

GPR44 is a pancreatic protein restricted to the human beta cell

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Abstract

Aims To address questions regarding onset and progression of types 1 and 2 diabetes (T1D/T2D), surrogate imaging biomarkers for beta cell function and mass are needed. Here, we assess the potential of GPR44 as a surrogate marker for beta cells, in a direct comparison with clinically used biomarker VMAT2.

Methods GPR44 surface availability was assessed by flow cytometry of human beta cells. RNA transcription levels in different pancreas compartments were evaluated. The density of GPR44 receptor in endocrine and exocrine tissues was assessed by the radiolabeled GPR44 ligand [³H]AZD 3825. A direct comparison with the established beta cell marker VMAT2 was performed by radiolabeled [³H]DTBZ.

Results GPR44 was available on the cell surface, and pancreatic RNA levels were restricted to the islets of

Langerhans. [³H]AZD 3825 had nanomolar affinity for GPR44 in human islets and EndoC-βH1 beta cells, and the specific binding to human beta cells was close to 50 times higher than in exocrine preparations. The endocrine-to-exocrine binding ratio was approximately 10 times higher for [³H]AZD 3825 than for [³H]DTBZ.

Conclusion GPR44 is a highly beta cell-specific target, which potentially offers improved imaging contrast between the human beta cell and the exocrine pancreas.

Keywords Beta cell imaging · Beta cell mass · GPR44 · VMAT2

Introduction

There is considerable interest in development of novel surrogate biomarkers for beta cells, due to the fact that current blood markers correlate to beta cell function rather than mass. Proposed novel plasma markers of beta cell death and function may alleviate this issue, but these will in the best case still constitute an indirect measure of beta cell mass [1–3]. Molecular imaging of the pancreas has been proposed as a solution for this clinical need. Positron emission tomography (PET) is a quantitative and essentially noninvasive technique with the sensitivity required for clinical in vivo imaging of beta cell mass.

The anatomical localization of the beta cells in humans sets strict requirements on putative beta cell imaging biomarkers. The beta cells constitute approximately 60 % of the islets of Langerhans, cell clusters of varying size (5–500 μm) which are distributed heterogeneously within the exocrine parenchyma. Additionally, there are pancreatic beta cells not embedded in islets of Langerhans. Clinical PET scanners have sufficient resolution

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(approximately 3 mm) to image the whole or parts of the pancreas, but not individual islets. Therefore, the aim is to measure the integrated pancreatic signal of a beta cell biomarker, which potentially correlates to the present beta cell mass in the individual.

Consequently, imaging biomarkers (for example a receptor) must combine high expression in beta cells with low expression in exocrine and other endocrine cells. Additionally, a suitable PET ligand must have high affinity to the target receptor, combined with low-to-negligible non-target interactions. Several target receptors and enzymes have previously been suggested as suitable biomarkers, most notably the vesicular monoamine transporter 2 (VMAT2) [4], the glucagon-like peptide-1 receptor (GLP-1R) [5] and DOPA decarboxylase [6]. However, despite promising pre-clinical data only few clinical studies on imaging of beta cells in T1D and the healthy condition have yet been published—two employing analogues of the VMAT2 high-affinity ligand dihydrotetabenazine (DTBZ) [7, 8], one using Indium-111 labeled Exendin4 [9] and one using the serotonin precursor [¹¹C]5-Hydroxy-Tryptophan [10]. Unexpectedly high radiotracer uptake was found in the beta cell-deficient T1D pancreas in each of these clinical studies, raising doubt on the beta cell selectivity of the respective target. Novel imaging targets are therefore required in order to increase the imaging contrast between the beta cells and the remaining pancreatic tissue [11, 12].

Recently the G coupled protein GPR44 that bind endogenous Prostaglandin D2 (PGD₂) has been identified as a beta cell-specific biomarker through proteomic screening [13]. GPR44 is also designated as the prostaglandin D2 receptor 2 (PTGDR2), chemoattractant receptor-homologous expressed on Th2 lymphocytes receptor (CRTH2) or CD294. Here, we have quantified the density of the GPR44 receptor in endocrine (isolated human islets and human and rodent beta cell lines) and exocrine tissues by the radiolabeled GPR44 ligand AZD 3825. To assess the potential of GPR44 as a surrogate marker for beta cells, we performed a direct comparison with radiolabeled DTBZ.

