimer, 0.8 μmol for IRDye 800CW trimer) was added and the reaction mixture was stirred at room temperature for 12 h followed by purification using SEC-HPLC at a flow rate of 1 mL/min phosphate buffered saline (PBS), pH 7.4. MALDI-TOF: 800CW dimer: m/z calculated: 9523, found: 9536; IRDye 800CW trimer: m/z calculated: 13745, found: 13758.

Cell culture and stable transfection cell line generation

NIT-1 cells were generously provided by Dr. Ralph Weissleder's Laboratory and used for *in vitro* receptor binding assays due to NIT-1 expression of GLP-1R. Cells were grown in F12K containing 10% (v/v) FBS, 50U/mL penicillin, 50 $\mu\text{g/mL}$ streptomycin, and 1.5 g/L sodium bicarbonate. The passage number for NIT-1 cells was between 4–8 for affinity measurements of all fluorescent exendin conjugates as well as internalization rate determination. For transfected cells, human embryonic kidney cells (HEK293) were grown in DMEM containing 10% (v/v) FBS, 50U/mL penicillin, and 50 $\mu\text{g/mL}$ streptomycin. To generate a stable cell line, HEK293 cells were first cultured in 6-well plates and transfected using Lipofectamine 2000 following the manufacturer's instructions to express GLP-1R (GFP-tagged, Origene) and then selected with 1 mg/mL G-418.

***In vitro* receptor binding assay**

NIT-1 cells were plated, grown for 48 hours, harvested with 0.05% trypsin-EDTA, washed, and resuspended in PBS with 0.1% bovine serum albumin (BSA). The cells were then aliquoted and suspended in binding buffer containing fluorescent exendin conjugates ranging in concentration on ice (0.025–250 nM). The cells were then washed two times with 0.1% BSA in PBS and then immediately analyzed using an Attune Acoustic Focusing Cytometer (Applied Biosystems) or a LICOR Odyssey CLx scanner (Lincoln, NE). Affinity curves and statistical analyses were carried out using Prism 6.0 software.

Internalization assay

The internalization protocol was adapted from a previously published method for antibodies and antibody fragments[49]. Briefly, NIT-1 cells were subcultured into 24-well tissue culture plates, allowed to adhere, and grown for 48 h. After washing once with media, cells were incubated in binding buffer containing 40 nM AF488 monomer in media and allowed to internalize continuously at 37°C for 3 h, while controls were kept at 4°C. At 15, 30, 60, 120, and 180 min, cells were washed once with chilled 0.1% FBS in PBS and placed on ice. Cells were then incubated with 100 μ L cell dissociation buffer (Gibco) for 5 min, lifted, and pelleted in microcentrifuge tubes. Lastly, cells were then resuspended in PBS or a dilution of 0.4% trypan blue in PBS (dilution factor 4) and analyzed using an Attune Acoustic Focusing Cytometer to differentiate between surface and internal fluorescence at various time points. Live cell time lapse images were collected at 37°C on an Olympus-IX81 spinning disk confocal microscope using a 488 nm laser line to corroborate the cytometry results. The cytometry results were fit to a multi-compartmental model with linear kinetics on MATLAB to calculate the internalization half-life of GLP-1R (SI).

Animals

All animal experiments were conducted in compliance with the University of Michigan University Committee on Use and Care of Animals (UCUCA). For determining plasma clearance half-life, IRDye 800CW monomer, IRDye 800CW dimer, IRDye 800CW trimer, or Cy7 monomer (0.5 nmol) was injected in the tail vein of C57BL/6 mice. Blood samples were collected at 1, 3, 5, 15, 30, 60, and 180 min; in the case of the Cy7 monomer, blood was also collected at 300 min. 10 μ L of whole blood was mixed with 20 μ L of 10 mM EDTA in PBS, and spun down to isolate the plasma. Plasma samples were quantified with controls using a LICOR Odyssey CLx scanner to determine absolute concentration in the blood. After the last time point, the mice were sacrificed and the organs resected and visualized macroscopically on the Odyssey CLx. To confirm *in vivo* specificity of the fluorescent exendin conjugates, a 15 nmol dose of non-fluorescent, wild-type exendin-4 was administered via the tail vein 45 min prior to injecting the fluorescent conjugate. All *in vivo* receptor expression and biodistribution experiments (blocked, unblocked for each probe) were done in triplicate.

