Fluorescent Probes

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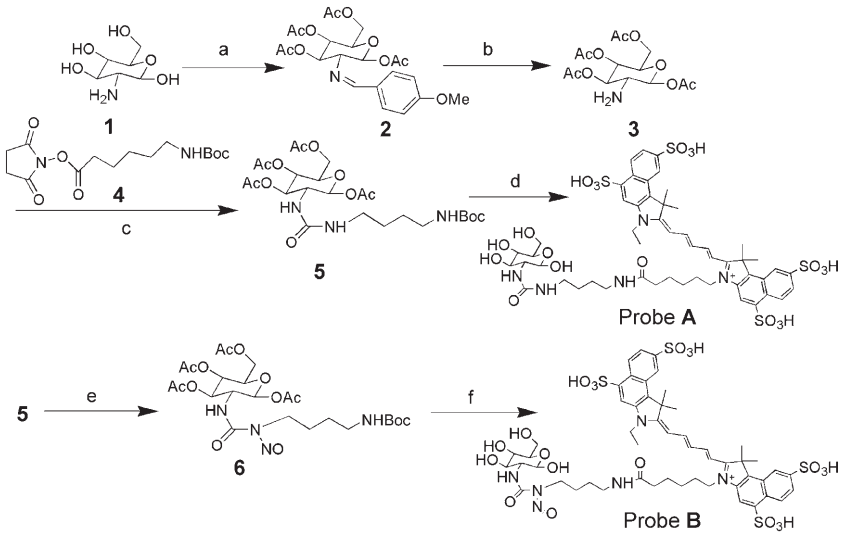
Synthesis and Testing of Beta-Cell-Specific Streptozotocin-Derived Near-Infrared Imaging Probes**

Chongzhao Ran, Pamela Pantazopoulos, Zdravka Medarova, and Anna Moore**

Pancreatic beta-cells are responsible for the production and release of insulin, a hormone that controls the level of glucose in the blood. Beta-cell mass is markedly reduced in both insulin-dependent and non-insulin-dependent diabetes mellitus.[1–5] The noninvasive estimation of beta-cell mass by imaging would have a significant impact on the management of clinical diabetes, pancreas- and/or islet-cell transplantation, and the understanding of the pathogenesis of the disease. Currently, measurements of insulin secretion and *c*-peptide production serve as surrogates for the assessment of beta-cell function.[6] However, these methods are not suitable for in vivo detection. Previously several attempts have been made by us and other investigators to image pancreatic-islet inflammation, rejection, and transplantation.[7–13] Here, we describe the synthesis and testing of near-infrared (NIR) probes for imaging beta-cells in pancreatic islets, based on the beta-cell-specific ligand streptozotocin (STZ), labeled with the fluorescent dye cyanine-5.5 (Cy5.5).



Scheme 1. Synthesis of the STZ-derived Cy5.5-labeled probes for beta-cell detection. a) 1. Anisaldehyde, NaOH; 2. acetic anhydride, pyridine; b) acetone, HCl; c) **4**, dichloromethane; d) 1. TFA; 2. Cy5.5–NHS; 3. NaOMe; e) *t*BuONO; f) 1. TFA; 2. Cy5.5–NHS; 3. NaOMe. Boc: *tert*-butoxycarbonyl; Cy5.5–NHS: cyanine-5.5–*N*-hydroxysuccinimide; TFA: trifluoroacetic acid.

Our probe design is based on the specificity of STZ for beta-cells and the following well-known facts. STZ is an FDA-approved drug for treating metastatic cancer of pancreatic-islet cells.[14] However, in the diabetes-research community, STZ is also widely used to establish mouse models of Type 1 diabetes, and this capability is ascribed to its specific toxicity to murine beta-cells.[15,16] It is believed that the selective targeting of STZ toward beta-cells is rooted in a specific interaction with the GLUT2 transporter,[17,18] which is only expressed in beta-cells (and not in alpha- or gamma-cells) within the adult pancreas and is essential for its glucose-sensing function.[19,20] Although the structure of STZ is very similar to glucose, it is not recognized by other glucose transporters such as GLUT1, GLUT3, and GLUT4.[17,18] This selectivity may arise from the nitrosourea moiety of STZ, which is the only part different from glucose. Therefore, in our probes **A** and **B** (Scheme 1), the 2-nitrosourea-glucose moiety of STZ was kept intact to maintain the probes' specificity for GLUT2 and beta-cells. On the other hand, in designing a high-quality imaging probe, it is mandatory to avoid STZ toxicity to beta-cells, which primarily arises from the alkylation of DNA and from the release of nitric oxide (NO).[21,22] Interestingly, for establishing mouse models of Type 1 diabetes, a widely used protocol requires that STZ be freshly pretreated in pH 4.5 citrate buffer. This requirement is consistent with the tendency of alkyl-nitrosourea compounds to generate nitric oxide (NO) and cause alkylation under acidic conditions.[21,22] These properties of STZ and the fact that it is an FDA-approved compound prompted us to speculate that if STZ and its derivatives are used under neutral conditions, their toxicity may decrease significantly and they could therefore safely be used as imaging probes. Moreover, to reduce the alkylation ability of STZ derivatives, we proposed the use of a longer chain to replace the methyl

