A number of exendin derivatives have been developed to target glucagon-like peptide 1 (GLP-1) receptors on beta cells in vivo. Modifications of exendin analogues have been shown to have significant effects on pharmacokinetics and, as such, have been used to develop a variety of therapeutic compounds. Here, we show that an exendin-4, modified at position 12 with a cysteine conjugated to a tetrazine, can be labeled with \(^{18}\text{F}-\)trans-cyclooctene and converted into a PET imaging agent at high yields and with good selectivity. The agent accumulates in beta cells in vivo and has sufficiently high accumulation in mouse models of insulinomas to enable in vivo imaging.

**Introduction**

The ability to visualize beta cells noninvasively could have far reaching implications for both biomedical research and clinical practice. Progressive loss of functional beta cell mass (BCM) is the underlying cause of autoimmune type 1 diabetes mellitus, and is also responsible for the secondary failure of clinical drugs in type 2 diabetes. It is widely believed that noninvasive imaging of beta cells could ultimately facilitate not only our understanding of the natural history of islet formation but also the pathophysiology of diabetes. In turn, we would have the capability to diagnose diabetes earlier, monitor the efficacy of widely used drugs, as well as advance the discovery of new therapies. Furthermore, beta cell-specific imaging approaches could be used to diagnose and localize insulinomas and aid the assessment of transplanted islets or pancreata.

In a previous report, we described the development and validation of near infrared fluorescent exendin-4 analogues for imaging beta cells at single cell resolutions,\(^{11}\) and for fiberoptic, endoscopic or intraoperative imaging.\(^{11}\) We showed that one lead agent, derived from exendin-4 (E4 K12-FL), had sub-nanomolar EC\(_{50}\) binding concentrations and high specificity. In addition, its binding could be inhibited by glucagon-like peptide 1 (GLP-1) receptor agonists. Following intravenous administration to mice, pancreatic islets could be readily distinguished from exocrine pancreas, achieving target-to-background ratios of 6:1. Serial imaging subsequently revealed rapid accumulation kinetics (with initial signal in the islets detectable within 3 min and peak fluorescence occurring within 20 min of injection). Such properties make this an ideal agent for in vivo imaging. Together with other reports of various exendins labeled with chelates,\(^{3–9}\) we hypothesized that \(^{18}\text{F}\)-labeled exendin-4 analogues could be used for noninvasive imaging with positron emission tomography-computed tomography (PET–CT). While two approaches of \(^{18}\text{F}\)-labeling have been recently reported,\(^{12,13}\) the \(^{18}\text{F}\)-conjugation methods used in these studies do not appear to have been used in concert with removal of unreacted material via bioorthogonal scavenging resins.\(^{12}\)

In this study, we started with a cysteine (C12) version of our previously validated exendin-4 (E4 K12), by exchanging the lysine at position 12 with a cysteine. Using bioorthogonal labeling strategies employing \(^{18}\text{F}-\)trans-cyclooctene (\(^{18}\text{F}-\)TCO) and tetrazine (Tz) modified molecules,\(^{13–15}\) we report the facile synthesis and purification of \(^{18}\text{F}\)-labeled exendin-4. The described reaction demonstrated fast reaction times (20 min), high purity as well as specific activity. Given that the ultimate goal is to translate this technology to the clinic, a lead \(^{18}\text{F}\)-labeled compound was subsequently applied to PET–CT imaging of insulinoma in a mouse model. Pharmacokinetic modeling, the plasma clearance and tracer-uptake data obtained from these experiments were subsequently used for extrapolation to humans.