***In vivo* GLP-1R quantification**

To measure the expression level of GLP-1R *in vivo*, a saturating dose of 1.2 nmol of AF647 monomer was injected in the tail vein of transgenic mice containing GFP under the control

of the mouse insulin promoter (MIP-GFP mice, Jackson Laboratory Strain B6.Cg-Tg(Ins1-EGFP)1Hara/J). At 30 min post-injection, the mice were sacrificed and the pancreas resected. Successful islet targeting was confirmed using an Odyssey CLx scanner and by verifying colocalization of GFP and AF647 signal using fluorescence microscopy. After confirming specific islet targeting, the pancreas was incubated at 37°C for 15 min in 1 mg/mL collagenase type XI in PBS with continuous mixing to obtain a single cell suspension. The cells were analyzed with an Attune Acoustic Focusing Cytometer to determine the fluorescence intensity of AF647 on the GFP-positive β -cells. After accounting for quenching effects of conjugating AF647 to exendin, a calibration curve generated with Quantum Alexa Fluor 647 MESF beads (Bangs Laboratories, Inc.) was used to determine the GLP-1R expression level *in vivo*.

Ex vivo biodistribution

The following protocol was modified from a previously published protocol for measuring uptake of fluorescent antibodies *ex vivo*[50]. Briefly, after animals were sacrificed, organs were resected, weighed, and incubated at 37°C in a mixture of RIPA buffer in PBS supplemented with 6 mg/mL collagenase type IV for 30 min. Next, 0.05% trypsin/EDTA was added and the digest solution was incubated for another 30 min at 37°C before being homogenized using an FB-120 Sonic Dismembrator. Each homogenized organ was then serially diluted in a 96-well plate and the fluorescence was quantified using an Odyssey CLx scanner. A calibration curve was generated using known amounts of fluorescent exendin in the digest solution and the %ID/g was calculated for each of the organs based on organ weight and homogenate volume.

Results

To test the effect of plasma clearance on beta cell TBR, both fast and slow clearing exendin conjugates were synthesized. Based on its position pointing away from the binding pocket of GLP-1R, the methionine at the 14th position (M-14) was substituted with azidohomoalanine (AHA) during solid phase peptide synthesis[51]. Novel heterobifunctional linkers were synthesized to simultaneously link multiple exendin molecules and enable fluorophore conjugation using copper catalyzed click chemistry (Fig. 1). Three conjugates of varying molecular weight—IRDye 800CW (800CW) monomer, 800CW dimer, and 800CW trimer—that rapidly clear from blood were synthesized. Careful consideration was taken when choosing dyes, since they can strongly influence the rate of extravasation and renal filtration due to interactions with albumin[52]. A highly charged and hydrophilic IRDye 800CW fluorophore was used to generate fast clearing multimers of exendin and a hydrophobic Cy7 fluorophore was selected to synthesize a slow clearing exendin monomer. Additionally, AF488 and AF647 conjugates were synthesized for internalization rate measurements and receptor expression quantification, respectively.

After synthesizing **1** and **2**, the linkers were conjugated with the fluorophores and peptides. All fluorescent exendin probes were purified using HPLC and characterized by MALDI-TOF (Fig. 2a). MALDI spectra for monomers were collected using reflectron positive mode; spectra for 800CW dimer and 800CW trimer were collected using linear positive mode.

Although crystallography for the receptor-ligand interaction for exendin indicate the M14 residue points away from the binding pocket[51], the affinity was measured to test the impact of fluorophore conjugation to the 14th position as well as confirm avidity effects of the 800CW dimer and trimer. Eleven or twelve-point affinity curves were generated in triplicate from binding assays using NIT-1 cells (Fig. 2b). Fluorophore conjugation had minimal impact on the affinity, and all fluorescent conjugates of exendin maintained high affinity for GLP-1R (Fig. 2c). For 800CW dimer and 800CW trimer, despite multivalency, the affinity decreased to values of $K_d = 8.3 \pm 1.2$ nM and $K_d = 20.9 \pm 2.5$ nM, respectively.

