Receptor-targeted photodynamic therapy of glucagon-like peptide 1

receptor positive lesions

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ABSTRACT

Treatment of hyperinsulinemic hypoglycemia is challenging. Surgical treatment of insulinomas and focal lesions in congenital hyperinsulinism (CHI) is invasive and carries major risks of morbidity. Medication to treat nesidioblastosis and diffuse CHI has varying efficacy and causes significant side effects. Here, we describe a novel method for therapy of hyperinsulinemic hyperglycemia, highly selectively killing beta cells by receptor-targeted photodynamic therapy (rtPDT) with exendin-4-IRDye700DX, targeting the glucagon-like peptide 1 receptor (GLP-1R).

A competitive binding assay was performed using Chinese hamster lung (CHL) cells transfected with the GLP-1R. The efficacy and specificity of rtPDT with exendin-4-IRDye700DX was examined *in vitro* in cells with different levels of GLP-1R expression. Tracer biodistribution was determined in BALB/c nude mice bearing subcutaneous CHL-GLP-1R xenografts. Induction of cellular damage and the effect on tumor growth were analyzed to determine treatment efficacy.

Exendin-4-IRDye700DX has a high affinity for the GLP-1R with an IC₅₀ value of 6.3 nM. rtPDT caused significant specific phototoxicity in GLP-1R positive cells (2.3 ± 0.8 % and 2.7 ± 0.3 % remaining cell viability in CHL-GLP-1R and INS-1 cells resp.). The tracer accumulates dose-dependently in GLP-1R positive tumors. In vivo rtPDT induces cellular damage in tumors, shown by strong expression of cleaved-caspase-3 and leads to a prolonged median survival of the mice (36.5 vs. 22.5 days resp. p<0.05).

These data show *in vitro* as well as *in vivo* evidence for the potency of rtPDT using exendin-4-IRDye700DX. This could in the future provide a new, minimally invasive and highly specific treatment method for hyperinsulinemic hypoglycemia.

Keywords: glucagon-like peptide 1 receptor, exendin, photodynamic therapy, hyperinsulinemic hypoglycemia

1 INTRODUCTION

2 Insulin production by pancreatic beta cells is usually a well-regulated process. However, 3 uncontrolled overproduction of insulin can arise, in most cases as a result of insulin-producing lesions. Such lesions cause major clinical symptoms and treatment can be challenging. In adults, 4 5 these lesions manifest in endogenous adult hyperinsulinemic hypoglycemia, most often caused 6 by an insulinoma, an insulin-producing neuroendrocrine tumor arising from pancreatic beta cells 7 (1). In 0.5% to 5% of cases, adult hyperinsulinemic hypoglycemia is caused by nesidioblastosis, 8 characterized by proliferation of abnormal beta cells throughout the pancreas (2). In neonates, the 9 most common cause of persistent hyperinsulinism is CHI (3). In diffuse CHI, there is diffuse 10 involvement of the pancreatic beta cells, while in focal CHI the disease is caused by focal 11 adenomatous islet cell hyperplasia (4). Episodic hypoglycemia due to endogenous 12 hyperinsulinism causes neuroglycopenic and autonomic symptoms. Prolonged hypoglycemia may 13 lead to seizures, loss of consciousness, permanent brain damage or brain death (5).

14 Insulinomas and focal CHI can be cured by surgical removal of the lesion (3,6). Enucleation 15 is possible in case of superficially localized lesions with sufficient distance to the pancreatic duct 16 (2-3 mm). Otherwise, a more extensive surgical procedure like partial or distal pancreatectomy 17 may be required. While such procedures can often be performed laparoscopically (7,8), they 18 remain challenging and may carry major risks of morbidity (9,10). The only surgical treatment 19 option for patients with nesidioblastosis and diffuse CHI not responding to medication is partial 20 pancreatectomy. Even after such an invasive procedure, hypoglycemic episodes often persist, 21 requiring continued treatment with medication and, in certain cases of CHI, total pancreatectomy 22 (2,4).

Because of these challenges, a novel, preferably minimally invasive treatment option for hyperinsulinemic hypoglycemia in adults as well as in children is warranted. In this study, we assess the feasibility of specific ablation of insulin-producing cells with PDT. PDT is based on inducing cell death by irradiation of a light-sensitive molecule, or photosensitizer (PS). The PS absorbs photons and is transferred to a higher energy state. By transfer of energy from the activated PS to the oxygen in the surrounding tissue, reactive oxygen species (ROS) are produced, which can cause cellular damage (*11*). To ensure efficient and specific delivery of the PS to the target tissue, the PS is coupled to a tumor-specific targeting moiety (*12*).

An attractive targeting moiety for rtPDT of insulin-producing cells is exendin-4. This peptide is a stable analogue of the hormone GLP-1. It specifically binds to the GLP-1R, which is expressed on pancreatic beta cells and in high levels in nearly 100% of benign insulinomas (*13*). GLP-1R imaging using ¹¹¹In- and ⁶⁸Ga-labelled exendin-4 has been shown to be a successful pre-operative imaging technique for insulinomas (*14-16*) and is also under investigation in CHI (clinicaltrials.gov; NCT03768518).

We have developed an approach for rtPDT of insulin producing lesions using the peptide exendin-4 coupled to the photosensitizer IRDye700DX. We hypothesize that this novel method will allow specific cell killing of GLP-1R positive cells.

40

41 MATERIALS AND METHODS

42 Reagents

Exendin-4-IRDye700DX was supplied by piCHEM (Graz, Austria). IRDye700DX NHS ester was 43 44 obtained from LI-COR Biosciences (Lincoln, Nebraska, U.S.A.). IRDye700DX absorbs and emits 45 light in the NIR range and has a higher extinction coefficient (2.1x10⁵ M⁻¹cm⁻¹ at 689 nm) than non-NIR PSs (12, 17). The N-epsilon amino group of lysine at position 40 was site specifically modified 46 47 during solid phase peptide synthesis with a mercapto-propionic acid, releasing an unprotected exendin-4 with a free thiol function after triisopropylsilane cleavage. IRDye700DX was modified 48 49 with a maleimide and coupling to exendin-4 was performed using a thiol reactive crosslinking 50 approach. The purity was >90%. Stock solutions of exendin-4-IRDye700DX were prepared in phosphate-buffered saline (PBS). The structure and amino acid sequence of the tracer are shown 51

in supplemental figure 1. Absorbance and emission spectra of exendin-4-IRDye700DX are shown
 in supplemental figure 2.

54 Cell culture

55 CHL cells stably transfected with the GLP-1R (18) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5g/L D-glucose and Glutamax, supplemented with 10% fetal calf serum 56 57 (FCS), 100 IU/mL penicillin G, 10mg/mL streptomycine, 1 mM sodium pyruvate, 0.1 mM non-58 essential amino acids and 0.3 mg/mL G418 geneticin. The rat insulinoma cell line INS-1 was 59 cultured in RPMI 1640 medium, supplemented with 10% FCS, 100 IU/mL penicillin G, 10mg/mL 60 streptomycine, 2 mmol/L L-glutamine, 1 mmol/L pyruvate, 10 mmol/L 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES) and 50 µmol/L 2-mercaptoethanol. The human pancreatic 61 62 tumor cell line PANC-1 was cultured in RPMI 1640 medium supplemented with 10% FCS, 100 63 IU/mL penicillin G, 10 mg/mL streptomycine and 2 mmol/L L-glutamine.

64 **Competitive binding assay**

The half-maximal inhibitory concentration (IC₅₀) of exendin-4-IRDye700DX and unlabeled exendin, as a reference, was determined using CHL-GLP-1R cells as described previously (*19,20*). 10⁶ cells/well were grown overnight in six well plates. Cells were washed twice with PBS and incubated for 4 hours on ice with 50.000 cpm ¹¹¹In-labelled exendin in the presence of increasing concentrations of exendin-4-IRDye700DX (0.1–300 nM). Cells were then washed with PBS, solubilized with 2 mL sodium hydroxide (NaOH), collected and the cell-associated activity was measured in a gamma-counter (Wizard 2480, PerkinElmer, Groningen, The Netherlands).

72 In vitro receptor-targeted photodynamic therapy

CHL-GLP-1R cells, INS-1 cells and PANC-1 cells were seeded into 24-well plates (Thermo Scientific) (150,000 cells/well) and grown overnight. Medium was replaced by binding buffer (medium with 0.1% bovine serum albumin (w/v) (BSA)) with exendin-4-IRDye700DX (300nM for CHL-GLP-1R cells and 400nM for INS-1 and PANC-1 cells (concentrations based on optimization experiments). As a control, cells incubated with binding buffer only were used. Separate wells

were incubated with an excess (15 µM for CHL-GLP-1R cells and 20 µM for INS-1 and PANC-1 78 79 cells) of unlabeled exendin-4 together with exendin-4-IRDye700DX. After incubation at 37°C 80 (CHL-GLP-1R cells 4 hours, INS-1 and PANC-1 cells 24 hours), cells were washed with binding 81 buffer. Subsequently, cells were irradiated with a NIR light-emitting diode (LED) (21) (emission 82 wavelength 670-710 nm, forward voltage: 2.6 V, power output: 490 mW) using 126 individual LED 83 bulbs ensuring homogenous illumination (21). CHL-GLP-1R cells were irradiated at 90 J/cm² (over 84 6 min). INS-1 and PANC-1 cells were irradiated at 150 J/cm² (over 10 min). Cells incubated with 85 exendin-4-IRDye700DX that were not irradiated were included as a control. All experiments were 86 carried out in triplicate.

Four hours after irradiation, during which the cells were kept at 37°C and 5% CO₂, the ATP content as a measure of cell viability was determined using a CellTiter-Glo[®] luminescent assay (Promega Benelux, Leiden, The Netherlands) according to the instructions of the manufacturer. Luminescence was measured using a TECAN infinite M200 Pro plate reader (PerkinElmer, Groningen, The Netherlands). The ATP content as a measure of cell viability was expressed as a percentage, determined by comparing the luminescent signal with the signal from untreated cells, which were considered 100% viable.