Method and materials

Chemicals

[³H]AZD 3825 (specific activity 22.2 Ci/mmol) and unlabeled AZD 3825 were synthesized by AstraZeneca, Mölndal, Sweden. [³H]DTBZ (specific activity 20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO, USA). Tetrabenazine (TBZ) was obtained from Biotrend AG (Zurich, Switzerland). [³H]PGD₂ (160 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA, USA).

Cell culture

The human beta cell line EndoC-βH1 [14] was obtained from Endocells (Paris, France) and cultured according to the provider in DMEM low glucose supplemented with 2 % BSA fraction V (Roche Diagnostics, Indianapolis, IN), 50 μM 2-mercaptoethanol (Sigma-Aldrich, St Louis, MO), 10 mM nicotinamide (VWR), 5.5 μg/ml transferrin and 6.7 ng/ml sodium selenite.

The rat insulinoma cell line INS-1 was obtained from Dr Hans Hohmeier (Duke University Medical Center, Durham, NC) and cultured in RPMI 1640 supplemented with 10 % FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol.

Homogenized Human Embryonic Kidney (HEK) 293 cells overexpressing GPR44 were used for a [³H]PGD₂ binding assay as described previously [15].

All cell culture reagents were from Life Technologies (Carlsbad, CA, USA) except when stated otherwise.

Human tissue

The use of human tissue was approved by the Uppsala Ethical Review Board (#2011/473, #Ups 02-577). Isolated pancreatic islets and exocrine tissue were obtained within the Nordic network for Clinical islet Transplantation laboratory in Uppsala, Sweden. Isolation procedures and culturing conditions have been described previously [15–17]. Fresh frozen tissue including two pancreatic samples with normal histology was obtained from Uppsala Biobank.

RNA analysis

RNA preparation and analysis were conducted within the Human Protein Atlas project [18].

Preparation of tissue

Fresh frozen tissue was imbedded in OCT compound (Sakura Finetek, Alphen aan den Rijn, the Netherlands) and used as source of RNA. Six sections of 10 μm each were cut using a cryotome and put into ice-cold RLT buffer (Qiagen, Hilden, Germany) and mixed by vortex. RNA from the islet and exocrine preparations was extracted without imbedding. Samples were stored in –70 °C until RNA extraction.

RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Disruption was conducted using a 3-mm steel grinding ball (VWR, Radnor, PA) and vortexing. Concentration and RNA integrity (RIN) were determined by Qubit 2.0 Fluorometer (Life Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), respectively. Purity of the

samples was confirmed by an A260/A280 value over 2.0 using Nanodrop (Thermo Scientific, Wilmington, DE). Samples with RIN values above 7.5 were sequenced by Illumina HiSeq 2000 and 2500 (Illumina, San Diego, CA) using the standard Illumina RNA-Seq protocol with a read length of 2×100 bases.

Analysis of data

The raw reads obtained from the sequencing system were trimmed for low-quality ends with the software sickle [19], using a phred quality threshold of 20. All reads shorter than 54 bp after the trimming were discarded. The processed reads were mapped to the GRCh37 version of the human genome with Tophat v2.0.3 [20]. Potential PCR duplicates were eliminated using the MarkDuplicates module of Picard 1.77 [21]. To obtain quantification scores for all human genes, FPKM (fragments per kilobase of exon model per million mapped reads) values were calculated using Cufflinks v2.0.2 [22], which corrects for transcript length and the total number of mapped reads from the library to compensate for different read depths for different samples. The gene models from Ensembl build 69 [23] were used in Cufflinks. In addition to Cufflinks, HTSeq v0.5.1 was run to calculate read counts for each gene, which were used for analyses of differentially expressed genes using the DESeq package [24]. All data were analyzed using R Statistical Environment [25] with the addition of package ‘gplots’ [26]. For analyses performed in this study where a log₂-scale of the data was used, pseudocounts of +1 were added to the data set.