**Results and Discussion**

We previously demonstrated that modification of the exendin-4 amino acid sequence at position 12 does not result in perturbation of the molecule’s intrapancreatic binding, selectivity or specificity for the GLP-1 receptor. In order to translate this finding into a noninvasive \(^{18}\text{F}\)-PET probe, we designed the cysteine-tetrazine (Tz) cross-linker, maleimide-Tz 3 (Scheme 1). The

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**References**

1. E. J. Keliher,* T. Reiner,* G. M. Thurber, R. Upadhyay, Prof. R. Weissleder
   Center for Systems Biology, Massachusetts General Hospital
   185 Cambridge St, CPZN 5206, Boston, MA 02114 (USA)
   Fax: (+1) 617-726-8226
   E-mail: rweissleder@mgh.harvard.edu

2. Prof. R. Weissleder
   Department of Systems Biology, Harvard Medical School
   200 Longwood Ave, Boston, MA 02115 (USA)

3. These authors contributed equally

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compound was synthesized from the literature-known Tz amine and the maleimide-NHS ester in 68% isolated yield. This crosslinker readily reacted with E4 C12, an exendin-4-related peptide in which the natural lysine at position 12 (K12) was exchanged for a cysteine (C12) yielding the bioorthogonally reactive Tz-labeled peptide E4 Tz12. Figure 1 shows liquid chromatography–mass spectrometry (LC–MS) traces of both maleimide-Tz (Figure 1A) and E4 Tz12 (Figure 1B), which confirm the identities of the cold precursors.

Similar to the techniques used for small-molecule radiolabeling, we subsequently incubated E4 Tz12 with 18F-trans-cyclooctene (18F-TCO). The radiolabeled bioorthogonally reactive prosthetic group 18F-TCO was synthesized in 46% decay-corrected radiochemical yield (dcRCY) by nucleophilic substitution of the tosylate precursor with 18F-fluoride in the presence of tetrabutylammonium bicarbonate (TBAB), as previously described. 18F-TCO (92 ± 12 µCi [3.40 ± 0.44 MBq]) via tail vein into transgenic mice that express enhanced green fluorescent protein (eGFP) under the control of the mouse insulin promoter (MIP)

Scheme 1. Synthetic scheme for the synthesis of radiolabeled 18F-E4 Tz12. Reagents and conditions: a) triethylamine, acetonitrile/dimethylformamide (4:1), 1 h, 68%; b) 1× PBS/dimethylformamide (20:1), 3 h, 29%; c) 18F-TCO, 1× PBS/DMSO (1:4), 20 min, 45% dcRCY.

Figure 1. Mass spectra analysis. LC-ESI-MS traces of A) maleimido-Tz and B) E4 Tz12.
green fluorescent protein (GFP). After 3 h, the mice were euthanized, and their pancreata excised. The pancreata were then imaged using surface reflectance imaging (to show the islet distribution) before being exposed for autoradiography (to show the distribution of \(^{18}\)F-E4\(_{12}\) \(_{17}\) ). Figure 3 shows good colocalization between the fluorescence of the GFP islet and the autoradiographic signal from \(^{18}\)F-E4\(_{12}\) \(_{17}\) with a Pearson’s coefficient of 0.83 \pm 0.04 (R\(_{coloc}\)). Based on micro-dissected specimens and target-to-background ratios, we calculated a concentration of approximately 40 %ID g\(^{-1}\) in the islets.

To determine the utility of \(^{18}\)F-E4\(_{12}\) \(_{17}\) for insulinoma detection, we tested it in different murine models: NIT-1, 916-1 or WTRT2 mouse insulinoma xenografts. These cell lines were chosen for their elevated GLP1R expression as verified by Western blot (Figure 4C). For tumors, uptake values of 2.5 %ID g\(^{-1}\) (916-1), 2.0 %ID g\(^{-1}\) (WTRT2) and 0.7 %ID g\(^{-1}\) (NIT-
1) were obtained, which allowed them to be detected by whole body PET imaging (Figure 4A and B). Tumor-to-muscle ratios from ex vivo scintillation counting data were 13.4, 10.5, and 14.6 for 916-1, WTRT2 and NIT-1, respectively. In all cases, preinjection of cold exenatide (250 μL, 60 μμ) resulted in a significant reduction of the standard uptake values (916-1: 82% reduction; WTRT-2: 54% reduction; NIT-1: 62% reduction). In contrast, muscle standard uptake values were not affected by preinjection with cold exenatide (0.11 %ID g⁻¹). This confirms the applicability and selective uptake of ¹⁸F-E⁴Tz12 ⁷ as a targeted probe for GLP-1 receptor-rich tissues.