Additionally, a co-culture of INS-1 and PANC-1 cells was plated in 24-well plates (70,000 94 95 and 40,000 cells/well, respectively). Before seeding, INS-1 cells were labeled with the fluorescent 96 dye DiO and PANC-1 cells with DiD dye according to the manufacturer's protocol (Life 97 Technologies, Thermo Fisher Scientific, Waltam, MA, USA). Cells were grown overnight and then 98 incubated with 400 nM exendin-4-IRDye700DX in binding buffer or binding buffer alone for 24 99 hours at 37°C and 5% CO₂. Subsequently, cells were irradiated with 150 J/cm² of NIR light. After 100 four hours, cells were incubated with 1 µg/mL propidium iodide (Thermo Fisher Scientific, 101 Waltham, MA, USA) in PBS for 15 minutes at room temperature. Cells were visualized using an 102 EVOS microscope (Thermo Fisher Scientific, Waltam, MA, USA).

103 Animal tumor model

Female BALB/c nude mice (Janvier, Le Genest Saint Isle, France), 6-8 weeks old, were housed in individually ventilated cages (6 mice per cage) under non sterile conditions with ad libitum access to chlorophyll-free animal chow and water. CHL-GLP-1R cells (5*10⁶ cells/ mouse in 200 µl DMEM with 4.5g/L D-glucose and Glutamax) were injected subcutaneously on the right flank of the mice.

109 In vivo biodistribution

110 Female BALB/c nude mice with CHL-GLP-1R xenografts were injected intravenously with 111 exendin-4-IRDye700DX in 200 µI PBS with 0.5% BSA (N=5 per group, 1, 3 and 10 µg exendin-4-112 IRDye-700DX). Four mice were injected with only PBS with 0.5% BSA. After 4 hours, mice were 113 sacrificed by CO₂ asphyxiation and the tumor and organs were removed and collected in Roche 114 MagNA Lyser tubes (F Hoffmann-La Roche Ltd., Basel, Switzerland). Radioimmunoprecipitation 115 assay (RIPA) lysis buffer (500 µL; 50mM (hydroxymethyl)aminomethane-hydrochloride (TRIS-116 HCI), pH7.4 with 150 mM sodiumchloride (NaCI), 1 mM ethylenediaminetetraacetic acid (EDTA), 117 1% Triton-X-100 and 1% sodium dodecyl sulfate (SDS)) was added to each tube. Organs were 118 homogenized using a Roche MagNA Lyser (F Hoffmann-La Roche Ltd., Basel, Switzerland) with 119 repeated cycles of 6000 rpm for 25 sec with cooling on ice for 1 minute between cycles. Organ 120 homogenates of the control mice (injected only with PBS with 0.5% BSA) were used to create 121 standard curves of exendin-4-IRDye700DX for each organ. 100 µl of homogenates were 122 transferred in triplicate to a black flat-bottom 96-well plate and fluorescence intensity was 123 measured using a TECAN infinite M200 Pro plate reader (PerkinElmer, Groningen, The 124 Netherlands) (excitation wavelength: 620 nm, emission wavelength: 700 nm). Standard curves 125 and tracer uptake were calculated using Microsoft Office Excel 2007.

126 Receptor-targeted photodynamic therapy in vivo; immunohistochemistry

Female BALB/c nude mice with subcutaneous GLP-1R positive xenografts (N=8 per group) were injected intravenously with 30 µg exendin-4-IRDye700DX in 200 µl PBS with 0.5% BSA or 200 µl PBS with 0.5% BSA only, and after 4 hours exposed to 100 J/cm² NIR LED light. One group was 130 treated only with exendin-4-IRDye700DX without NIR light exposure. 2 or 24 hours after NIR light 131 exposure, mice were sacrificed by CO_2 asphyxiation. Tumors were harvested, fixated in 4% 132 buffered formalin, embedded in paraffin and sectioned at 4 µm thickness. Slices were 133 deparaffinized with xylene and rehydrated in ethanol. Antigen retrieval was performed with 10 mM 134 citrate pH 6.0 in a PT-Module (Thermo Fisher Scientific, Waltam, MA, USA) (10 min, 96°C). 135 Endogenous peroxidase activity was quenched with 3% H₂O₂ for 10 min. Slices were incubated 136 with 20% normal goat serum for 30 min and subsequently with rabbit-anti-cleaved-caspase-3 137 (1:4000 in PBS + 1% BSA, ASP175, Cell Signaling Technology, Leiden, The Netherlands) in a 138 humidified chamber at 4°C overnight in the dark. Slides were then washed 3 times with 10 mM 139 PBS and incubated with goat-anti-rabbit-biotin (1:200 in PBS + 1% BSA, Vector Laboratories, 140 Peterborough, UK) for 30 min at room temperature. After washing with PBS, slides were incubated 141 with Vectastain Elite ABC kit (Vector Laboratories, Peterborough, UK) for 30 min at room 142 temperature. The bound antibodies were visualized using diaminobenzine (DAB, Bright DAB, 143 BS04 Immunologic, VWR, Dublin, Ireland). Slides were counterstained with 3 times diluted 144 hematoxylin (Klinipath, Olen, Belgium) for 5 seconds and mounted with a cover slip (permount, 145 Fisher Scientific, Waltam, MA, USA).

The immunohistochemical staining was independently analyzed by two blinded observers. Scores were allocated to each slide following an ordinal 6-point scale ranging from 0 (no staining), 1 (very weak staining), 2 (weak staining), 3 (intermediate staining), 4 (intense staining) to 5 (very intense staining). The scores of the two observers were averaged.

150 **Receptor-targeted photodynamic therapy in vivo; survival**

Female BALC/c nude mice with CHL-GLP-1R xenografts were randomized into 2 groups of 8 animals based on tumor size. When tumors were at least 30 mm³, mice were injected intravenously with 30 μ g exendin-4-IRDye700DX in 200 μ I PBS with 0.5% BSA or PBS with 0.5% BSA only. After 4 hours, mice were exposed to 150 J/cm² of NIR LED light under inhalation anesthesia (2,5% isoflurane mixed with 100% O₂ (1 L/min)). Kidneys were protected from exposure by covering them with gauze and aluminum foil. Tumor diameters were measured by a

157 blinded observer three times per week in three dimensions using a caliper. Mice were euthanized

158 by CO_2 asphyxiation when tumor volume reached more than 1000 mm³ (tumor volume was

- 159 calculated by $1.25^{*}\pi^{*}$ (((length + width + height) / 6) ^3)). Overall survival was defined as the day
- 160 that tumors reached a size of 1000 mm³.

161 Statistics

- 162 Statistical calculations were performed using GraphPad Prism (GraphPad Software, La Jolla, CA,
- 163 USA). IC₅₀ values were calculated by fitting the data with non-linear regression using least squares
- 164 fit with GraphPad Prism. In vitro cell viability after various treatments, assessed by a CellTiter-
- 165 Glo[®] assay, were compared by two-way ANOVA with post-hoc Bonferroni tests. Tracer uptake in
- 166 various tumors was compared between the different injected doses by one-way ANOVA.
- 167 Survival curves were compared with the log-rank (Mantel-Cox) test using GraphPad Prism168 (version 5.03).

169 Study approval

- All animal experiments have been approved by the institutional Animal Welfare Committee of the
 Radboud University Medical Centre and were conducted in accordance to the guidelines of the
 Revised Dutch Act on Animal Experimentation.
- 173

174 **RESULTS**

175 Exendin-4-IRDye700DX binds the GLP-1R with high affinity

The IC₅₀ values of exendin-4 and exendin-4-IRDye700DX, were 2.54 nM (95% CI; 1.32–4.90) and 6.25 nM (95% CI; 3.07–12.74), respectively (Fig. 1). While the binding affinity of the labeled peptide is significantly lower compared to the unlabeled peptide (p < 0.0001), it binds with a high affinity to the GLP-1R in the nanomolar range.

180 In vitro receptor-targeted PDT with exendin-4-IRDye700DX and NIR light causes specific

181 GLP-1R positive cell death.

182 rtPDT with exendin-4-IRDye700DX caused significant phototoxicity in cells with high GLP-1R 183 expression (CHL-GLP-1R cells) and the rat insulinoma cell line INS-1 cells, with GLP-1R 184 expression comparable to human insulinomas. Remaining cell viabilities were 2.3±0.8 % and 185 2.7±0.3 % respectively (Fig. 2). In PANC-1 cells no cellular phototoxicity was observed under 186 these conditions (96.1±1.2 % viable cells). Co-incubation with an excess of unlabeled exendin-4 187 abolished the phototoxic effect in CHL-GLP-1R cells as well as in INS-1 cells (99.3±1.3 and 188 98.4±2.1 % cell viability respectively). NIR light irradiation alone did not cause cellular phototoxicity 189 in any of the cell types (106.6±1.2 %, 102.5±5.9 % and 102.0±1.8 % viable cells in CHL-GLP-1R, 190 INS-1 and PANC-1 cells, respectively). No dark toxicity of the tracer was observed (103.3±6.7 %, 191 105.2±4.7 % and 103.6±1.4 % cell viability without irradiation in CHL-GLP-1R, INS-1 and PANC-192 1 cells, respectively). Incubation of a co-culture of INS-1 and PANC-1 cells with exendin-4-193 IRDye700DX followed by irradiation specifically caused cell death in INS-1 cells, as shown by co-194 localization of the red and green nuclei (Fig. 3). Absence of p.i. signal upon rtPDT indicated that 195 exendin-4-IRDye700DX alone or NIR light alone did not cause cell death in either cell type.

196 **Exendin-4-IRDye700DX** accumulates in GLP-1R positive tumors.

197 Relative uptake of exendin-4-IRDye700DX in subcutaneous GLP-1R tumors in mice was 3.9 ± 1.9 198 % injected dose (ID)/g for 1 µg tracer dose and diminishes slightly to 3.3 ± 0.6 %ID/g for 3 µg tracer 199 dose and 2.5 ± 0.8 %ID/g for 10 µg tracer dose (p = 0.25) (Fig. 4). As a result, the absolute tumor 200 uptake increases with increasing injected tracer doses to 25.0 µg/g with 10 µg tracer injection. 201 Highest uptake of exendin-4-IRDye700 was observed in the kidneys, due to renal clearance.

In vivo receptor-targeted PDT causes cell death in GLP-1R positive tumors and improves survival

Analysis of the immunohistochemical staining revealed a low expression of cleaved-caspase-3 in the control groups. In both treatment groups the expression of cleaved-caspase-3 was higher than in the control groups. While the intensity of cleaved-caspase-3 staining was variable at 2 hours after treatment, the intensity of the staining was high and uniform in the tumors 24 hours after treatment, showing a significant induction of apoptosis in the tumors. The expression of cleavedcaspase-3 was slightly increased in control group receiving only NIR light irradiation, showing that the light itself induces some cell death, most likely due to the heat produced by the LED light source (Fig. 5).