[*] Dr. C. Ran, P. Pantazopoulos, Dr. Z. Medarova, Prof. A. Moore
Molecular Imaging Laboratory
Athinoula A. Martinos Center for Biomedical Imaging
Massachusetts General Hospital/Harvard Medical School
13th Street, Building 149, Room 2301, Charlestown MA 02129 (USA)
Fax: (+1) 617-726-7422
E-mail: zmedarova@partners.org
amoore@helix.mgh.harvard.edu

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group of STZ, since a longer chain has a much lower alkylating ability toward DNA.[23] In our probes **A** and **B**, the butyl chain was chosen as both the replacement of the methyl group and the linker between the STZ moiety and a fluorescent dye. Furthermore, to observe the effects of the nitroso (N=O) group for specific targeting and toxicity, we synthesized probe **A** without an NO group, while probe **B** had an NO group attached. Since imaging in the NIR region has minimal tissue autofluorescence, which dramatically improves target/background ratios, we employed the well-documented NIR dye Cy5.5–NHS as the fluorescent reporter for our probes **A** and **B**.

To synthesize probes **A** and **B**, we started from glucosamine (**1**), which was converted into **2** by protecting the 2-amino group with anisaldehyde and acetylating the 1,3,4,6-hydroxy groups with acetic anhydride. After removal of the anisylidene protecting group with hydrochloride solution,[24] **3** was obtained with a high yield and was treated with **4** to afford the key intermediate **5**. Synthesis of probe **A** was achieved by removing the BOC group of **5** with TFA and by subsequent reaction with Cy5.5–NHS and deacetylation in methanol. To prepare probe **B**, **5** was first transformed into **6** with *t*BuONO in dichloromethane, and then probe **B** was obtained from **6** by a similar procedure to that used for **A** (Scheme 1).

To test the specificity of the synthesized probes, we chose the insulinoma INS-1E cell line, which is known to mimic the behavior of pancreatic beta-cells.[25] For controls, we used the pancreatic adenocarcinoma CAPAN-2 cell line and the embryonic kidney HEK 293 cell line, which is nontumorigenic.

Our flow cytometry studies showed that probes **A** and **B** labeled almost all INS-1E cells (92.5 and 96.0%, respectively) but only 18.7 and 32.7% of CAPAN-2 cells and 18.3 and 20.1% of HEK293 cells, respectively (Figure 1). Confocal microscopy corroborated the results of the fluorescence-activated cell sort (FACS) and showed a significantly higher uptake of both probes by INS-1E cells compared to that by CAPAN-2 and HEK 293 cells and relative to the results with Cy5.5 dye (see the Supporting Information).

Further studies with INS-1E cells demonstrated that both probes were taken up by cells in a dose-dependent fashion (Figure 2A) and that this uptake reached a plateau after 8 h of incubation (Figure 2B). Since the specificity of STZ toward beta-cells originates from the particular targeting of GLUT2 on the beta-cells,[17,18] it is essential to test whether the modified-STZ-based probes **A** and **B** retain the specific interaction with the GLUT2 transporter on INS-1E cells. We conducted these experiments by incubating INS-1E cells with the GLUT2 inhibitor phloretin[26] for 8 h and then with probe **A** or **B** for 24 h. This was followed by measurement of the NIR-fluorescence intensity in cell lysates or by flow cytometry. There was a concentration-dependent inhibition of the uptake of both probes. Flow cytometry revealed a significant reduction of the mean fluorescence intensity following treatment with phloretin (34.9% with probe **A** and 38.3% with probe **B**; see the Supporting Information). From these results, we concluded that the cellular uptake of the probes was mediated by the GLUT2 transporter.