Ultimately, these agents are being developed for their clinical application. While their clearance is very rapid in mice (80% with a 1.9 min half-life and 20% with a 26.8 min half-life for ¹⁸F-E⁴Tz12 ⁷), we were interested in determining the optimal clearance kinetics in humans. A compartmental pharmacokinetic model was thus developed to extrapolate our results from mice. The advantage of this model is that some of the parameters (e.g., plasma clearance) that vary between species can be scaled up, while others (e.g., the binding rate constants and radioactive decay half-life) are kept constant.

Using clinical data available for exenatide, the plasma concentration after continuous infusion ¹⁵ was fit to a two-compartmental model, in order to predict the percent clearance of a bolus imaging dose. The results indicated that 73% of the imaging agent dose redistributes to peripheral tissues with a rapid 1 min half-life, while the remaining 27% clears with a 63 min half-life. This is close to the percent clearance observed with insulin in humans following an intravenous bolus injection (76% with a 10 min half-life and 24% with a 86 min half-life ²⁰); the model therefore provides a reasonable estimate of clearance.

The exchange rate of the compound between the plasma and extracellular space was subsequently estimated from literature values ²¹ and adjusted to fit our experimental results in mice (Figure 5A). The results in Figure 5B show estimates of human uptake and clearance, based on clinical data, and using mechanistic rate constants from mice. In both cases, the specific uptake of the compound in islets is significantly higher than in the exocrine pancreas due to its specific target binding.

The GLP-1 receptor is highly expressed in beta cells within the islets of Langerhans as well as in functioning beta cell islet tumors (insulinosmas). GLP-1 analogues are a new class of peptide-based drugs used for the treatment of diabetes. Exenatide, the first FDA approved GLP-1 analogue, is a synthetic version of exendin-4. It is a 39-amino acid peptide isolated from the saliva of the Gila monster (Heloderma suspectum) and contains 53% sequence homology with GLP-1. A recent crystal structure of the extracellular domain of the GLP-1 receptor showed the binding mode of exendin-4 (amino acids 9–39). ²² From this crystal structure, it was clear that lysine 12 (K12) is not involved in binding to the GLP-1 receptor domain. Moreover, it explains why K12-modified exendins retain high affinity for the receptor. ¹⁻¹ Our results further demonstrate that K12 modification with tetrazines are not only stable but allow rapid site-specific and high-yield fluorinations. Tetrazine functionalization of the peptide also allows removal of unreacted starting material with the complimentary trans-cyclooctene beads, an option not available to other current ¹⁸F or metal chelation labeling strategies. The resulting compounds exhibit appropriate pharmacokinetics for PET imaging of beta cells in a mouse model.

In an effort to predict the compound’s kinetics in humans, we applied pharmacokinetic modeling and allometric scaling. ²³,²⁴ In the mouse, the synthesized compound had a weighted half-life (t½w) of 6.8 min. Using our modeling and scaling approach, we predicted a t½ value of 18 min in human. Importantly, we found that this molecule size has a beta phase clearance half-life of 63 min. Agents with clearance rates that are much slower than the radioactive half-life could have a background that is too high during the imaging window. Conversely, agents that clear much faster than the radioactive half-life could have inefficient accumulation within the target tissue. Given that the decay of ¹⁸F is 109.8 min, our modeling indicates that this compound would have close to ideal clearance for human imaging. The pharmacokinetic modeling also indicates that further improvements in linker modification could reduce exocrine uptake and improve detection sensitivity. For example, by using bioorthogonal chemistry, which allows facile modulation of the linkers, further improvements in the reaction kinetics, stability and biocompatibility of the compound could be achieved ²⁵,²⁶.