At the start of the survival experiment, sizes of the subcutaneous GLP-1R were very variable, although mean tumor sizes were similar between the groups $(161\pm205 \text{ mm}^3)$ (35-657 mm³) in the exendin-4-IRDye700DX group and 171±144 mm³ (36-480 mm³) in the control group. Upon light exposure, tumor growth was slower in the group which received exendin-4-IRDye700DX leading to a significantly longer median survival in this group compared to the control group (36.5 vs. 22.5 days resp. p<0.05) (Fig. 6).

218

219 **DISCUSSION**

Treatment of hyperinsulinemic hypoglycemia is challenging. To address this issue, a treatment strategy which specifically destroys GLP-1R positive cells with rtPDT was developed as an alternative treatment option for all forms of hyperinsulinemic hypoglycemia.

We show effectivity of rtPDT with exendin-4-IRDye700DX *in vitro* and *in vivo*. The specific cytotoxic effect demonstrates that rtPDT with exendin-4-IRDye700DX could enable destruction of GLP-1R positive lesions without causing damage to the surrounding pancreatic tissue.

This is the first evidence of the effectiveness of a peptide-based agent for rtPDT *in vivo* to date. In the current development of tracers for rtPDT, the most widely used carrier molecules are mAbs and nanoparticles, because of their slow clearance from the circulation and high uptake in target organs. A single previous study examining rtPDT using various targeting peptides was limited to *in vitro* studies and showed no efficient cytotoxic effect (*22*).

We believe that rtPDT with exendin-4-IRDye700DX has the potential to be used as a minimally invasive technique to destroy insulin-producing cells with minimal morbidity. Upon delivery of the tracer, NIR light can be administered interstitially using diffuser fibers which are

234 placed into the target tissue. Using this method of so-called interstitial PDT (iPDT), it is feasible to 235 deliver light to deeply seeded lesions/tissues. Successful results of iPDT have been obtained in 236 for example prostate cancer (23), head and neck cancer (24) and importantly pancreatic tumors 237 (25). An optimal treatment result depends on optimization of the number of light sources as well 238 as their specific placement and power output (26-28). With percutaneous delivery, areas up to 23 239 cm² can be treated (29), making it suitable for treatment of CHI and nesidioblastosis. Alternatively, 240 the less invasive endoscopic delivery of a fiber can be applied for treatment of small lesions, since 241 a single fiber can be applied using this technique (30,31).

The data in this paper do not show 100% cell killing. Since these experiments were performed in an immunocompromised mouse model, they did not take into account the possible added effect on cell killing of the immune response elicited by PDT, as has been shown for other tumor types (*32*). Additionally, because of the minimal invasiveness of PDT, treatment can easily be repeated if hypoglycemia persist. Of interest, in a clinical situation, killing of enough cells to prevent overproduction of insulin will be sufficient, eliminating the need for 100% cell killing.

248 The receptor-targeted approach of PDT with exendin-4-IRDye700DX enables specific killing of GLP-1R expressing cells without damaging the surrounding tissue, and the focused 249 250 irradiation of the tissue of interest avoids a risk of damaging the kidneys. Since treatment of 251 nesidioblastosis and diffuse CHI will involve irradiation of a larger part of the pancreas, this risks 252 development of impaired glucose tolerance. However, rtPDT has advantages over near-total 253 pancreatectomy, since it avoids the risk of exocrine pancreatic insufficiency and is much less 254 invasive. Also, localization and quantification of the insulin-overproducing cells based on pre-255 operative PET images using radiolabeled exendin-4 could be used for planning of the rtPDT to 256 optimize the treatment and minimize side effects.

We believe that the data presented here, together with the advances in the technology of interstitial PDT, can provide a basis towards clinical translation of rtPDT using exendin-4-IRDye700DX. For this, verification of efficient targeting to human tissues as well as the potential

treatment efficacy by ex-vivo analysis of human tissues will be necessary before initiation of a first
 clinical trial.

262

263 CONCLUSION

Here, we show the feasibility of rtPDT with exendin-4-IRDye700DX, which is also the first demonstration of efficient PDT using small molecules *in vivo*. In the future, ablating insulinproducing cells using rtPDT with exendin-4-IRDye700DX could provide a new, minimally invasive treatment method for patients with hyperinsulinemic hypoglycemia. Since this treatment could be applied to a specific site of the pancreas in the case of insulinomas or focal CHI or to a larger pancreatic area in the case of nesidioblastosis or diffuse CHI, it clearly has the potential to be effective to normalize blood glucose regulation in all forms of hyperinsulinemic hypoglycemia.

271

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275

276 AUTHOR CONTRIBUTIONS

M. Boss, S. van Lith, M. Buitinga, M. Brom and M. Gotthardt designed the study. M. Boss, D. Bos,
C. Frielink, G. Sandker and P. Bronkhorst conducted the experiments. M. Boss, D. Bos and C.
Frielink collected and analyzed the data. All authors discussed the results and implications and
commented on the manuscript at all stages. M. Gotthardt is the guarantor of this work and, as
such, had full access to all the data in the study and takes responsibility for the integrity of the
data and the accuracy of the data analysis.

284 KEY POINTS

- 285 **Question:**
- 286 Does rtPDT with exendin-4-IRDye700DX enable effective and specific cell killing of GLP-1R
- 287 positive cells?
- 288 **Pertinent findings:**
- 289 rtPDT with exendin-4-IRDye700DX causes specific phototoxicity in GLP-1R positive cells. The
- 290 tracer accumulates in GLP-1R positive tumors and in vivo rtPDT causes cellular toxicity resulting
- in slower tumor growth.

292 *Implications for patient care:*

- 293 rtPDT with exendin-4-IRDye700DX could provide a new, minimally invasive treatment method for
- 294 patients with hyperinsulinemic hypoglycemia.

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409 Figures





- 412 IRDye700DX. ¹¹¹In-DTPA-exendin-4 was used as a tracer.
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Figure 2. ATP content as a measure of cell viability of CHL-GLP-1R cells, INS-1 cells and PANC-1 cells following incubation with binding buffer (control), exendin-4-IRDye700DX or exendin-4-IRDye-700DX combined with an excess of unlabeled exendin-4 and with or without NIR light irradiation. Experiments were performed in triplicate Data are presented as mean ± SD. * indicates p<0.001.</p>



421

422 Figure 3. Fluorescence microscopy of INS-1 cells labeled with the fluorescent dye DiO (green) and PANC-

423 1 cells labeled with the fluorescent dye DiD (cyan), co-cultured and incubated with propidium iodide (red),

424 after incubation of exendin-4-IRDye700DX or only binding buffer and with and without NIR irradiation with

425 a radiant exposure of 150 J/cm². The scale bar denotes 100 μ m.



Figure 4. Biodistribution of exendin-4-IRDye700DX (1 µg, 3 µg and 10 µg, N=5 mice per group) in tumors,
spleen, pancreas, kidneys and liver of female BALB/c nude mice 4 hours after tracer injection. (A) Relative
uptake expressed as % of the injected dose per gram of tissue. (B) Absolute uptake expressed as µg of
exendin-4-IRDye700DX per gram of tissue.

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Figure 5: Representative examples of cleaved-caspase-3 and HE staining of CHL-GLP-1R tumors. A) Control tumors after i.v. administration of exendin-4-IRDye700DX. B) Control tumors after only illumination. C) Tumors after i.v. administration of exendin-4-IRDye700DX and illumination, dissected after 2 hours. D) Tumors after i.v. administration of exendin-4-IRDye700DX and illumination dissected after 24 hours. E) Intensity scores of capase-3 staining for tumor sections of all mice.

Е

Intensity of the staining

- 0 = no staining
- 1 = very weak staining
- 2 = weak staining 3 = intermediate staining
- 4 = intense staining
- 5 = very intense staining





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449

450 **Figure 6.** Kaplan-Meier plot of survival of BALB/c nude mice with GLP-1R positive tumors after injection of

451 30 µg exendin-4-IRDye700DX or PBS (control), followed by illuminaton with a radiant exposure of 150

452 J/cm².

Supplementary data







Supplemental Figure 2: Absorbance and emission spectra of exendin-4-IRDye700DX



Receptor-targeted photodynamic therapy of glucagon-like peptide 1 receptor positive lesions

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ORIGINAL ARTICLE



Comparison of glucagon-like peptide-1 receptor (GLP-1R) PET/CT, SPECT/CT and 3T MRI for the localisation of occult insulinomas: evaluation of diagnostic accuracy in a prospective crossover imaging study

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Abstract

Purpose Benign insulinomas are the most prevalent cause of endogenous hyperinsulinaemic hypoglycaemia (EHH) in adults, and because of their small size are difficult to localise. The purpose of the study was to test the diagnostic accuracy and clinical impact of glucagon-like peptide-1 receptor (GLP-1R) PET/CT using ⁶⁸Ga-DOTA-exendin-4 in consecutive adult patients referred for localisation of insulinomas. The results were compared with ¹¹¹In-DOTA-exendin-4 SPECT/CT, study-MRI and previously performed external CT and/or MRI (prior external CT/MRI).

Methods We prospectively enrolled patients with neuroglycopenic symptoms due to EHH. GLP-1R PET/CT, SPECT/CT and study-MRI were performed in a randomised, crossover order within 3–4 days. The reference standard was surgery with histology and treatment outcome.

Results From January 2014 until March 2017, 52 patients were recruited. All imaging and invasive procedures before recruitment identified suspicious lesions in 46.2% of patients. GLP-1R PET/CT, SPECT/CT and study-MRI detected suspicious lesions in 78.8%, 63.5% and 63.4% of patients, respectively. In 38 patients, conclusive histology was available for final analysis.

Accuracy (95% confidence interval) for PET/CT, SPECT/CT, study-MRI and prior external CT/MRI was 93.9% (87.8–97.5%), 67.5% (58.1–76.0%), 67.6% (58.0–76.1%) and 40.0% (23.9–57.9%), respectively (all *P* values < 0.01, except comparison of SPECT/CT and study-MRI with a *P* value = 1.0). Impact on clinical management was 42.3%, 32.7% and 33.3% for PET/CT, SPECT/CT and study-MRI, respectively. Percentage reading agreement was 89.5%, 75.7%, and 71.1% for PET/CT, SPECT/CT and study-MRI, respectively.