Flow cytometry

Surface expression of GPR44 on EndoC-βH1 cells was analyzed by flow cytometry. Cells were harvested enzymatically using accutase. Cells in suspension were stained by a rabbit polyclonal GPR44 antibody (HPA14259, Atlas Antibodies, Stockholm, Sweden) for 30 min at +4 °C. Staining of HLA-I (GTX61891, GeneTex, Irvine, CA) was used as a positive control of the detection antibody. An alexa-488 conjugated goat anti-rabbit antibody was used for detection (Life Technologies). Cells were analyzed on a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA).

Preparation of tissue for in vitro binding studies

50–100 mg isolated endocrine (75–95 % islet purity) and exocrine tissues as well as EndoC-βH1 cells were homogenized in ice-cold 0.32 M sucrose by hand using a Dounce glass homogenizer to a final concentration of 6 mg/ml. 50–100 mg INS-1 was homogenized using a

polytron tissue homogenizer (Polytron® PT 3000, Kinematica AG, Littay-Luzern, Switzerland) in ice-cold 0.32 M sucrose at a concentration of 6 mg/ml and then by hand using a Dounce glass homogenizer. Protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. Aliquots of the homogenates were stored at –80 °C until used.

AZD 3825 binding to GPR44

Potency of binding of AZD 3825 to GPR44 was determined by a [³H]PGD₂ displacement assay on membrane fractions from HEK 293 cells overexpressing GPR44, over a range of concentrations as described previously [15]. The assay readout was the concentration of AZD 3825 giving 50 % of maximum inhibitory effect (IC₅₀) on [³H]PGD₂ binding.

In vitro binding assays for [³H]AZD 3825 and [³H]DTBZ

Two milligrams of homogenized endocrine, exocrine and insulinoma tissue, and 1 mg of homogenized EndoC-βH1 cells were incubated for 3 h in room temperature with 4 nM [³H]AZD 3825 (dissolved in ethanol, final concentration 1 %) and 50 mM TRIS (pH7.4) in a final incubation volume of 1 ml. Twenty micrograms of unlabeled AZD 3825 dissolved in ethanol (final concentration 0.5 %) was added for determination of non-specific binding. The samples were filtered using a Brandel M-48 cell harvester with Whatman GF/C filter (presoaked with 50 mM TRIS) and washed four times with 3 ml 50 mM TRIS (room temperature). Filters were put into scintillation vials and dried for 30 min before adding 10 ml Ultima Gold scintillation fluid (Perkin Elmer, Waltham, MA). The filters were shaken for 90 min and then counted in a β-counter (Packard Scintillator Tri-Carb 2100TR). Similar experiments with 4 nM [³H]DTBZ in ethanol were performed using 20 μM TBZ to estimate the non-specific binding. The K_d and B_{max} values for [³H]AZD 3825 and [³H]DTBZ binding were determined using concentrations of the tritiated ligand from 1 to 15 nM. The specific binding was calculated by subtracting the non-specific binding from the total binding, and expressed as fmol/mg protein. All measurements were performed in triplicates. The binding data were analyzed with curve-fitting software (GraphPad Prism version 5.04) to obtain K_d and B_{max} values.

Statistical analysis

When reported on group level, results are given as mean ± SEM. Differences between groups were assessed

by nonparametric Mann–Whitney rank test using a 95 % confidence level.

Results

RNA analysis

RNA analysis of different pancreas compartments shows higher transcripts levels of GPR44 in islets (5.2 FPKM) compared to exocrine tissue (0.2 FPKM) (Table 1). Similar levels as in islets were observed in small intestine and colon, while stomach had a slightly higher RNA expression of 8.2 FPKM. Transcript levels in other tissues were generally below 1 FPKM. Taken together, RNA analysis of a large panel of human tissues shows that there is a selective

Table 1 RNA values of GPR44 in various tissue

Tissue	FPKM
Islets	5.2
Exocrine tissue	0.2
Whole pancreas	0.2
Stomach	8.2
Small intestine	5.5
Colon	5.2
Duodenum	3.7
Heart	2.0
Brain	1.3
Fallopian tube	1.1
Placenta	1.0
Fat	0.8
Esophagus	0.7
Appendix	0.7
Lung	0.6
Gall bladder	0.6
Urinary bladder	0.6
Breast	0.6
Skin	0.4
Spleen	0.4
Cervix	0.3
Liver	0.2
Kidney	0.2
Testis	0.2
Prostate	0.2
Salivary gland	0.1
Ovary	0.1
Lymph node	0.1
Thyroid gland	0
Adrenal gland	0
Bone marrow	0

transcriptional expression of GPR44 restricted to islets in the pancreas and some cells in the gastrointestinal tract.