**Experimental Section**

**Chemistry**

**General:** Unless otherwise noted, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. Exendin-4 (exenatide, Byetta®) was obtained from Amylin/Eli Lilly (San Diego, CA, USA). E4C12 (4163 g mol⁻¹; HGEGTTTSDLLSQMEEAVRFLIEWLNGGPSGAPPSPS) was obtained from Genscript (Piscataway, NJ, USA). [¹⁸F]:Fluoride (n.c.a.) was purchased from PETNET Solutions (Woburn, MA, USA). 3-maleimido-propanoic acid succinimidyl ester 1, tetrazine (Tz) amine 2 and ¹⁸F-trans-cyclooctene (¹⁸F-TCO) 4 were synthesized as described else-
where. High performance liquid chromatography-electro-spray ionization mass spectrometry (HPLC-ESI-MS) analyses and HPLC purifications were performed on a Waters LC-MS system (Milford, MA, USA). For LC-ESI-MS analyses, a Waters X Terra C18 5 mm column was used. For preparative runs, an Atlantis Prep OBC 2 mm column was used. High-resolution ES mass spectra were obtained on a Bruker Daltonics APEX IV 4.7 Tesla Fourier Transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) in the Department of Chemistry Instrumentation Facility at the Massachusetts Institute of Technology. Proton nuclear magnetic resonance (1H NMR) spectra were recorded on a Varian AS-400 (400 MHz) spectrometer. Chemical shifts for protons are reported in parts per million (ppm) and are referenced against the [D6]acetone lock signal (1H, 2.05 ppm). NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constants (Hz) and integration.

3-maleimido propanamide-tetrazine (maleimido-Tz): A solution of 3-maleimido-propanoic acid succinimidyl ester (5 mg, 19 μmol, 20 mg/mL) in DMSO was added to a solution of E4Tz12 (5 mg, 19 μmol) in MeCN (1 mL), and the resulting reaction mixture stirred at RT for 1 h. Volatiles were removed in vacuo and the crude product purified using HPLC to give compound 3 as a pink solid (4.4 mg, 13 μmol, 68%): 1H NMR (400 MHz, D2O; [D6]acetone): δ = 10.43 (s, 1H), 8.52 (d, 8JNH = 3.2, 2H), 7.78 (m, 1H), 8.58 (d, 8JNH = 8.2, 2H), 6.86 (s, 2H), 4.52 (d, 8JNH = 6.0, 2H), 3.80 (t, 8JCH = 7.4, 2H), 2.59 ppm (t, 8JCH = 7.4, 2H); LC-ESI-MS+ (m/z): 339.2 (100) [M + H]+, 677.4 (29) [2M + H]+; LC-ESI-MS− (m/z): 371.7 (29) [M−H]−, 383.1 (100) [M + HCOO−]−, 721.3 (27) [2M + HCOO−]; HRMS-ESI: m/z [M−H]+ calcd for [C18H14N6O3Na] 361.1020, found 361.1013 (100); [M+H]− calcd for [C18H14N6O3Na] 383.1013, found 383.1009 (100). E4Tz12: A solution of maleimido-Tz (50 μL 10 mM) in DMF was added to a solution of E4Tz12 (3 mg, 0.7 μmol) in 1×PBS (1000 μL) and the resulting solution was stirred at RT for 3 h. The reaction mixture was purified using an Amicon Ultra 3 kDa centrifugal filter (Millipore, Carrigtwohill, Ireland) before being subjected to HPLC purification, yielding compound 5 as a rose-colored solid (0.8 mg, 0.2 μmol, 29%): LC-ESI-MS+: m/z (%): 1125.9 (100) [M+4H]4+; 1501.3 (51) [M+3H]3+; LC-ESI-MS−: m/z (%): 1498.7 (100) [M−3H]−.

18F-E4Tz12: 2-(4-[18F]Fluoro)-3-(2-Fluoroethoxy)cyclooct-1-ene (18F-TCO) was prepared in a similar manner to previously described procedures employing a Synthra RN Plus automated synthesizer (Synthra GmbH, Hamburg, Germany) operated by SynthraView software in an average time of 102 min. The synthesizer reagent vials were prepared in a similar manner to previously described procedures employing a Synthra RN Plus automated synthesizer (Synthra GmbH, Hamburg, Germany) operated by SynthraView software in an average time of 102 min.