Conclusion ⁶⁸Ga-DOTA-exendin-4 PET/CT performed significantly better than ¹¹¹In-DOTA-exendin-4 SPECT/CT and MRI in the localisation of benign insulinomas and should be considered in patients where localisation fails with CT/MRI (ClinicalTrials. gov, NCT02127541).

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Keywords Insulinoma · Glucagon-like peptide-1 receptor · GLP-1R PET/CT · GLP-1R SPECT/CT · MRI · 68 Ga-DOTA-exendin-4

Introduction

Benign insulinomas are neuroendocrine tumours (NET) usually located in the pancreas. They are the most prevalent cause of endogenous hyperinsulinaemic hypoglycaemia (EHH) in adult patients [1]. At present, surgery remains the only curative treatment. Pancreas-preserving surgery such as limited segmental resection or enucleation is considered the treatment of choice [1-3]. Therefore, the exact preoperative localisation of insulinomas is critical in order to plan surgical strategy and improve postoperative outcome.

The small size of insulinomas (usually $\leq 2 \text{ cm}$) [4] challenges the detectability by conventional imaging techniques such as contrast-enhanced computed tomography (CT), contrast-enhanced magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS). The selective arterial calcium stimulation and venous sampling (ASVS) approach exhibits high sensitivity [5] but detects only the vascular bed of the insulinoma, not the insulinoma itself, and can be associated with relevant risk for complications [5–7]. A recent systematic review of 2379 cases reported mean sensitivity of 85%, 76%, 58% and 54% in accurate detection of insulinomas by ASVS, EUS, MRI and CT, respectively [8], indicating that there is still an unmet need for a more sensitive non-invasive tool.

In vitro studies using autoradiography have shown that almost all benign insulinomas express glucagon-like peptide-1 receptors (GLP-1R) at high density [9]. The GLP-1R is a G protein-coupled peptide hormone receptor expressed mainly in the alimentary tract, particularly in the pancreatic islet cells, where it mediates the release of glucagon-like peptide-1 (GLP-1) from the small intestine in response to food intake. An insulinoma consists mainly of islet cells. Previously, we and others have shown that targeting GLP-1R using the specific ligands ¹¹¹In-DOTA-exendin-4 [10], ¹¹¹In-DTPA-exendin-4 [11] or ^{99m}Tc-HYNIC-exendin-4 [12] as radiotracers for single-photon emission computed tomography (SPECT) is a very sensitive, non-invasive method to localise benign insulinomas. However, all of the aforementioned radiotracers are exclusively SPECT tracers, with limitations in comparison to positron emission tomography (PET). PET possesses higher sensitivity and spatial resolution than SPECT, the radiation exposure is lower than SPECT with ¹¹¹In-labelled compounds, and accurate quantification of radiotracers is better established with PET than with SPECT [13, 14].

In a proof-of-principle study, GLP-1R PET and SPECT were compared in five patients with EHH after the injection of ⁶⁸Ga-DOTA-exendin-4 ([Nle¹⁴,Lys⁴⁰(Ahx-DOTA-⁶⁸Ga)NH₂]exendin-4) and ¹¹¹In-DOTA-exendin-4

([Nle¹⁴,Lys⁴⁰(Ahx-DOTA-¹¹¹In)NH₂]exendin-4), with excellent image quality for the PET modality [15], consistent with a recent clinical study using ⁶⁸Ga-NOTA-exendin-4 [16].

At present, GLP-1R PET has not been compared with either GLP-1R SPECT or contrast-enhanced MRI to determine the most sensitive non-invasive morphological imaging modality [8, 17].

We therefore tested the diagnostic accuracy and clinical impact of ⁶⁸Ga-DOTA-exendin-4 PET/CT in a multiinstitutional series of consecutive adult patients referred for localisation of insulinomas. The results are compared with ¹¹¹In-DOTA-exendin-4 SPECT/CT, study 3-Tesla MRI and previously performed external CT and/or MRI (prior external CT/MRI) according to the Standard for the Reporting of Diagnostic Accuracy (STARD) guidelines (Supplementary Table 1).

Materials and methods

Study design and patients

For this prospective, single-centre, crossover imaging study, 52 consecutive patients (Table 1 and Fig. 1) were recruited from different centres in Europe and the United States between January 2014 and March 2017 and referred to the University Hospital Basel (ClinicalTrials.gov, NCT02127541). Inclusion criteria were biochemically proven EHH with neuroglycopenic symptoms, a positive Whipple triad defined as (1) attacks of fainting, dizziness and sweating on fasting, (2) hypoglycaemia present during attacks, and (3) relief of symptoms after administration of carbohydrates and negative results on sulfonylurea. Exclusion criteria were evidence of a malignant insulinoma on conventional imaging, pregnancy or breastfeeding in women, and renal insufficiency (serum creatinine >140 µmol/L).

Procedures

Imaging and invasive procedures before recruitment included prior external CT/MRI, EUS with or without biopsy, somatostatin receptor imaging (⁶⁸Ga-DOTATOC PET/CT or OctreoScan®), ¹⁸F-DOPA (¹⁸F-fluorodopa) PET/CT, ASVS and/or surgery using intraoperative ultrasound as locally available. Prior external CT/MRI was performed by the referring centres not more than 2 months before the beginning of the study.

Patients received one $^{68}\text{Ga-DOTA-exendin-4}$ PET/CT and two $^{111}\text{In-DOTA-exendin-4}$ SPECT/CT scans (4 and 72 h

Table 1	Baseline	characteristics
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Participants ($n = 52$)		
Age	49 (38–57)	
Sex		
Female	40/52 (77%)	
Male	12/52 (23%)	
Biochemical assessments at the end of the fasting	test	
Duration of fasting, h	17 (11–35)	
Plasma glucose, mmol/L	2.1 (1.8–2.3)	
C-peptide, nmol/L	0.83 (0.50-1.02)	
Insulin, mU/L	12 (5–18)	
External imaging before recruitment	Number of scans ^a	Number of positive scans ^b
CT/MRI	49/52 (94%)	14/49 (29%)
EUS	33/52 (63%)	13/33 (39%)
Somatostatin receptor imaging	18/52 (35%)	5/18 (28%)
¹⁸ F-DOPA PET/CT	8/52 (15%)	0/8 (0%)
All scans	52/52 (100%)	24/52 (46%)
Invasive procedures before recruitment	Number of invasive procedures	Number of positive invasive procedures ^c
ASVS	10/52 (19%)	4/10 (40%)
EUS with biopsy	5/52 (10%)	0/5 (0%)
Surgery with intraoperative ultrasound	6/52 (12%)	0/6 (0%)
All invasive procedures	16/52 (31%)	4/16 (25%)

Data are median (IQR) or number/total (%). ^a Number of patients who had a prior external scan with the respective imaging modality. ^b Number of patients with a suspicious lesion in the respective imaging modality. ^c Number of patients with histologically proven insulinoma or positive intra-arterial calcium stimulation and venous sampling with the respective invasive procedure

scans) in a randomised crossover order within 3–4 days. A standardised study-MRI scan was performed between PET/CT and SPECT/CT scans. The reference standard was successful surgery with histological evaluation and treatment outcome (monitoring glucose levels for at least 4 weeks after surgery) in all patients.

Detailed information about synthesis and labelling of ⁶⁸Ga-DOTA-exendin-4 and ¹¹¹In-DOTA-exendin-4, coadministration of glucose infusion, and image acquisition of standardised PET/CT, SPECT/CT and study-MRI, as well as prior external CT/MRI, are summarised in the Supplementary Material. Adverse events were recorded and graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 protocol.

Evaluation

In order to localise insulinomas in a standardised manner, the pancreas was categorised into three regions, namely head, body and tail with the portal vein and the superior mesenteric artery serving as anatomic landmarks (Supplementary Fig. 1).

PET/CT, SPECT/CT and study-MRI scans were randomised and independently assessed by three boardcertified nuclear medicine physicians (GN, CR, FK) for PET/ CT and SPECT/CT scans or three board-certified radiologists (EM, CZ, DB) for MRI each with >10 years of experience in PET/CT and SPECT/CT or MRI reading. All readers were unaware of the patients' identity, other imaging results or the patient's clinical history. A non-blinded nuclear medicine physician measured tracer uptake in the tumour and normal pancreas parenchyma (background) as well as the kidneys by drawing volumes of interest and measuring maximal standardised uptake values (SUV) in attenuation- and scattercorrected PET images or count statistics in 4-h and 72-h attenuation- and scatter-corrected SPECT images. Tumour size was derived by measurements on the T1-weighted MRI images by a non-blinded radiologist and by the surgeons/pathologists.

The impact on clinical management was defined as follows: identifying patients with negative or inconclusive finding on all imaging and invasive procedures before recruitment (prior external CT/MRI, EUS with or without biopsy, somatostatin receptor imaging, ¹⁸F-DOPA PET/CT, ASVS and surgery as locally available) and positive findings in the standardised prospective investigations (PET/CT, SPECT/ CT and study-MRI) that allowed surgery planning/imageguided surgery.

Histopathologic diagnosis was made at the local referring institution where surgery was performed. The pathologists were blinded to the results of other diagnostic tests but were aware of the patient's clinical history. In the case of controversial findings, central histological reading was available at the tertiary institution (Institute of Pathology, University Bern). Fig. 1 Study profile. ^a One patient (patient 43) did not receive study-MRI due to pacemaker implantation. ^b Three patients did not have a CT or MRI scan. Instead, they had an EUS



Statistical analysis

The number of detected lesions (% of total) reflects the average of the three readers' results. Only tests that showed consistency between imaging, surgery and histological analysis (positive for insulinoma or nesidioblastosis) were considered as true positives. In patients with more than one lesion, the imaging test was regarded as true-positive if at least one insulinoma was correctly localised (per-patient analysis).

As measures of diagnostic accuracy for each method, sensitivity, overall accuracy and positive predictive value (PPV) were estimated with exact binomial 95% confidence intervals. Except for single readings of prior external CT/MRI, estimates were first derived for each reader separately and then averaged for all readers per method, using the R add-on package *epiR* (Supplemental Material, Supplementary Table 2). Pairwise comparisons of diagnostic measures were made between ⁶⁸Ga-DOTA-exendin-4 PET/CT and ¹¹¹In-DOTA-exendin-4 SPECT/CT, and between study-MRI and each of the other methods. For each comparison, a separate unconditional mixed-effects logistic regression model was fitted, including rater as random effect. *P* values for the corresponding main effect (method) are reported.