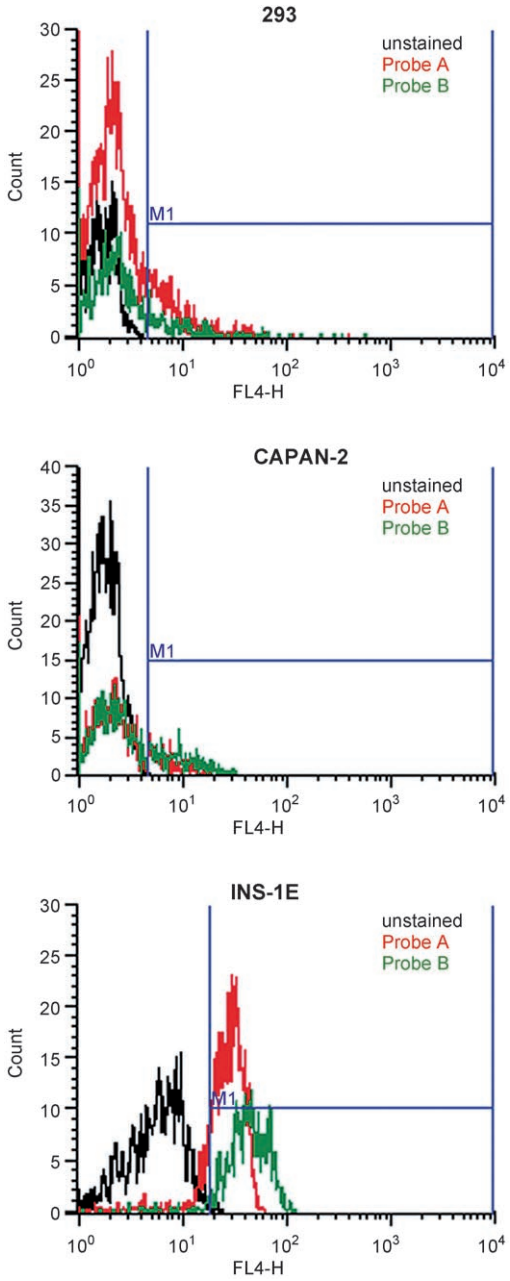

Figure 1. Cell labeling with probes **A** and **B**. Note that the probes labeled almost all of the INS-1E cells.

Since the ultimate goal of our studies is to image pancreatic islets in the native organ, we tested whether islets could be labeled with the probes. To that end, we incubated purified human islets with probe **A** or **B** for 24 h at a concentration of 6.0 μM and then subjected the islets to confocal microscopy after fixation in 4% formaldehyde. Untreated human islets served as a control. As anticipated, we observed a bright fluorescence signal consistent with intracellular accumulation of both probes (Figure 3). These results hold promise for further use of these probes in in vivo animal model studies.

For the successful development of a molecular imaging probe, low toxicity to the target is also a key factor. Since STZ

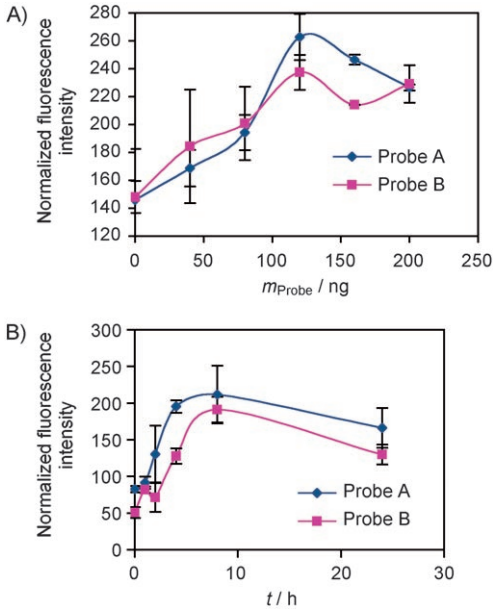

Figure 2. A) INS-1E cells take up probes **A** and **B** in a dose-dependent fashion. B) Cellular uptake reached a plateau after eight hours of incubation; m_{Probe}: amount of probe added.

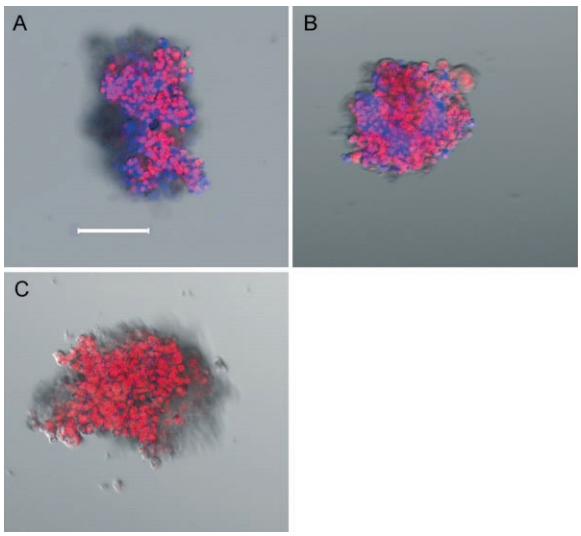

Figure 3. Confocal microscopy of human pancreatic islets treated with probe **A** (A) and probe **B** (B). No signal in the Cy5.5 channel was observed in the control of nontreated islets (C). Blue: Cy5.5; red: propidium iodide. Images were overplayed onto Nomarski optics; scale bar: 100 μm.