Surface expression of GPR44 in EndoC- β H1

To verify surface expression of GPR44, EndoC- β H1 was stained with an antibody against GPR44 and evaluated by flow cytometry. The majority of the cells were detected by the GPR44 antibody (Fig. 1). As a positive control, HLA-I was used, which gave a slightly higher positivity than GPR44, 84 % compared to 71 %. The secondary fluorescent antibody gave negligible background signal. This demonstrates that GPR44 is expressed in and available at the cell surface of EndoC- β H1.

AZD 3825 binding to GPR44

AZD 3825 inhibited [3 H]PGD₂ binding on membrane fractions from HEK 293 cells overexpressing GPR44. The AZD 3825 displayed high potency for interacting with human GPR44 through competing with [3 H]PGD₂, with an IC₅₀ of 0.98 nM (Table 2).

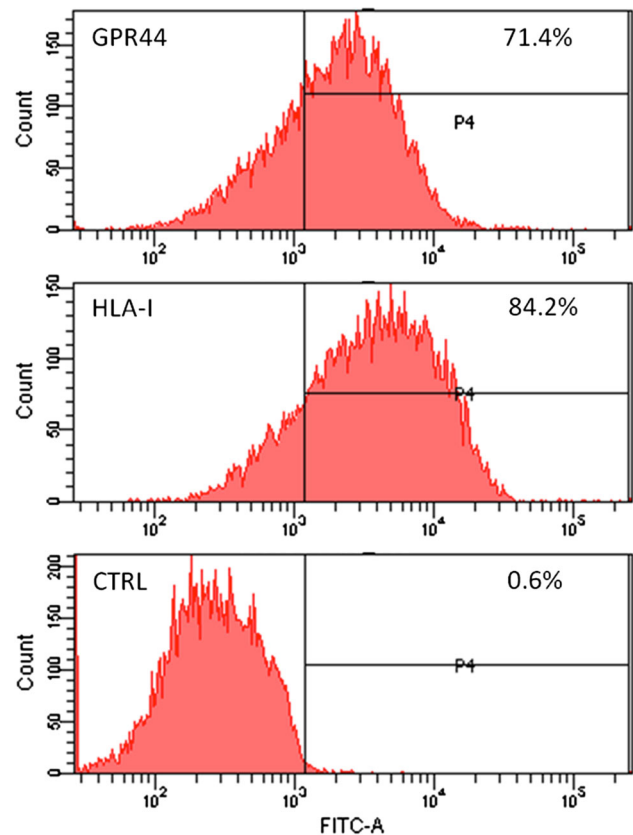


Fig. 1 Surface expression of GPR44 on EndoC- β H1 cells evaluated by flow cytometry. The majority of the cells were positive for GPR44 (*upper histogram*), slightly less than the HLA-1 control (*middle histogram*). The *lower histogram* shows the negative control represented by the secondary detection antibody alone

Initial experiments for optimizing in vitro binding of [³H]AZD 3825 and of [³H]DTBZ

Initial experiments with [³H]AZD 3825 and [³H]DTBZ in homogenates from endocrine tissue were performed to find optimal incubation time and amount of tissue needed. Binding reached a maximum between 150 and 180 min with [³H]AZD 3825 and after 90 min with [³H]DTBZ (data not shown). An incubation time of 180 min was chosen for all experiments. There was a linear increase in specific [³H]AZD 3825 and [³H]DTBZ binding using 0.5–2 mg tissue ($r = 0.997$ and 0.976 , respectively). Binding to homogenates corresponding to 2 mg tissue was used in the final experiments. In comparison with different tissues (endocrine, exocrine, EndoC- β H1 cells and INS-1 insulinoma), a concentration of 4 nM [³H]AZD 3825 and [³H]DTBZ was chosen.