Cell biological evaluation: We chose three different insulinoma tumor cell lines (NIT-1, WRT2, 916-1), to correlate imaging findings and to elucidate how 18F-E4Tz12 behaves in different insulinoma tumor environments. Both WRT2 and 916-1 were generously provided by Johann Joyce (Memorial Sloan–Kettering Cancer Center, New York City, USA). NIT-1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). WRT2 and 916-1 were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with fetal bovine serum (10%), l-glutamine, penicillin (100 IU), and streptomycin (100 μg/mL). NIT-1 were cultured in F-12k medium (Kaighn’s Modification of Ham’s F-12 Medium, ATCC, Manassas, VA, USA) supplemented with fetal bovine serum (10%), sodium bicarbonate (2%), l-glutamine, penicillin (100 IU), and streptomycin (100 μg/mL). All cell lines were cultured at 37 °C and 5% CO2.

Western blot: 916-1, WRT2, and NIT-1 cells seeded into six-well plates were washed twice with ice-cold 1×PBS and lysed on ice for 10 min with ice-cold RIPA lysis buffer (100 μL) supplemented with a 100-fold dilution of protease inhibitor cocktail for mammalian cells (Sigma–Aldrich). The lysate was centrifuged (10 min, 10,000 g) and the supernatant collected. Protein concentrations were determined using bicinchoninic acid (BCA) protein assays (Pierce, Rockford, IL, USA). Cell lysates (10 μg) were subjected to SDS-PAGE, followed by immunoblotting using anti-GLP-1R antibody (#39072, Abcam, Cambridge, UK), goat-antibody secondary (Jackson ImmunoResearch, West Grove, PA, USA), and detection with chemiluminescence (PicoWestern ECL substrate, Pierce). Blots were stripped using Restore Stripping Buffer (Thermo Scientific), labeled with anti-GAPDH antibody (AF 5718, R&D Systems) followed by detection with chemiluminescence.

Mice: Experiments were performed in Nu/Nu mice (from Massachusetts General Hospital, Boston, MA; for tumor implantations and imaging; n = 6), C57BL/6 (B6) mice (from The Jackson Laboratory, Bar Harbor, ME; for biodistribution and pharmacokinetics; n = 8), or B6.Cg-Tg(Ins1-EGFP)1Hara/J mice (from The Jackson Laboratory, Bar Harbor, ME; for autoradiography/surface reflectance imaging; n = 3). B6.Cg-Tg(Ins1-EGFP)1Hara/J mice express the enhanced green fluorescent protein (eGFP) in the islets under the control of the mouse insulin 1 promoter (MIP-GFP). For all surgical procedures and imaging experiments, mice were anesthetized with 2% isoflurane in O2 at 2.0 L/min. For imaging experiments last-
ing longer than 1 h, the isoflurane flow rate was reduced to –1.0% isoflurane in O₂ at 2.0 L/min. Surgeries were conducted under sterile conditions with a zoom stereomicroscope (Olympus S261). All procedures and animal protocols were approved by the Massachusetts General Hospital subcommittee on research animal care.

Whole pancreas islet imaging: B6.Cg-Tg(Ins1-EGFP)1Hara/J (MIP-GFP) mice were administered ¹⁸F-E4Tz12 (7 (92 ± 12 μCi (3.40 ± 0.44 MBq)) via intravenous tail-vein injection, and the GLP-1 receptor-specific probe was allowed to accumulate and clear for 3 h. Mice were then euthanized, their organs perfused using 1× PBS (30 mL) and the pancreata harvested. They were subsequently weighed and activity measured using a Wallac Wizard 3″ 1480 Automated Gamma Counter. Blood sampling was performed by retro-orbital puncture using heparinized capillary tubes. Samples were subsequently processed and their radioactivity counted using a Wallac Wizard 3″ 1480 Automated Gamma Counter. Statistical analysis was performed using ImageJ 1.45 software.