Glucagon-like peptide-1 receptor, a member of the seven-transmembrane family of GPCRs, has been shown to internalize GLP-1 peptide with a rapid half-life of 2–3 min and a recycle half-life of 15 min [48]. To verify that M14 exendin has no impact on receptor activation and subsequent endocytosis, internalization rates were measured on NIT-1 cells using a modified protocol from Schmidt et al[49]. After cells were allowed to internalize continuously for predetermined amounts of time, the total intensity per cell was compared to the surface quenched intensity per cell. The intensities plotted against time (Fig. 3a) were then fitted to a compartmental binding and internalization model to yield a rapid, early internalization half-life of 5.7 ± 0.9 min and a slower, long-term internalization half-life of 220 ± 10 min (Fig. 3c). The biexponential fit indicates that the early, rapid binding and internalization phase accounts for 68% of fluorescent intensity in the first 3 h of GLP-1R internalization. Additionally, by comparing the quenching of surface receptors incubated on ice (no internalization) with those after extended internalization, we estimate that the surface expression of GLP-1 receptor is downregulated between 55–60%. The slower internalization that is apparent at longer times (>30 min) is likely a result of GLP-1R downregulation and slower receptor dynamics. Qualitatively, live cell time-lapse imaging of NIT-1 (Fig. 3b) and HEK-293 cells transfected with GFP-GLP-1R (SI, video) agree with the results of the internalization assay. Surface binding and internalization occur rapidly at early times, while images at longer times (60 and 180 min images) show small increases in fluorescence intensity.

To measure *in vivo* clearance and biodistribution of the 800CW monomer, dimer, trimer, and Cy7 monomer, 0.5 nmol of fluorescent probe was injected via tail vein in C57BL/6 mice and allowed to circulate. Blood samples were taken at multiple time points, and the half-lives of exendin conjugates in plasma were calculated by fitting the data to a biexponential decay (Fig. 4a). As anticipated, the 800CW conjugates cleared rapidly. We observed a fast decay of the majority of probe within a few minutes followed by a slower (~30 min) clearance phase for each (Fig. 4c). The clearance half-lives for all molecular weight variants were rapid and there was no significant difference among the different constructs (fast clearance $p = 0.68$; slow clearance $p = 0.69$). Cy7 monomer had much slower (> 7-fold) plasma clearance due to the hydrophobic nature of the fluorophore and plasma protein binding[52]. After the last blood sample was taken, the animals were sacrificed and the organs were weighed, homogenized, serial-diluted, and imaged to quantify normalized uptake (Fig. 4b). Uptake was higher in the kidney for the dimer and trimer compared to the 800CW and Cy7 monomers, and the Cy7 monomer was higher in the liver than the 800CW probes ($p = 0.03$). No significant differences were detected in the total pancreatic uptake between all constructs. With a blocking injection, non-specific/exocrine pancreatic uptake for the slower-

clearing Cy7 monomer measured 0.55 ± 0.04 %ID/g whereas 800CW monomer was 0.3 ± 0.1 %ID/g. Subtracting %ID/g of blocked pancreata from experiments without blocking, the specific uptake in the islets was quantified to be 0.5 ± 0.1 %ID/g and 0.5 ± 0.4 %ID/g for the 800CW monomer at 3 h and Cy7 monomer at 5 h, respectively (Table S2, SI).

An AlexaFluor-647 monomer was injected intravenously in MIP-GFP mice to quantify receptor expression. *Ex vivo* labeling of isolated beta cells resulted in inconsistent signal intensity, so *in vivo* saturation was used to quantify expression levels. Macroscopic pancreas scans (Fig. 5a) show AF647 signal in punctate spots several hundred microns in diameter, and confocal imaging of the pancreas from MIP-GFP mice indicate colocalization between the extendin-647 monomer and GFP expression (Fig. 5b). Macroscopic images of the pancreas from mice injected with 800CW monomer, 800CW dimer, 800CW trimer, and Cy7 monomer (Fig. 5c) indicate similar islet specific targeting across all constructs tested. Digestion of the pancreas into a single-cell suspension followed by fluorescence intensity measurements using flow cytometry indicated GLP-1R expression levels at $54,000 \pm 3000$ receptors/cell *in vivo*. A GLP-1R expression measurement with NIT-1 cells indicated $17,000 \pm 4000$ receptors/cell. Cells incubated with collagenase for 15 min had < 5% change in cell surface labeling.