is a known inducer of diabetes in mice, we tested whether our probes caused toxicity in purified human pancreatic islets. The results of our TUNEL assay (see the Supporting Information) revealed that there was no significant cell death after incubation with either of the probes. There was no difference in the number of apoptotic cells between untreated islets and islets treated with probe **A** or **B**. Control islets treated with STZ had similar levels of apoptosis, which is anticipated when the low STZ toxicity towards human islets is taken into account.[27,28] These results are most likely indicative of the fact that STZ and probes **A** and **B** could be safe if used under neutral conditions (pH 7.4), instead of with a pH 4.5 citrate buffer pretreatment. Since the NO-releasable probe **B** has a very low NO-generation potency under neutral conditions, it is not surprising that both probes showed similar results.

Cheng et al. reported that direct conjugation of Cy5.5 with D-glucosamine at the 2-position produced Cy5.5–2DG conjugates, but these products did not show specific interaction with GLUTs.[29] They ascribed this nonspecificity to the large size of the Cy5.5–2DG conjugates. By contrast, our probes **A** and **B** have displayed considerable selectivity for the GLUT2 transporter, probably due to the higher similarity of **A** and **B** to the GLUT2-specific STZ molecule and the presence of a longer linker between Cy5.5 and the STZ moiety in our probes.

In summary, we have successfully developed two NIR probes for specific labeling of beta-cells through their interaction with the GLUT2 transporter. These two probes have displayed dose- and time-dependent behavior, low toxicity towards their target, and excellent selectivity for the beta-cell-derived INS-1E cell line over other cell lines. Furthermore, we confirmed that cell labeling by these probes was mediated by GLUT2. In addition, our data have demonstrated that our probes are not only able to label a beta-cell-like cell line but also have the capability to label human islets. We believe that our probes could be useful for pancreas- and/or islet-cell transplantation, monitoring, and the understanding of the pathogenesis of diabetes.

Experimental Section

Probes **A** and **B**: Triethylamine (40.4 mg, 0.4 mmol) and **4** (32.9 mg, 0.1 mmol) were added to a suspension of **3** (38.7 mg, 0.1 mmol) in an acetonitrile/water (10:1) solvent mixture (5.0 mL), and the resulting mixture was stirred at room temperature overnight. The solvent was evaporated to give a semisolid residue, which was subjected to flash silica gel chromatography with dichloromethane/ethanol (15:1) to give **5** (54.8 mg, 97.6%). TFA (100 μL) was added to a solution of **5** (4.4 mg, 8.0 μmol) in dichloromethane (100 μL), and the resultant mixture was stirred at room temperature for 2 h. After evaporation of the solvent and TFA, an oily residue was obtained. Cy5.5–NHS (1.0 mg), water (100 μL), acetonitrile (100 μL), and triethylamine (10.0 mg) were added to this residue. The resulting mixture was stirred at room temperature for 4.0 h, and this was followed by addition of sodium methoxide (108.0 mg) in methanol (100 μL). The resultant mixture was stirred at room temperature overnight, and the solvent was removed under vacuum to give an oily residue. This oily residue was subjected to a reversed-phase flash C18 column to give probe **A** (0.6 mg, 55.3%); ESI-MS: m/z 1192.9. Probe **B** (55.8%) was synthesized via **6** by a similar procedure to that for probe **A**; ESI-MS: m/z 1222.3.

INS-1E, CAPAN-2, and HEK293 cells were seeded on a 12-well plate and treated with increasing concentrations of probe **A** or **B** (for cell uptake studies) or with a fixed concentration of **A** or **B** (for confocal microscopy and flow cytometry (FACS)). For GLUT-2 specificity studies, the cells were pretreated with inhibitor (phloretin) before addition of the probes. The cells were then washed and fixed by using conventional procedures for FACS analysis and confocal microscopy. Islet sample preparation was similar to the cell sample preparation. Confocal microscopy was performed by using an

Axiovert 200M inverted microscope (Carl Zeiss). FACS analysis was performed with a FACSCalibur instrument (Becton Dickinson, San Diego, CA).

Purified human islets treated with probe **A**, probe **B**, or STZ (2 μM). After washing with phosphate-buffered saline and fixation in 4% formaldehyde, TUNEL staining was performed according to the manufacturer's protocol (DeadEnd Colorimetric TUNEL Assay, Promega, Madison, WI). Untreated islets served as a control.

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