¹⁸F-E4Tz12 biodistribution studies: C57BL/6 (B6) mice were used for blood half-life determinations. Mice were administered ¹⁸F-E4Tz12 (68 ± 12 μ Ci (2.52 ± 0.44 MBq)) via intravenous tail-vein injection. Blood sampling was performed by retro-orbital puncture using heparinized capillary tubes. Samples were subsequently weighed and activity measured using a Wallac Wizard 3″ 1480 Automatic Gamma Counter (PerkinElmer). Blood half-life data were fitted to a biexponential model using Graphpad Prism 4.0c software (GraphPad Software Inc., San Diego, CA), and results were reported as the weighted average of the distribution and clearance phases. For biodistributions, (B6) mice were intravenously injected via tail vein with ¹⁸F-E4Tz12 (7 (131 ± 18 μ Ci (4.85 ± 0.67 MBq))). Mice were euthanized at 3 h and their organs perfused using 1× PBS (30 mL). Tissues were subsequently harvested, weighed and their radioactivity counted using a Wallac Wizard 3″ 1480 Automatic Gamma Counter. Statistical analysis was performed using Graphpad Prism 4.0c.

MicroPET-CT imaging: Mice were imaged by PET-CT using an Inveon small animal microPET scanner (Siemens Medical Solutions). Mice were injected with ¹⁸F-E4Tz12 (557 ± 38 μ Ci (20.61 ± 1.41 MBq)) via tail-vein injection under isoflurane anesthesia (see above). Acquisition for static microPET images started 2 h post injection and acquisition took approximately 30 min. For dynamic microPET imaging, mice were injected approximately 30 s after the start of microPET acquisition, and data was collected for 2 h. The radioactivity concentration for a tissue was determined by measuring within regions of interest (ROIs) for a given tissue with the units of Bq/mL·min⁻¹. A tissue density of 1 g mL⁻¹ was assumed and ROIs were converted to Bq g⁻¹·min⁻¹ and divided by the injected activity to obtain an imaging ROI-derived %ID·g⁻¹. For GLP-1 receptor blocking experiments, unlabeled exenatide (250 μL, 60 μm) was preinjected 45 min prior to injection of ¹⁸F-E4Tz12. A high-resolution Fourier rebinning algorithm was used, followed by a filtered back-projection algorithm using a ramp filter, to reconstruct 3D images without attenuation correction. The image voxel size was 0.796×0.861×0.861 mm, for a total of 128×128×159 voxels. Peak sensitivity of the Inveon account for 11.1% of positron emission, with a mean resolution of 1.65 mm. The total counts acquired was 600 million per PET scan. Calibration of the PET signal with a cylindri-{

dral phantom containing ¹⁸F was performed before all scans. CT images were reconstructed using a modified Feldkamp reconstruction algorithm (COBRA) from 360 cone-beam X-ray projections (80 kVp and 500 μA X-ray tube). The isotropic voxel size of the CT images was 60 μm. The reconstruction of data sets, PET-CT fusion, and image analysis were performed using Inveon Research Work-{

place (IRW) software (Siemens). 3D visualizations were produced using a digital imaging and communications in medicine (DICOM) viewer (OsiriX Foundation, Geneva, Switzerland).

Modeling

A compartmental model was used to extrapolate results from mouse-imaging studies to humans. The model includes biexponential loss from the plasma compartment (due to redistribution and clearance), and separate compartments for the endocrine and exo-{

crine pancreas. Exchange with the endocrine tissue (islets) was esti-{

mated as a function of the vascular surface area-to-volume ratio (measured at 505 ± 146 cm⁻¹ using CD31 stained histology slides), and permeability was estimated at 30 μm s⁻¹ (for this sized molecule in the fenestrated capillary bed).

Exocrine pancreas was modeled in a similar manner, while the exchange param-

eters were adjusted to fit experimental data. Within the compart-

ments, the imaging agent is able to bind the target, dissociate, in-

ternalize, and be degraded and washed out. These rate con-

stants were assumed constant between species. For plasma clear-

ance in humans, the rate constants for exchange and clearance from a two-compartmental model were fit to experimental data taken from patients undergoing an intravenous infusion of exenata-

tide using a least-squares fitting algorithm in Matlab (Mathworks, Natick, MA, USA). Estimates for humans were obtained by entering the plasma clearance values from human clinical data into the model together with the microscopic transport rates obtained from mouse experiments.

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