Interrater reliability is indicated as the percentage agreement for three blinded readers. This measure is not corrected for chance agreement. Nevertheless, it was chosen since Fleiss' kappa—which would have been the chance-corrected measure of choice—can reach paradoxically low values when the observed proportions, e.g. sensitivity, are very high (the so-called paradox of kappa statistics) [18]. All analyses were conducted using R version 3.3.3 statistical software, using two-sided statistical tests and a significance level, α , of 0.05. No adjustment for multiple testing was made.

There were no clinical GLP-1R PET/CT data available at the start of this study. In this situation, sample size calculations can only be based on crude estimates. It was therefore decided that the results of 40 operated patients should deliver a meaningful statement for a first larger clinical evaluation of ⁶⁸Ga-DOTA-exendin-4 PET/CT. The choice of this sample size was based on a feasibility rationale and experience.

Results

Imaging and invasive procedures before recruitment

Baseline characteristics of all 52 participants as well as the results of all imaging and invasive procedures before recruitment are summarised in Table 1 and Supplementary Table 3. Forty-nine of 52 patients underwent prior external CT/MRI, in

which 14 patients (29%) had one or more suspicious lesions compatible with an insulinoma.

PET/CT, SPECT/CT and study-MRI

All 52 patients underwent ⁶⁸Ga-DOTA-exendin-4 PET/CT and ¹¹¹In-DOTA-exendin-4 SPECT/CT. study-MRI was performed in 51/52 patients (Fig. 1). Patient 43 did not receive study-MRI due to pacemaker implantation. ⁶⁸Ga-DOTAexendin-4 PET/CT, ¹¹¹In-DOTA-exendin-4 SPECT/CT and study-MRI identified suspicious lesions in 78.8%, 63.5% and 63.4% of patients (average reading), respectively.

The highest radiotracer (68 Ga-/ 111 In-DOTA-exendin-4) uptake was noted in tumours and kidneys (Figs. 2 and 3). Median (interquartile range) tumour-to-background ratios were 3.6 (2.3–5.8) for 68 Ga-DOTA-exendin-4 PET and 2.2 (1.5–3.2) for 111 In-DOTA-exendin-4 SPECT. Blood sampling of 68 Ga-DOTA-exendin-4 revealed a biexponential blood clearance, with a half-time of 12.2 ± 1.9 min and 41 ± 4.9 min. 111 In-DOTA-exendin-4 also revealed a biexponential blood clearance with a half-time of 13.6 ± 2.6 min and 118 ± 25.4 min (data are mean ± SD). Both compounds showed a plasma clearance of about 50% in the α -phase. The clearance occurred exclusively via the kidneys.

Nausea and sporadic vomiting are known side effects of exendin-4 radiotracers. Twenty-seven percent (14/52) of patients experienced nausea and 2% (1/52) of patients experienced vomiting after injection of ⁶⁸Ga-DOTA-exendin-4.

Fifty-two percent (27/52) of patients experienced nausea and 44% (23/52) of patients experienced vomiting after injection of ¹¹¹In-DOTA-exendin-4. In this study, these side-effects were grade 1 according to CTCAE 4.03, confined to the first hour after injection and more pronounced with ¹¹¹In-DOTA-exendin-4 in comparison to ⁶⁸Ga-DOTA-exendin-4. No other adverse effects were observed. No severe hypoglycaemic episode occurred after injection of 11.6–23.8 μ g ⁶⁸Ga-DOTA-exendin-4 and 11.0–16.9 μ g ¹¹¹In-DOTA-exendin-4 as all patients received an exogenous glucose (1000 mL, 10%) infusion for 5 h starting just before injection of the radiotracer.

Surgery and histological assessment

Surgical planning was based on all available imaging results. The median (interquartile range) number of days between study imaging and surgery was 46 (24–88). Taking all available preoperative imaging (all imaging and invasive procedures before recruitment as well as study imaging) together, one or more highly suspicious lesions were detected in 43/52 patients (83%). In these patients, surgery was recommended. In four patients (11, 22, 25, 28), none of the imaging modalities or invasive procedures detected a suspicious lesion. In five patients (30, 35, 47, 49, 52), contradicting findings between procedures or readers were found which did not justify surgery. Three patients (4, 12, 19) declined surgery despite unequivocal findings—for example, patient 12 with high suspicion for an ectopic insulinoma (Supplementary Fig. 2).



Fig. 2 Patient 48 with EHH and negative prior external MRI. Transaxial PET/CT 2.5 h after injection of ⁶⁸Ga-DOTA-exendin-4 (a), transaxial SPECT/CT 72 h after injection of ¹¹¹In-DOTA-exendin-4 (b), T1-weighted contrast-enhanced transaxial study-MRI (c) and T2-weighted transaxial study-MRI (d). ⁶⁸Ga-DOTA-exendin-4 PET/CT (a) shows a clear focal uptake in the transition zone between pancreatic head and

body (see arrow). ¹¹¹In-DOTA-exendin-4 SPECT/CT (b) and study-MRI (c, d) were interpreted as negative scans by all readers. Based on PET/CT findings, surgery was performed, and histological evaluation confirmed the PET/CT reading, which was correctly interpreted by all readers: benign insulinoma of 8 mm



Fig. 3 Patient 26 with EHH but without evidence of an insulinoma in the prior external scans (MRI, ¹⁸F-DOPA PET/CT). Study transaxial contrast-enhanced T1-weighted MRI (a), transaxial PET/CT 2.5 h after injection of ⁶⁸Ga-DOTA-exendin-4 (b), transaxial SPECT/CT 4 h (c) and 72 h (d) after injection of ¹¹¹In-DOTA-exendin-4. study-MRI (a) and 4-h SPECT/CT (c) were interpreted as negative scans by several readers.

PET/CT (b) shows a clear focal uptake in the head of the pancreas and the 72-h SPECT/CT (d) shows a more diffuse but more intense uptake at the same location than the 4-h SPECT/CT (c) (see arrows). Based on PET/CT findings, surgery was performed and histological evaluation confirmed a benign insulinoma measuring 9 mm

Altogether 77% of patients (40/52) underwent surgery. In 18/40 patients (45%), the tumour was resected through minimally invasive enucleation. In two patients (patients 23 and 33), symptoms of EHH ceased after surgery, but local and central histological assessment did not confirm the diagnosis of a benign insulinoma or nesidioblastosis. Both patients were excluded from evaluation, as the final diagnosis remained unclear. Consequently, 38 patients had a histological evaluation and hence were included in the main assessment (Fig. 1). Thirty-seven of 38 patients showed a normalisation of blood glucose levels. One patient (patient 31) was operated on according to study-MRI and SPECT/CT findings with enucleation of a lesion in the head of the pancreas, but histology results were negative for an insulinoma, and hypoglycaemia persisted. One or multiple benign insulinomas (median size 12 mm; range 5–23 mm) were confirmed in 36 patients by histology (Figs. 2 and 3), including six patients with a confirmed germline mutation of multiple endocrine neoplasia type 1 (MEN-1). One patient (patient 5) was diagnosed with an adult focal nesidioblastosis. The scan results for this patient, including histopathological confirmation by central assessment, were published previously as a case report [19].

Main outcomes

Table 2 and Supplementary Table 2 summarise averaged accuracy, sensitivity and PPV, as well as percentage reading agreement, for ⁶⁸Ga-DOTA-exendin-4 PET/CT, ¹¹¹In-DOTA-exendin-4 SPECT/CT, study-MRI and prior external CT/MRI in all 38 patients with histological confirmation. Prior external CT/MRI was performed in 35/38 patients. In three patients (patients 6, 7 and 8), prior external CT/MRI assessment was not performed because EUS was available. ⁶⁸Ga-DOTA-exendin-4 PET/CT had the highest accuracy, sensitivity and percentage reading agreement of all tested methods.

Change of clinical management: among the 52 patients, surgery planning/image-guided surgery became possible after initially (before recruitment) negative or inconclusive findings in 42.3%, 32.7 and 33.3% of patients after ⁶⁸Ga-DOTA-exendin-4 PET/CT, ¹¹¹In-DOTA-exendin-4 SPECT/CT and study-MRI, respectively (Table 2). All of these patients underwent surgery and showed normalisation of blood glucose (cure) after surgery. The only exceptions were patients 4 and 19 who refused surgery and patient 31 with false-positive imaging. Furthermore, PET/CT localised the insulinoma or nesidioblastosis in 81% of patients with initially (before recruitment) negative invasive procedures (ASVS, EUS with biopsy and surgery with intraoperative ultrasound) (Table 1 and Supplemental Table 3).

Discussion

The main findings in our study can be summarised as follows: (1) GLP-1R PET/CT has significantly higher accuracy and sensitivity, and influences surgical planning/image-guided surgery more than MRI and SPECT/CT. (2) GLP-1R PET/

	⁶⁸ Ga-DOTA-exendin-4 PET/CT ($n = 38$)	¹¹¹ In-DOTA-exendin-4 SPECT/CT ^a $(n = 38)$	Study MRI $(n = 37)$	Prior external CT/MRI $(n = 35)$	Test for superiority ^b
Accuracy	93.9% (87.8–97.5)	67.5% (58.1–76.0)	67.6% (58.0–76.1)	40.0% (23.9–57.9)	P<0.001
Sensitivity	94.6% (88.6–98.0)	68.5% (59.0-77.0)	69.4% (59.8–77.9)	38.2% (22.2–56.4)	P < 0.001
Positive predictive value (PPV)	99.1% (94.9–100)	97.4% (91.0–99.7)	96.2% (89.2–99.2)	100% (75.3–100)	P > 0.2
Percentage reading agreement	89.5%	75.7%	71.1%	NA	NA
Impact for surgery planning	42.3%	32.7%	33.3%	_	_

 Table 2
 Comparison of GLP-1R imaging, study-MRI and prior external CT/MRI in patients with suspected insulinoma and available reference standard (surgery and normalisation of blood glucose levels)

Imaging performance is given as the averages (95% confidence interval) of three readings by three independent readers except prior external MRI and CT which were done and interpreted at referring centres. ^a SPECT/CT results are based on 24-h and 72-h readings. ^b *P* values for comparisons of ⁶⁸ Ga-DOTA-exendin-4 PET/CT versus ¹¹¹ In-DOTA-exendin-4 SPECT/CT and study-MRI, n = 38 and 37 patients, respectively. Accuracy and sensitivity were not significantly different between ¹¹¹ In-DOTA-exendin-4 SPECT/CT and study-MRI (P = 1.0 and P = 0.88). There was a significant difference in accuracy and sensitivity between study-MRI and prior external MRI (P = 0.004 and P = 0.002). Impact in surgery planning/image-guided surgery was evaluated for each study image modality separately. NA = not applicable

CT showed the highest reader agreement compared to study-MRI or GLP-1R SPECT/CT. (3) Standardisation of MRI improved accuracy, sensitivity and impact on surgery planning. MRI performs equally well as GLP-1R SPECT/CT if MRI is performed meticulously and is read by experienced radiologists.