To quantitatively analyze the impact of plasma clearance, molecular weight, binding affinity, and receptor internalization and down-regulation on targeting, we used a previously validated tissue transport simulation[24, 33, 53–54] incorporating the parameter values measured in this work (SI). The results showed this partial differential equation simulation can be simplified to an ordinary differential equation (compartmental) model without loss in accuracy (Fig. 6). In short, the imaging agent extravasates out of the pancreatic vasculature and is either specifically internalized after binding to GLP-1R, nonspecifically internalized by the exocrine pancreas, or washes out of the tissue. Both specific uptake in the islets and the internalization in the exocrine pancreas and nearby non-target organs such as liver and kidney are important for determining the target to background ratio in the pancreas. Additionally, kidneys express GLP-1R[55–56] but signal is dominated by non-specific uptake due to scavenger receptors and transporters in the proximal tubule. In the pancreas, GLP-1R is weakly expressed in acinar cells of the exocrine pancreas whereas endocrine expression dominates[57–58]. With the experimentally determined time-dependent internalization rates as inputs for the compartmental model, we demonstrate TBR is maximized with rapidly cleared agents and decreases as uptake in non-target tissue increases. This is primarily due to the down-regulation in receptor internalization at longer times and increased non-target tissue uptake for slow clearing agents.

Discussion

Despite growing interest in developing GLP-1R imaging agents, the impact of plasma clearance and receptor kinetics on targeting efficiency have not been fully elucidated. The plasma clearance of exenatide has been manipulated through peptide/protein fusions[59–61] and PEGylation[46–47] for drug treatment. However, ideal pharmacokinetics for imaging agents are typically very different than effective therapeutics. For example, rapid internalization of the GLP-1 receptor has been demonstrated upon peptide binding[24, 48].

While this can be problematic for therapeutics as endocytic pH changes and protease activity may rapidly degrade the molecule[62], internalization is often beneficial for imaging agents by trapping a pH-tolerant residualizing label within the cell[63]. To quantify the impact of plasma clearance and internalization on the molecular imaging of GLP-1 receptors, we generated a series of exenatide-based probes, measured GLP-1 receptor expression and internalization rates, and tested probe pharmacokinetics and imaging properties *in vivo*.

To investigate the effects of molecular weight on uptake in target and non-target tissue, a bifunctional linker was created to simultaneously crosslink multiple exendin peptides and conjugate a highly charged and fast clearing 800CW dye (Fig. 1). Additionally, a slower clearing exendin conjugate was synthesized using a more hydrophobic Cy7 dye that binds to plasma proteins[52]. All conjugates were synthesized with moderate yield (32–70%) and maintained high affinity (Fig 2). Unexpectedly, the 800CW dimer and 800CW trimer molecules resulted in weakened affinity upon crosslinking. This is likely due to steric effects that outweigh any benefit from avidity. A longer linker between the peptides may reduce this effect, but the cellular kinetics mitigate any benefit. The rapid internalization of GLP-1 receptor results in most of the probe being internalized before it can dissociate (SI), similar to many antibodies[64].

Literature reports on the internalization of GLP-1 receptor ligands vary in both rate and time-dependence, with some showing linear uptake over time[65] and others indicating rapid internalization followed by down-regulation[48, 66]. To determine the potential amplification of cell labeling by internalization and recycling, NIT-1 cells were incubated with excess probe at 37°C to allow for continuous uptake. The surface signal was quenched at each time point to decouple surface-bound and internalized probe. Fast surface labeling and internalization indicated by punctate vesicles in the cytosol occurred within minutes, consistent with previous observations. A time-lapse video (SI) along with live cell images at short times (<5 min) indicate the presence of numerous punctate, intracellular spots indicative of receptor trafficking upon internalization. This initial phase of uptake was followed by a much slower rate of continual internalization (half-life 220 min versus 5.7 min, Fig. 3). This 38-fold reduction is predicted to lower the impact of sustained targeting on islet signal.

Additionally, we sought to measure the endogenous expression level of GLP-1 receptor in mice. Reports of absolute receptor expression levels are rare and often variable in the literature. Levels on the RIN-m5f cell line have been reported at 1,107 receptors/cell[67]. However, our results *in vivo* indicate a higher expression based on signal intensity[24]. The reduced expression on the insulinoma cell line may be a result of adaptation to cell culture. Because labeling islet cells *ex vivo* resulted in variable signal, we injected a saturating dose (1.2 nmol) of AF647 monomer into MIP-GFP mice and harvested the pancreas after only 30 min to minimize continuous uptake. After obtaining a single cell suspension, the cells were run on flow cytometry and compared to quantitative beads. The results indicate 54,000 receptors per cell in B6 mice and 17,000 receptors per cell for the NIT-1 cell line.