Comparison of [³H]AZD 3825 and [³H]DTBZ binding to endocrine and exocrine pancreatic tissues and EndoC- β H1 cells

For both [³H]AZD 3825 and [³H]DTBZ, there was higher amount of displaceable binding in endocrine compared to exocrine pancreatic tissue ($p < 0.001$, Fig. 2a).

The specificity in endocrine tissue was not significantly different between [³H]AZD 3825 and [³H]DTBZ at 4 nM (56 ± 3 and 49 ± 5 %, respectively, Fig. 2a). In exocrine tissue, the percentage-specific binding was approximately 3 times lower for [³H]AZD 3825 compared to [³H]DTBZ (6.3 ± 2 and 22 ± 2 %, respectively, Fig. 2a). Specific binding of 4 nM [³H]AZD 3825 was almost 20 times higher in endocrine tissue compared to exocrine tissue (Fig. 2b, $p < 0.001$), while the specific binding of 4 nM [³H]DTBZ was only 2.2 times higher in endocrine tissue compared to exocrine tissue (Fig. 2b, $p < 0.05$). Accordingly, the important endocrine-to-exocrine uptake ratio was approximately 10 times higher for [³H]AZD 3825 (Fig. 2c). The absolute specific binding of [³H]DTBZ was significantly higher than binding of [³H]AZD 3825 in both endocrine ($p < 0.001$) and exocrine ($p < 0.05$) tissue. The specific binding of 4 nM [³H]AZD 3825 in EndoC- β H1 cells was significantly higher ($p < 0.05$) in comparison with endocrine tissue, whereas the specific binding of 4 nM [³H]DTBZ was similar in endocrine tissue and in EndoC-

β H1 cells (Fig. 2d). The uptake ratio of [³H]AZD 3825 between the pure beta cells and the exocrine tissue was in excess of 90 (Fig. 2c). Very low or non-existent specific binding of [³H]AZD 3825 (0.88 ± 0.18 fmol/mg protein) and [³H]DTBZ (9.70 ± 1.55 fmol/mg protein) was detected in homogenates of INS-1 insulinoma xenografts (Fig. 2d).

Characteristics of [³H]AZD 3825 and [³H]DTBZ binding to endocrine and exocrine pancreatic tissue

Saturation experiments with 1–15 nM [³H]AZD 3825 and [³H]DTBZ were performed with homogenates of endocrine, exocrine tissue and EndoC- β H1 cells (representative graphs are shown in Supplementary Fig. 1). Nonlinear regression analysis of data for [³H]AZD 3825 binding to endocrine and exocrine tissue gave average K_d values of 1.39 and 1.87 nM and B_{max} values of 31.9 and 5.0 fmol/mg protein, respectively (Table 3). The affinity of [³H]DTBZ to endocrine and exocrine tissue was approximately 6 and 9 times lower than that for [³H]AZD 3825, while the B_{max} values were approximately 7 and 97 times higher in endocrine and exocrine tissues, respectively (Table 3). K_d obtained from saturation experiments with EndoC- β H1 cells was 1.18 nM for [³H]AZD 3825, which was similar to the value estimated for endocrine tissue, whereas B_{max} was approximately 7 times higher in the EndoC- β H1 cells (Table 3).

Discussion

Efforts to find a biomarker for beta cell mass that can be targeted and studied noninvasively by imaging have yielded a few potential candidates. Analogues of DTBZ were the first PET ligands putatively targeting beta cells that were evaluated in prospective clinical studies aimed at visualizing native pancreatic beta cell mass. VMAT2 that DTBZ targets is, however, not completely restricted to beta cells as demonstrated in subjects with long-standing T1D where a decrease in binding of the ligand of not more than 15 % was observed [7]. Naively, one would expect receptor-specific radioligand binding to be decreased by close to 100 % in T1D, accompanying the near total loss of beta cells. Similarly when targeting the GLP-1R, a large overlap between groups of non-diabetic subjects and subjects with T1D was seen [9]. Assessment of serotonergic biosynthesis by DOPA decarboxylase in pancreas by PET could differentiate between non-diabetic subjects and subjects with T1D without overlap, but the serotonergic metabolic pathway is present in all pancreatic endocrine tissue and therefore not beta cell specific [10].