The superior accuracy and sensitivity of GLP-1R PET/CT in comparison to SPECT/CT can be attributed to the three following factors: higher spatial resolution, higher scanner sensitivity and higher tumour-to-background ratio aiding visual assessment. Notably, in our patient collective, in which the median tumour size was 12 mm, the higher spatial resolution and scanner sensitivity of PET/CT offered a considerable benefit in comparison to SPECT/CT and MRI. The latter is limited in the detection and characterisation of small lesions through motion artefacts, such as respiratory motion, cardiac pulsation, and bowel peristalsis [20]. However, small insulinomas \leq 10 mm in diameter can be missed even with ⁶⁸Ga-DOTA-exendin-4 PET/CT, as the single false-negative PET/CT finding was identified in a patient with an insulinoma measuring $5 \times 5 \times 10$ mm (patient 37).

The physiologic high kidney uptake of GLP-1R-specific radiotracers and the inherent high partial volume effect is a limitation of GLP-1R imaging [15]. The better spatial resolution of GLP-1R PET/CT is a substantial advantage over GLP-1R SPECT/CT perceivable in three patients. PET/CT was able to delineate the benign insulinoma in the distal portion of the pancreatic tail in close proximity to the left kidney, whereas SPECT/CT was not able to discriminate the lesions from the kidney uptake (patients 1, 6 and 42).

The superior biexponential blood clearance of ⁶⁸Ga-DOTA-exendin-4 compared to ¹¹¹In-DOTA-exendin-4 and the lower partial volume effect of PET compared to SPECT [13] lead to a higher tumour-to-background ratio with PET/ CT. This fact may explain the higher reader agreement of PET/CT reading, surpassing that of SPECT/CT and studyMRI, making it a reliable imaging technique. An additional advantage of ⁶⁸Ga-DOTA-exendin-4 in comparison to ¹¹¹In-DOTA-exendin-4 is the shorter half-life of ⁶⁸Ga (68 min vs. 67 h) which results in a lower radiation burden for patients [14]. Furthermore, PET/CT scans are performed 2.5 h after injection of ⁶⁸Ga-DOTA-exendin-4 which is more convenient for patients than ¹¹¹In-DOTA-exendin-4 SPECT/CT which should be performed at later time points, e.g. at 24 and 72 h (Fig. 3) [21].

Sensitivity of GLP-1R SPECT/CT and previously performed external CT/MRI were lower in the current study than in our previous published study [11]. This can be attributed to the referral of particularly challenging cases with prior negative or inconclusive imaging procedures. This is reflected in the smaller tumours size in comparison to our previous study (median size; 12 vs. 15 mm) and the fact that 13/52 patients had previous unsuccessful invasive procedures such as ASVS, surgery or biopsy.

The study imaging procedure, in particular PET/CT, significantly influenced the clinical management of patients, defined as successful image-guided surgery in the presence of previous (before recruitment) negative or ambiguous imaging findings or negative invasive procedures. This suggests that the surgical strategy takes into account the correct preoperative localisation, allowing for a laparoscopic or focused pancreatic resection approach [1-3], with 45% of all operated patients receiving enucleation (Supplementary Table 3). In addition, GLP-1R PET/CT has a high impact on the surgical management of patients with EHH in the context of MEN-1. MEN-1 patients with EHH often present multiple pancreatic lesions [22] and MRI cannot differentiate insulin-producing from other neuroendocrine tumours. Finally, GLP-1R PET/ CT can localise focal nesidioblastosis (patient 5) [19], thereby influencing surgical management in these patients.

Nine of 52 patients did not undergo surgery mainly due to negative or inconclusive imaging results (Supplementary

Table 3). This is attributable to the fact that resecting an insulinoma without knowing its exact localisation is associated with extensive pancreatic exploration, including palpation and intraoperative sonography with its associated morbidity [1-3]. There may be several reasons for a negative scan in the presence of a documented EHH: (1) The size of the insulinoma is below the detection rate of a given imaging modality. (2) A different aetiology of EHH, in particular nesidioblastosis, may be the cause of a negative scan in the presence of EHH. Previous in vivo and in vitro work suggests that GLP-1Rs are overexpressed in adult nesidioblastosis but to a lesser extent than in benign insulinoma [19]. Indeed, in three patients the GLP-1R imaging showed generally increased uptake, but was considered negative for a focal lesion. One of the patients (patient 11) had an additional ¹⁸F-DOPA PET/CT scan that was also compatible with a possible nesidioblastosis. Unfortunately, there was no focal lesion, and the patients thus refused a surgical intervention. All three patients were treated medically (diaxozide or somatostatin analogues). (3) The documentation of EHH (fasting test) was performed by the referral centres, and since it was not standardised, an inadequate procedure cannot be completely excluded.

There is one case that needs particular attention: Patient 31 was the only patient who was operated upon our recommendation and proved to be false-positive. MRI depicted a small lesion of 9 mm in the pancreatic head with a corresponding diffuse uptake in the SPECT/CT scan and a questionable focus in the PET/CT scan spotted by one reader. Surgery was performed and a 3.5-mm lesion was removed in this location which showed possible neuroendocrine tissue on frozen section. However, histological and immunohistochemical evaluation including insulin staining was negative, and the reason for persistence of hypoglycaemia after surgery remained unclear. The same patient had previously undergone a pancreatic tail resection without evidence of an insulinoma or nesidioblastosis. The reason for the false-positive imaging finding remained unclear, but previous studies indicate that the Brunner glands of the duodenum (which homogeneously express GLP-1Rs at high density) might have interfered [11].

Somatostatin receptor PET/CT (e.g. ⁶⁸Ga-DOTATATE or ⁶⁸Ga-DOTATOC) and ¹⁸F-fluorodopa (¹⁸F-DOPA) PET/CT are alternative molecular imaging modalities used for the localisation of insulinomas. Prasad et al. detected insulinomas or nesidioblastosis in 11/13 patients (85%) with ⁶⁸Ga-DOTATATE or ⁶⁸Ga-DOTATOC PET/CT [23]. In their study, CT was nearly as good as somatostatin receptor PET/CT, with a detection rate of 77% (10/13 patients), indicating that these were not particularly difficult cases. In our study, ⁶⁸Ga-DOTATOC PET/CT was performed in nine patients, with histological proof in six patients (patients 1, 5, 21, 24, 34 and 36), resulting in the detection of two insulinomas in six patients (33%). In those six patients, ⁶⁸Ga-DOTA-exendin-4 PET/CT

detected the insulinoma in 94% of patients. Reubi et al. quantified GLP-1R and somatostatin receptor subtype 2 (the main target of ⁶⁸Ga-DOTATOC and ⁶⁸Ga-DOTATATE PET/CT) in 26 insulinoma tissue samples using in vitro autoradiography [9]. GLP-1R was expressed in 24/26 samples (92%) at a high density, whereas somatostatin receptor subtype 2 was expressed in 18/26 samples (69%) at a moderate to high density. As a result, GLP-1R PET/CT is likely to perform better than somatostatin receptor PET/CT in the detection of insulinomas.

Kauhanen et al. detected insulinomas or nesidioblastosis in 9/10 patients (90%) with ¹⁸F-DOPA PET [24]. Other groups could not repeat the initially excellent results of Kauhanen et al. For example, Nakuz et al. detected the insulinoma in 5/10 patients (50%) with ¹⁸F-DOPA PET [25]. Imperiale et al. showed somewhat better results with cabidopa pretreatment that seems to reduce the physiological uptake of ¹⁸F-DOPA in the pancreas: insulinoma detection rate of 8/11 patients (73%) [26]. In our patient collective, ¹⁸F-DOPA PET/CT without carbidopa pretreatment detected none of five insulinomas correctly. In those five patients, ⁶⁸Ga-DOTA-exendin-4 PET/CT detected the insulinoma in 93% of patients. As a result, GLP-1R PET/CT is likely to perform better than ¹⁸F-DOPA PET/ CT in the detection of insulinomas. Taken together, the evidence level for comparative PET/CT studies is scarce and controversial in patients with EHH, and only a direct prospective comparison between the different tracers would allow a firm conclusion.

This study has limitation, as follows: (1) Intentionally, the diagnostic performance of GLP-1R imaging was compared to a study-MRI protocol and not to EUS and/or ASVS, since the latter procedures are not widely available (ASVS) or are more investigator-dependent (EUS, ASVS) than MRI. We, therefore, cannot compare the accuracy and sensitivity of standardised EUS and ASVS with GLP-1R imaging. (2) The inclusion criteria of a pathological fasting test with neuroglycopenic symptoms confirming EHH is, despite possible false-positive results, a rather specific criterion for an insulinoma [27]. Consequently, we cannot evaluate the specificity and the negative predictive value of the tested imaging modalities. (3) Furthermore, ⁶⁸Ga-DOTA-exendin-4 PET/CT, which was the most sensitive and accurate method (sensitivity of 94.6%), detected at least one suspicious lesion in only 78.8% of all 52 patients. Consequently, diagnostic accuracy and sensitivity is overestimated with the chosen reference standard (histology and clinical outcome). A positive fasting test with neurogylcopenic symptoms is a rather specific criterion for an insulinoma, but it is not suitable as a reference standard, since the purpose of the study was the correct localisation of insulinomas that can best be verified with surgery including histology and treatment outcome. For example, in five patients (9.6% of patients, Fig. 1), imaging findings were inconclusive (different location of lesions), and did not justify surgery. In these patients, the fasting test would not be able to differentiate between true-positive and false-positive findings, which is essential for the evaluation of accuracy and sensitivity.

Conclusion

⁶⁸Ga-DOTA-exendin-4 PET/CT performs significantly better than ¹¹¹In-DOTA-exendin-4 SPECT/CT and MRI in the preoperative localisation of insulinoma and nesidioblastosis and changes clinical management. It is also more convenient than GLP-1R SPECT/CT, with a lower radiation burden and a shorter investigation time. It should be recommended in the case of negative or inconclusive results with conventional CT or MRI.