To test whether slow clearance can be used to increase islet labeling, the different constructs were injected into B6 mice, and the plasma clearance and biodistribution were measured

(Fig. 4). For plasma clearance, no statistically significant difference occurred for the monomer (5.3 kDa), dimer (9.5 kDa), and trimer (13.7 kDa) labeled with 800CW. This is consistent with molecules that are well below the ~60 kDa cut-off for renal filtration[68]. However, the monomer with a lipophilic Cy7 dye showed over a 7-fold increase in AUC due to plasma protein binding and reduced renal clearance[52].

The targeting of islets for each probe was confirmed by imaging the pancreas of each mouse, with islets readily apparent as bright, punctate foci ~100–300 microns in diameter (Fig. 5). Using a MIP-GFP mouse, which expresses GFP exclusively in the beta cells, colocalization confirmed islet specificity (Fig. 5b). All 4 constructs showed excellent islet targeting (Fig. 5c), but no statistically significant differences occurred in the pancreas %ID/g. The lack of higher uptake from the more slowly cleared agent is likely due to the down-regulation and slower net internalization of GLP-1 receptors upon sustained exposure to exendin peptide (Fig. 3). Since the initial rapid internalization is responsible for the majority of specific beta cell signal, a slow clearing imaging agent with detectable concentrations in the blood at longer times is not advantageous when the goal is to maximize the TBR.

Off-target uptake is equally important in designing effective imaging agents as demonstrated by the biodistribution. Despite similar plasma clearance rates, the renal uptake of the dimer and trimer are 3-fold higher than the monomer. After filtration by the glomerulus, degradation and scavenger receptors in the renal proximal tubule recycle amino acids and peptides, preventing excretion in the urine[69]. Uptake of the dimer and trimer in the proximal tubule by scavenging receptors appears to be more efficient than the monomer and highlights a drawback of multimerization. Our results here are consistent with other reports[70–71] showing higher renal uptake for multimers, motivating the development of high affinity monovalent constructs[68, 72–73] rather than using avidity to compensate for low affinity. Although not statistically significant in all cases, the dimer and trimer generally have higher signal in other tissues as well. Non-specific internalization in off-target tissues is a major determinant of probe TBR, particularly for rapidly cleared low molecular weight agents. The rapid plasma clearance allows imaging after the majority of probe is removed from the blood (< 0.5% in the case of the monomer, dimer, and trimer). However, due to the residualizing IRDye 800CW label[74], both specifically and non-specifically internalized IRDye 800CW label wash out of tissue extremely slowly, negating any benefit of later imaging times.

The lipophilic Cy7-conjugate had ~10-fold slower clearance than the IRDye 800CW monomer, but tissue differences were more modest when the majority of probe had cleared from the plasma. One significant difference was the higher liver uptake of the Cy7 monomer. An alternative approach to slow clearance is to add an albumin binding peptide sequence through solid-phase synthesis [60], but increased liver uptake due to longer plasma exposure and/or scavenger uptake[75–76] and down-regulation of GLP-1R may not provide any targeting benefit over the more rapidly cleared monomer. In the pancreas, total uptake of 800CW and Cy7 monomers was not statistically different ($p = 0.26$). To measure the specific uptake in the pancreas (versus total uptake), mice were given a large dose of wild-type exendin-4 45 minutes prior to injection of either 800CW monomer or Cy7 monomer. The higher non-specific uptake in the pancreas ($p = 0.024$), despite having 5 h to clear (versus 3

h for 800CW), is consistent with the internalization conclusions: the majority of specific uptake in the target tissue occurs on the time scale of minutes given the rapid internalization rate of GLP-1R. Slower clearance does not increase specific uptake as demonstrated by similar specific %ID/g between the Cy7 monomer and 800CW monomer. Considering the fact that islets only make up ~1–2% of the pancreas mass, specific uptake of 0.5% ID/g on a whole pancreas basis would mean the efficiency of uptake within the islets must be 25–50% ID/g. Although the high blood flow, fenestrated capillaries, and large capillary surface area provide excellent delivery to the islet, this analysis suggests equal consideration should be paid to reducing non-specific uptake in the tissue due to charge and hydrophobic interactions[77–79]. Additionally, ratio imaging may help normalize the non-specific uptake to better quantify specific targeting[80].

Ultimately, a radiolabeled probe is required for clinical imaging given the depth restrictions with near-infrared fluorescence. The advantage of fluorescent tags during probe development is the tissue and cellular resolution, which enables *in vivo* receptor expression, quantification of rapid cellular trafficking, and high-resolution histology for islet colocalization as reported here. NIR fluorescence can also provide whole animal biodistribution and plasma clearance similar to radiolabeled probes. One major drawback is the plasma protein binding of the fluorescent tag, which can differ depending on the radiolabel and significantly impact non-specific uptake and plasma clearance.