Herein, we have therefore quantified the expression and density of the recently identified beta cell biomarker

Table 2 Inhibition by AZD3825 of radioligand binding to membranes from HEK cells expressing human GPR44

Cell system	Assay readout	pIC ₅₀ (mean \pm SEM)	IC ₅₀ (nM)
HEK 293 cells (GPR44)	[³ H]PGD ₂ displacement	9.5 \pm 0.05 ($n = 25$)	0.98

Fig. 2 [^3H]AZD 3825 and [^3H]DTBZ evaluated for specific and non-displaceable with binding at 4 nM ligand concentration. Non-displaceable binding was assessed by co-incubation with unlabeled ligand in excess. **a** Specificity of [^3H]AZD 3825 and [^3H]DTBZ in human pancreatic endocrine and exocrine preparations. **b** Amount of receptor bound [^3H]AZD 3825 or [^3H]DTBZ per mg protein in endocrine and exocrine preparations. **c** Comparison of endocrine-to-exocrine and EndoC- βH1 -to-exocrine uptake ratio between [^3H]AZD 3825 and [^3H]DTBZ. **d** Amount of receptor bound [^3H]AZD 3825 or [^3H]DTBZ per mg protein in human EndoC- βH1 and rodent INS-1 cells

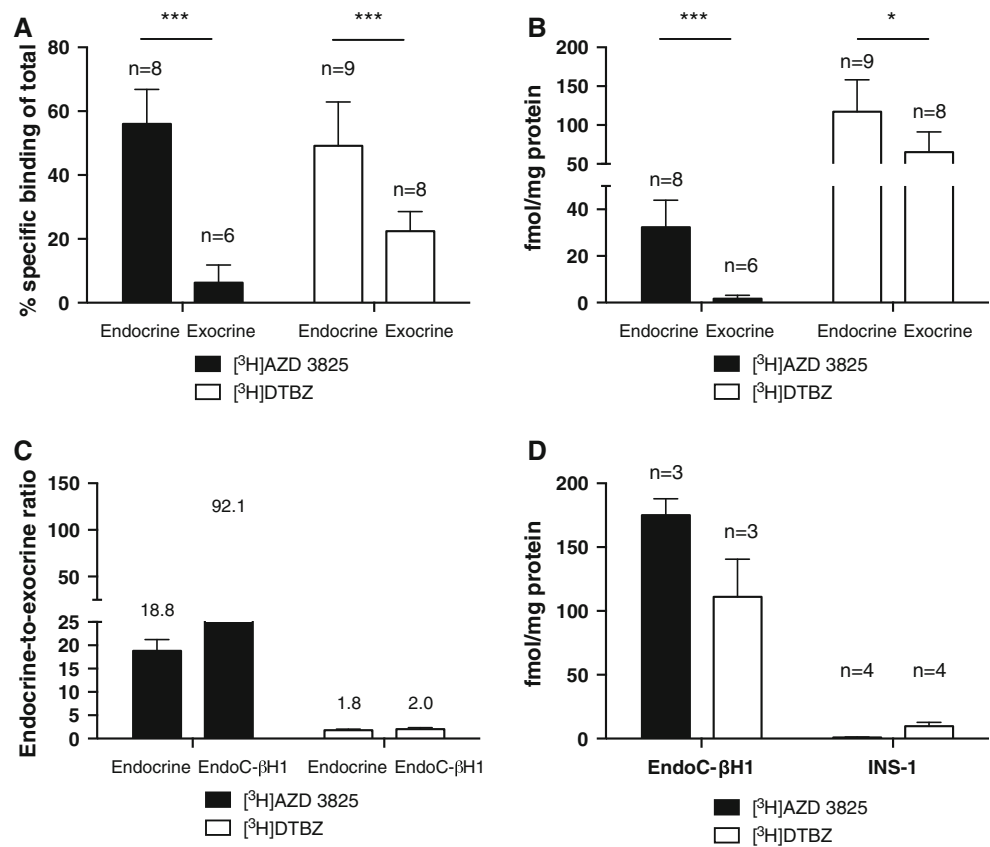


Table 3 Binding properties of [^3H]AZD 3825 and [^3H]DTBZ in endocrine and exocrine tissue of human pancreas and EndoC- βH1 cells, as assessed by saturation-binding experiments