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Compliance with ethical standards

Disclosure of potential conflict of interest The authors declare that they have no conflict of interest relevant to this article.

Ethical approval The study was approved by the regional scientific ethics committee, and all procedures performed in studies involving human participants were in accordance with the ethical standards of the regional scientific ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Glucagon-like peptide-1 receptor imaging for the localisation of insulinomas: a prospective multicentre imaging study

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Summary

Background Small benign insulinomas are hard to localise, leading to difficulties in planning of surgical interventions. We aimed to prospectively assess the insulinoma detection rate of single-photon emission CT in combination with CT (SPECT/CT) with a glucagon-like peptide-1 receptor avid radiotracer, and compare detection rates with conventional CT/MRI techniques.

Methods In our prospective imaging study, we enrolled adults aged 25–81 years at centres in Germany, Switzerland, and the UK. Eligible patients had proven clinical and biochemical endogenous hyperinsulinaemic hypoglycaemia and no evidence for metastatic disease on conventional imaging. CT/MRI imaging was done at referring centres according to standard protocols. At three tertiary nuclear medicine centres, we used whole body planar images and SPECT/CT of the abdomen up to 168 h after injection of ¹¹¹In-[Lys₄₀(Ahx-DTPA-¹¹¹In)NH₂]-exendin-4 (¹¹¹In-DTPA-exendin-4) to identify insulinomas. Consenting patients underwent surgery and imaging findings were confirmed histologically.

Findings Between Oct 1, 2008, and Dec 31, 2011, we recruited 30 patients. All patients underwent ¹¹¹In-DTPAexendin-4 imaging, 25 patients underwent surgery (with histological analysis), and 27 patients were assessed with CT/MRI. ¹¹¹In-DTPA-exendin-4 SPECT/CT correctly detected 19 insulinomas and four additional positive lesions (two islet-cell hyperplasia and two uncharacterised lesions) resulting in a positive predictive value of 83% (95% CI 62–94). One true negative (islet-cell hyperplasia) and one false negative (malignant insulinoma) result was identified in separate patients by ¹¹¹In-DTPA-exendin-4 SPECT/CT. Seven patients (23%) were referred to surgery on the basis of ¹¹¹In-DTPA-exendin-4 imaging alone. For 23 assessable patients, ¹¹¹In-DTPA-exendin-4 SPECT/CT had a higher sensitivity (95% [95% CI 74–100]) than did CT/MRI (47% [27–68]; p=0.011).

Interpretation ¹¹¹In-DTPA-exendin-4 SPECT/CT could provide a good second-line imaging strategy for patients with negative results on initial imaging with CT/MRI.

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Introduction

Benign insulinomas are small neuroendocrine tumours that are nearly always located in the pancreas and are the most common cause of endogenous hyperinsulinaemic hypoglycaemia in adult patients without diabetes.¹ Conventional imaging (ie, CT/MRI) and, if available, endoscopic ultrasound form the basis of localisation and diagnosis of suspected insulinoma.²⁻⁵ However, because of the small size of these tumours, localisation with conventional procedures has notable diagnostic difficulties.⁶ Methods such as selective intra-arterial calcium stimulation and venous sampling (ASVS) have a better sensitivity than do conventional techniques, but are invasive procedures with a concomitant risk of complications.⁶⁻⁸

As somatostatin receptor expression is low in benign insulinomas,⁹ detection rates with somatostatin receptor scintigraphy are low, and thus development of a new receptor targeted imaging technique for this type of neuroendocrine tumour will be important. Data from

in-vitro studies suggest that benign insulinomas have a very high density of glucagon-like peptide-1 receptors (GLP-1R) that might be used as specific targets for in-vivo receptor imaging.¹⁰

GLP-1 or its analogues, liraglutide and exenatide, enhance insulin secretion by β cells and have been introduced for treatment of type 2 diabetes.^{11,12} Several GLP-1-like radioligands retaining high binding affinity to GLP-1R have been developed, including the specific ligand [Lys40(Ahx-DOTA)NH2]exendin-4 and [Lys40(Ahx-DTPA) NH,]exendin-4 labelled with indium-111 (111In-DOTAexendin-4 and 111In-DTPA-exendin-4) and [Lys40(Ahx-HYNIC)NH,]exendin-4 labelled with technetium-99m (99^mTc-HYNIC-exendin-4).^{13–17} In a proof-of-principle study,18 111In-DOTA-exendin-4 was given to six patients with endogenous hyperinsulinaemic hypoglycaemia. Single-photon emission CT in combination with CT (SPECT/CT) detected the lesion in all six patients whereas conventional imaging appropriately located the tumour in only one patient and endosonography was positive in four CrossMark

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Correspondence to: Prof Damian Wild, Division of Nuclear Medicine, University of Basel Hospital, Petersgraben 4, CH-4031 Basel, Switzerland damian.wild@usb.ch patients. Notably, in-vitro GLP-1R autoradiography of the surgical specimen showed a high density of GLP-1R in all six insulinomas.¹⁸ Similarly, ^{99m}Tc-HYNIC-exendin-4 showed promising results in a recent report.¹⁵

A curative treatment approach for insulinoma relies on surgical removal of the tumour. Therefore, identification of the precise preoperative location of the insulinoma is crucial to reduce the extent of the surgical intervention and allow for preservation of pancreatic tissue.^{3,6} The results of the proof-of-principle study¹⁸ showed that GLP-1R imaging is capable of localising insulinomas in vivo.

Our study aimed to determine the insulinoma detection rates of ¹¹¹In-DTPA-exendin-4 SPECT/CT and conventional imaging, and to assess whether ¹¹¹In-DTPAexendin-4 SPECT/CT changes clinical management in patients with negative conventional imaging.

Methods

Study design and patients

In our prospective multicentre imaging study, we screened patients with suspected hyperinsulinaemic hypoglycaemia (positive Whipple triad) at tertiary referral centres in Switzerland (University of Basel Hospital, University of Berne Hospital, Kantonsspital Lucerne, and Kantonsspital St Gallen), Germany (University Hospital Freiburg), and the UK (Royal Free Hospital) according to the present guidelines.19 We enrolled adults aged 25-81 years with biochemically proven endogenous hyperinsulinaemic hypoglycaemia in the fasting state (ie, neuroglycopenic symptoms in the fasting state with low plasma glucose, inappropriately high serum insulin and C-peptide concentrations, and a negative screening for sulfonylurea).20 In addition, to exclude patients with evidence of malignant insulinoma, local conventional imaging (CT/MRI) had to have shown no or only one suspicious lesion. We excluded pregnant women, patients with allergies to exendin-4, and patients with renal (blood insufficiency creatinine concentrations >140 µmol/L). All patients who fulfilled inclusion criteria (reviewed by EC) were referred to one of the three tertiary nuclear medicine referral centres (University of Basel Hospital, Basel, Switzerland; University Hospital Freiburg, Freiburg, Germany; University College London Hospital, London, UK) for 111In-DTPA-exendin-4 imaging. All three centres used the same inclusion and exclusion criteria and the same radiotracer and SPECT/CT imaging protocol. The Swiss study was registered with ClinicalTrials.gov, number NCT00937079.

The study was approved by the local institutional review board of each participating institution, and patients provided written consent in accordance with provisions of the Declaration of Helsinki.

Procedures

Conventional imaging was done by the referring centres, and included triple phase multidetector CT and MRI

with 1.5 T or 3.0 T systems and a dedicated circular polarised body array for signal reception. Minimum pulse sequence requirements were multiplanar (axial and coronal) fast spin echo T2-weighted images and axial multiphasic T1-weighted gradient echo images before and after the administration of a gadolinium-containing contrast agent. Additional (optional) pulse sequences included axial gradient dual echo images and axial diffusion weighted images. The appendix summarises CT and MR imaging procedures undertaken at the referral centres.

We did GLP-1R imaging within 2 months of conventional imaging. Synthesis and labelling of ¹¹¹In-DTPA-exendin-4 has been published elsewhere.¹³ We monitored blood sugar concentrations 15 min, 40 min, 60 min, 120 min, 180 min, and 240 min after the injection of ¹¹¹In-DTPA-exendin-4 and glucose (5%) infusion was administered if needed.

Total-body planar images and single-photon emission CT (SPECT) in combination with CT scans of the abdomen were acquired at 4 h and 3–5 days after injection of 8–14 µg (80–128 MBq) ¹¹¹In-DTPA-exendin-4. The radiopharmaceutical was intravenously injected over 4–5 min. We did imaging with a combined SPECT/CT unit (Symbia T2 [Siemens Medical Systems, Erlangen, Germany], Infinia Hawkeye [GE Healthcare, Chalfont St Giles, UK], or Bright View XCT [Philips Healthcare, Best, Netherlands]) equipped with a medium-energy, parallel-hole collimator.¹⁸ We used low-dose CT imaging (130 kVp, 40 mAs) to correct for attenuation effects and to provide better anatomical localisation of SPECT findings.

We regarded histological diagnosis as the gold standard for detection of insulinomas. All conventional scans were independently reported by experienced dedicated radiologists at the referral centres. Two experienced nuclear medicine physicians (DW and FF) visually assessed GLP-1R scans. All tissue samples with evidence of adult nesidioblastosis were reviewed by an experienced pathologist (AP). The radiologists, nuclear medicine physicians, and pathologists were masked to the results of other diagnostic tests but were aware of the patients' clinical histories.

Statistical analysis

We regarded positive imaging tests that showed consistency between imaging, surgery, and histological analysis (positive for insulinoma) as true positives. The only exception was made in patients with several lesions. In such patients, the imaging test was regarded as true positive if at least one insulinoma was correctly localised (per patient-based analysis).

For point estimates of sensitivity, specificity, and positive predictive value (PPV), we calculated 95% CI according to the method by Agresti and Coull.²¹ For point estimates of the diagnostic odds ratio, we derived 95% CI assuming an approximate normal distribution of the logarithm of the odds ratio.²²

See Online for appendix

To compare differences between imaging techniques, on the basis of a small pilot study¹⁸ of six patients we assumed ¹¹¹In-DTPA-exendin-4 would have a 25% increased detection rate of insulinoma than conventional CT/MRI imaging. With a power of 80% and α of 5%, we planned to enrol 30 patients assuming a dropout rate of 10%.