To determine the impact of down-regulation and plasma clearance on imaging, a mechanistic, quantitative, and predictive model was used to examine the interdependent parameters. No experimental data were fit to the kinetic model, allowing for an independent comparison of islet targeting. A full distributed-parameter simulation[53–54] was first conducted to show that the rapid diffusion and highly vascularized islet results in relatively homogeneous islet distribution, enabling the use of a compartmental analysis (SI). The kinetic analysis using the receptor expression, internalization kinetics/downregulation, and biodistribution parameters from this work indicate a rapidly cleared agent provides higher TBR than a slowly cleared agent (SI). The predicted uptake values were within experimental error of the measured uptake values. However, quantitative analysis of islet receptor expression indicates that a significant portion of the specific signal may be from low-level exocrine cell expression (SI), and plasma protein binding of the fluorescent-exendin derivatives was an important parameter in the simulations (SI). Being able to quantify plasma protein binding, blood clearance rate, and uptake rate in the exocrine pancreas as well as nearby organs such as kidney and liver will aid in imaging agent design when scaling to the clinic.

Sweet et al. estimated that the signal from islets would need to be 10-fold higher than non-specific exocrine signal for accurate BCM quantification[32]. Since beta cells form ~1% of the pancreas, the beta cell signal needs to be 1,000-fold higher than exocrine cells. This is a challenging task, particularly if a significant fraction of exocrine cells express low levels of GLP-1R[57–58]. A quantitative and mechanistic understanding of other targeting molecules[81–82] rather than exclusively biodistribution data will help make rational comparisons between agents. These quantitative simulations can also focus design efforts for improvements in the development of new agents to achieve the necessary specificity.

Conclusions

We investigated the impact of plasma clearance and receptor down-regulation using four different GLP-1 receptor imaging agents varying in molecule weight and plasma clearance. The high affinity of the monomeric probe was sufficient for specific targeting, whereas higher non-target tissue uptake, particularly in the kidney, highlights the drawbacks of imaging with multimeric peptides. Targeting of a plasma protein binding probe was mitigated by the rapid down-regulation of the receptor. Our results indicate the ideal BCM imaging agent clears quickly from blood to benefit from early rapid internalization while avoiding non-target uptake at later times. High levels of receptor expression ($> 50,000$ receptors per cell), rapid internalization, and an easily accessible cell surface location make GLP-1 receptor an attractive target for BCM quantitation, and simulations help identify the most important areas for improvement. Lowering non-specific interactions through peptide stabilization[43–44] and/or accounting for this signal through ratio imaging[80] will help increase the precision and sensitivity of beta cell mass measurements for the early detection of type 1 diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design and synthesis of fast clearing IRDye 800CW conjugates and a slow clearing Cy7 monomer. Di-alkyne and tri-alkyne linkers were used to simultaneously generate exendin multimers and conjugate IRDye 800CW. Exendin crystal structures (PDB 3C59) are shown with the modified residues highlighted in red.

Figure 2. MALDI-TOF traces of purified imaging agents demonstrating successful synthesis (a). Affinity curves indicate a strong affinity of all conjugates to GLP-1R (b) with fitted K_d values (c).

Figure 3.

In vitro internalization assay indicates rapid surface binding and internalization followed by slower internalization and GLP-1R downregulation (a). Live cell imaging of NIT-1 cells labeled with AF488 monomer (green intensity, b). Cells nuclei stained with Hoechst 33342 (blue). Fitted values for internalization and receptor expression (c).

Figure 4.

Plasma clearance is rapid for all the hydrophilic IRDye 800CW conjugates, while the lipophilic agent clears slowly (a). Biodistribution shows higher non-specific uptake of multimers in the kidney and Cy7 uptake in the liver (b). Clearance rates were not significantly different between the monomer, dimer, and trimer (c).

Figure 5.

Macroscopic pancreas scans indicate punctate spot formation for all exendin conjugates (a, c). Confocal microscopy demonstrates islet specificity using a transgenic MIP-GFP mouse through colocalization of GFP and probe (b).

Figure 6.

Compartmental analysis of transient probe concentrations using receptor expression, binding rates, internalization kinetics, and biodistribution parameters. At later times, the majority of the probe is predicted to be in the internalized compartments for both target and non-target tissue, highlighting the importance of minimizing non-specific internalization.