Ligand	Tissue	K_d (nM)	B_{max} (fmol/mg protein)	BP (/mg protein)
[^3H]AZD 3825	Endocrine	1.76	27.4	15.57
[^3H]AZD 3825	Endocrine	0.54	29.3	54.26
[^3H]AZD 3825	Endocrine	1.88	38.9	20.69
Mean \pm SEM		1.39 \pm 0.43	31.87 \pm 3.56	30.17 \pm 12.13
[^3H]AZD 3825	Exocrine	1.79	4.43	2.47
[^3H]AZD 3825	Exocrine	2.84	5.17	1.82
[^3H]AZD 3825	Exocrine	0.97	5.44	5.61
Mean \pm SEM		1.87 \pm 0.54	5.01 \pm 0.30	3.30 \pm 1.30
[^3H]AZD 3825	EndoC- βH1	1.36	227.7	167.00
[^3H]AZD 3825	EndoC- βH1	1.01	225.2	222.90
Mean \pm SEM		1.18 \pm 0.17	226.4 \pm 1.25	194.5 \pm 27.9
[^3H]DTBZ	Endocrine	4.91	161	32.79
[^3H]DTBZ	Endocrine	16.9	363	21.47
[^3H]DTBZ	Endocrine	12.8	283	22.11
[^3H]DTBZ	Endocrine	4.18	280	66.98
[^3H]DTBZ	Endocrine	3.61	273	75.62
Mean \pm SEM		8.48 \pm 2.68	272 \pm 32	43.79 \pm 11.49
[^3H]DTBZ	Exocrine	21.6	490	22.68
[^3H]DTBZ	Exocrine	13.7	318	23.21
[^3H]DTBZ	Exocrine	19.0	733	38.58
[^3H]DTBZ	Exocrine	11.8	410	34.74
Mean \pm SEM		16.5 \pm 2.27	487 \pm 89	29.80 \pm 4.03

GPR44 in pancreatic tissue [13], in order to investigate its potential future use for radionuclide imaging.

RNA analysis of a panel of human tissues showed that GPR44 is specific at the transcriptional level for endocrine cells in pancreas. Similar levels were observed in stomach and intestines, and further protein profiling using immunohistochemistry showed weak staining in stomach, negative staining in intestines whereas the staining of islet cells were strong (data not shown). We therefore believe that other organs in the abdominal tract will not interfere with signals from the pancreas in imaging applications. The same antibody was used to verify surface availability of the target in the recently established human beta cell line EndoC- β H1 [14] by flow cytometry. Since the majority of the cells were positive in this setting, it is likely that GPR44 is targetable at the surface, i.e., internalization of ligands is unnecessary.

Binding assays using the tritiated GPR44 ligand AZD 3825 shows preferential accumulation in the human endocrine pancreas, with minor binding to exocrine preparation. The affinity to endocrine and exocrine preparations did not differ, indicating a structurally similar or identical target protein. This is in contrast to DTBZ binding which could be found in high density mainly in endocrine, but also exocrine pancreatic preparations. Furthermore, the affinity in exocrine tissue was higher, indicating displaceable interactions to target proteins other than VMAT2. For example, [18 F]FP-DTBZ has been shown to interact with Sigma receptors with low affinity but high capacity especially in the rat exocrine pancreas [27]. We previously found similar results when assessing two Fluorine-18 labeled DTBZ analogues for beta cell specificity [28, 29]. These observations, and those of other reports [30, 31], may partially explain the difficulties encountered in visualizing human beta cells by targeting VMAT2.

The binding studies were performed on islet and exocrine homogenates. This assay does not rule out also binding to exposed intracellular epitopes. Repetition of these studies using intact viable human islets could rule out this possibility.

Furthermore, the endocrine pancreas also contains nervous tissue such as Schwann cells. The assay using homogenates of endocrine pancreatic homogenates does not exclude the presence of receptors also on non-islet nervous tissue. However, the IHC staining in the HPA database was negative for GPR44 in most CNS tissue including glial cells [32]. Quantitative *in vitro* autoradiography using AZD 3825 labeled by a positron emitting nuclide, with corresponding insulin staining of consecutive sections, must be performed in order to verify the extent of overlap between tracer receptor binding and the Islets of Langerhans.

Only minor uptake of AZD 3825 was observed in the rat cell line INS-1. This can have several explanations; first, the amino acid sequences of GPR44 have 79 % similarity between human and rat (according to BLAST) which means that the ligand could target a sequence that is dissimilar; second, the INS-1 cells are derived from insulinoma that implicates that many genes might have altered expression levels. Similarly, low binding of DTBZ was observed in the rodent-derived INS-1 cell line. These “false negatives” in DTBZ and AZD3825 binding (in relation to human islets) underline the importance of utilizing humanized animal models or human beta cell lines such as EndoC- β H1 in the preclinical evaluation of imaging agents in diabetic research.

A further requirement for a reliable and accurate biomarker of beta cell mass is low variability in expression between individual beta cells within an individual, as well as between beta cells in different individuals. The receptor densities shown in Table 3 are all acquired from islet preparations from different individuals. This indicates quite low variation of GPR44 between individuals both in their endocrine and exocrine pancreatic tissues. However, since these are tissue homogenates, it does not yield information on the variability between individual beta cells. This must be further explored by, for example, quantitative autoradiography using a positron emitting GPR44 ligand.

The major issue in visualizing biological processes or receptors by molecular imaging is that of contrast. In the case of beta cell imaging, the contrast requirements are immense due to the volumetric difference between the beta cells and the non-endocrine pancreatic parenchyma. Here we show that GPR44 is highly preferential for the endocrine pancreas and the beta cell in particular. The contrast between human beta cells and human exocrine preparations was 45.2 (B_{\max} ratio) or 59.1 [Binding Potential (BP) ratio]. This is in contrast to VMAT2 or other molecular entities targeted by DTBZ that were found in both endocrine and exocrine pancreatic preparations.

Our ability to visualize and quantify a receptor by *in vivo* molecular imaging is interplay between ligand affinity and receptor density. We have here demonstrated excellent *in vitro* affinity to GPR44 by the tritiated ligand AZD 3825, as well as improved contrast for the endocrine pancreas compared to DTBZ. GPR44 was expressed in relatively low densities compared to VMAT2 in the endocrine pancreas, which potentially limits the possibilities of generating a strong enough signal. However, the binding potential, or the receptor density divided by the dissociation constant, was similar to that of DTBZ (approximately 30 % lower). Given that the entire DTBZ binding in pancreas *in vivo* is displaceable and therefore receptor specific [33] and readily quantifiable in primates and humans, we foresee no insurmountable problems in

quantifying the GPR44 in the endocrine pancreas as soon as a PET ligand is available.

A final criterion, which applies to PET tracers in general, is that the agent should be pharmacologically neutral, i.e., the tracer molecule itself should not induce a physiological response even at high doses. This is preferred but not an absolute requirement, as pharmacologically active agonists have been successfully employed in clinical imaging research such as [¹¹C]Carfentanil [34] or radiolabeled Exendin4 [9]. Therefore, the pharmacological effect of GPR44 agonism or antagonism must be further explored before selecting a GPR44 binder for PET labeling with the aim of clinical translation.

Conclusion

The GPR44 protein has been confirmed as beta cell restricted by proteomics, transcriptomics as well as by a radiolabeled small molecule ligand. Given the development of a suitable PET ligand, this target potentially offers improved contrast between endocrine and exocrine pancreas compared to currently available biomarkers.

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Compliance with ethical standards

Conflict of interest Paul Czernichow is an employee of EndoCells. The remaining authors report no conflicts of interest.

Ethical standard All work involving human tissue was conducted according to the principles expressed in the Declaration of Helsinki and in the European Council's Convention on Human Rights and Biomedicine. Consent for organ donation (for clinical transplantation and for use in research) was obtained from the relatives of the deceased donors by the donor's physicians and documented in the medical records of the deceased subject. The study was approved by the Regional Ethics Committee in Uppsala, Sweden (<http://www.epn.se>) according to the Act concerning the Ethical Review of Research Involving Humans (2003:460), Permit Number: Dnr 2009/371 (from April 10, 2013).

Human and animal rights disclosure This article does not contain any studies with human or animal subjects performed by any of the authors.

Informed consent None.

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