We assessed significance of the difference in sensitivity and specificity between ¹¹¹In-DTPA-exendin-4 SPECT/CT and CT/MRI by use of an exact binomial test for dependent proportions as introduced by Mosteller.²³ For comparison of PPVs, we applied the generalised score test.²⁴ Because estimation of odds ratios was either very imprecise (for the GLP-1R imaging) or not possible (for CT/MRI), we did not consider a comparison of the odds ratios. All analyses were done with R, version 2.15.3.

Role of the funding source

The sponsor of the study had no role in study design, data collection, analysis, interpretation, or writing of the report. The corresponding author, EC, and FF had full access to all the data in this study and had final responsibility for the decision to submit the manuscript.

Results

Between Oct 1, 2008, and Dec 31, 2011, we recruited 30 consecutive patients in neuroendocrine tertiary referral centres (table 1) and referred them to three tertiary nuclear medicine centres. All patients underwent a fasting test. After 4–72 h of fasting, all patients had symptoms of neuroglycopenia (eg, confusion or unconsciousness up to seizure) with low plasma glucose concentrations and inadequately high concentrations of insulin and C-peptide (table 1). 25 (83%) of 30 patients had definite surgical histological diagnosis and were included in the main assessments (figure 1).

CT/MRI assessments were done for 23 (92%) of these 25 patients (figure 1). In two patients, CT and MRI assessments were not done because endoscopic ultrasound was available. Insulinoma was correctly diagnosed by CT/MRI in nine of 19 patients (47% sensitivity, 95% CI 27–68), with a PPV of 100% (95% CI 66–100). Figure 2 shows the contingency table for conventional imaging and histology.

11 (44%) of 25 patients had endoscopic ultrasound, which identified lesions in eight patients. Endoscopic ultrasound localised the insulinoma in seven (88%, 95% CI 66–100) of these patients and islet-cell hyperplasia in the other patient (ie, a false positive). Two patients had a true negative result (islet-cell hyperplasia and no final histological diagnosis) and one patient had a false negative result with endoscopic ultrasound.

Seven (28%) of 25 patients underwent ASVS. The correct vascular territory of the insulinoma was detected in five (71%) of these patients. Two patients with positive results after ASVS did not show any evidence for an insulinoma during or after surgery (false positive). Histological assessment showed islet-cell hyperplasia in one patient and no pathological findings in the other patient.

The labelling yield of ¹¹¹In-DTPA-exendin-4 was more than 95% with a specific activity of 90 GBq/µmol and a radiochemical purity of 92%. The median decrease in blood glucose level was 1.3 mmol/L (IQR 0.8-2.1; range 0.0-2.6 mmol/L) after injection of ¹¹¹In-DTPA-exendin-4. The nadir of blood glucose concentrations occurred 40 min after injection. 20 (67%) of 30 patients required an exogenous glucose infusion (5%) for a maximum of 90 min. No serious episodes of hypoglycaemia occurred. The appendix shows a summary of blood glucose findings of each patient before and after injection of ¹¹¹In-DTPAexendin-4. We noted no other side-effects.

The longest residence times of ¹¹¹In-DTPA-exendin-4 were noted in the tumour and kidneys (figure 3), and the clearance of the radiotracer occurred exclusively via the kidneys. For 20 (80%) of 25 patients, GLP-1R imaging

	Participants (n=30)
Age, years	55 (39–75; 25–81)
Sex	
Male	11 (37%)
Female	19 (63%)
Biochemical assessments at the end of the fasting test	
Duration of fasting, h	24 (13-32; 4-72)
Plasma glucose, mmol/L	1.9 (1.7–2.2; 1.0–3.0)
C-peptide, nmol/L	1.11 (0.44–1.90; 0.23–2.50)
Insulin, mU/L	11.0 (6.7–22.0; 1.9–38.0)
Data are median (IQR; range) or n (%).	



Figure 1: Study profile

¹¹¹In-DTPA-exendin-4=¹¹¹In-[Lys⁴⁰(Ahx-DTPA-¹¹¹In)NH₃]-exendin-4. SPECT/CT=single-photon emission CT in combination with CT. ASVS=selective intra-arterial calcium stimulation and venous sampling.

A 23 p	atients		Histology		B 25 p	atients	Histology			
		+	-	Total			+	-	Total	
2	+	9	0 9		<u>د</u> م	+	19	4	23	
CT/M	-	10	4	14	GLP-l magi	-	1	1	2	
	Total	19	19 4 23			Total	20	5	25	

showed intense focal ¹¹¹In-DTPA-exendin-4 uptake 4 h after injection. In five patients (patients 14, 19, 25, 26, and 30) only late scans at or after 3 days showed more

Figure 2: Contingency tables of conventional imaging and histology (A) and GLP-1R imaging with histology (B) GLP-1R=glucagon-like peptide-1 receptor. +=positive. ==negative.



Figure 3: Whole-body planar image (A) and SPECT/CT images (B and C) from patient 29, 4 h after injection of 108 MBq ¹¹¹In-DTPA-exendin-4.

Focal ¹¹¹In-DTPA-exendin-4 uptake in the head of pancreas (arrowhead) and in the body of the pancreas (arrows). Surgery confirmed an insulinoma in head of pancreas (17 mm) and in the body of pancreas (33 mm). In the tail of pancreas a glucagon-producing neuroendocrine tumour (25 mm) was not detected with GLP-1R imaging. Additional small tumour lesions <10 mm (insulinoma and gastrinomas) were also not detected. ¹¹¹In-DTPA-exendin-4=¹¹¹In-[Lys⁴⁰(Ahx-DTPA-¹¹¹In)NH,]-exendin-4.

	¹¹¹ In-DTPA-exendin-4 SPECT/CT (n=25)	¹¹¹ In-DTPA-exendin-4 SPECT/CT (n=23*)	CT/MRI (n=23*)	p value*
Sensitivity	95% (75–100)	95% (74–100)	47% (27–68)	0.011
Specificity	20% (2-64)†	25% (3–71)†	100% (45–100)†	1.0†
Positive predictive value	83% (62–94)	86% (65–96)	100% (66–100)	0.069
Diagnostic odds ratio	4.8 (0.24-93)	6.0 (0.29–124)	N/A	N/A

Data are n (95% CI). N/A=not applicable because of missing false-positive results. ¹¹¹In-DTPA-exendin-4=¹¹¹In-[Lys⁴⁰(Ahx-DTPA-¹¹¹In)NH2]-exendin-4. SPECT/CT=single-photon emission CT in combination with CT. *Based on 23 patients for whom complete imaging was available. †Estimates based on five patients in the ¹¹¹In-DTPAexendin-4 SPECT/CT group and four patients in the CT/MRI group.

Table 2: Comparison of GLP-1R imaging and conventional imaging in patients with suspected insulinoma

conclusive results. Histological evaluation in these five patients showed small insulinomas (7–11 mm) in four patients and no conclusive result in one patient.

GLP-1R imaging was done in all 25 evaluated patients, showing focal radiotracer uptake in 23 patients (92%). ¹¹¹In-DTPA-exendin-4 SPECT/CT correctly detected the insulinoma in 19 of 20 patients (95% sensitivity, 95% CI 75–100). The technique had four false positive results (two adult nesidioblastosis and two uncharacterised lesions) resulting in a PPV of 83% (95% CI 62–94; table 2). ¹¹¹In-DTPA-exendin-4 SPECT/CT was more sensitive than CT/MRI (table 2). Table 2 and figure 2 summarise sensitivity, specificity, PPV, and diagnostic odds ratios for CT/MRI and ¹¹¹In-DTPA-exendin-4 SPECT/CT.

25 (83%) of 30 patients had a surgical procedure with histological analysis (figure 1, table 3). Surgical planning was based on all available imaging results. All patients had surgery less than 5 weeks after imaging. 20 insulinomas (median size 14 mm [IQR 10–16]) were confirmed histologically, including two patients with multiple endocrine neoplasia type 1 (MEN1) and two patients with malignant insulinoma. Both patients with malignant insulinoma had only one local lymph-node metastasis. In the remaining five patients, changes compatible with adult nesidioblastosis (islet-cell hyperplasia) were diagnosed (three patients), or a definite diagnosis (two patients) could not be established despite use of intraoperative ultrasound, palpation, and biopsy sampling.

Two patients had a confirmed germline mutation of MEN1. For both patients, GLP-1R imaging was positive, with one lesion identified in the tail of the pancreas (15 mm) in one patient and two positive lesions identified in the other patient (33 mm and 17 mm; figure 3). Both patients underwent successful operations on the basis of preoperative localisation. Because of the localisation of the two lesions in the second patient, a simultaneous Whipple procedure and a left-sided partial pancreatectomy was done, whereas in the first patient the positive lesion in the tail of the pancreas was removed. Positive lesions noted on GLP-1R imaging in both patients were confirmed as insulinomas on histopathological examination. In addition, histological assessment in the first patient detected two microadenomas of less than 2 mm with insulin staining, which were not detected on GLP-1R imaging or other imaging modalities. In the second patient (patient 29), one further insulin-staining microadenoma (4 mm), one glucagon-staining tumour (25 mm), and two gastrin-staining tumours (9 mm and 6 mm) were identified, which were not identified on GLP-1R imaging or other imaging strategies.

Five patients did not undergo a surgical intervention (figure 1, table 3). Two (patients 18 and 28) had negative conventional imaging and GLP-1R imaging results. Another patient (patient 2) had a negative GLP-1R scan, positive ASVS, and diffusely enhanced uptake in the pancreas with ¹⁸F-fluorodopa (¹⁸F-DOPA)-PET imaging (data not shown), suggesting islet-cell hyperplasia

	CT/MRI	EUS	ASVS	GLP-1R imaging	Surgery and histology	Final diagnosis	Tumour localisation	Dimension of tumours
Patient 1	TP	Not done	ТР	TP	Done	Malignant insulinoma	Uncinate process of pancreas and one local lymph node metastasis	16 mm primary, 15 mm metastasis
Patient 2	-	-	+	-	Not done	No diagnosis	N/A	N/A
Patient 3	TP	Not done	Not done	TP	Done	Benign insulinoma	Head of pancreas	15 mm
Patient 4	ТР	ТР	Not done	FN	Done	Malignant insulinoma	Tail of pancreas and one local lymph node metastasis	50 mm primary, 11 mm metastasis
Patient 5	TN	Not done	Not done	FP	Done	Histology negative for tumours	N/A	N/A
Patient 6	TN	Not done	Not done	TN	Done	Islet-cell hyperplasia	N/A	N/A
Patient 7	FN	Not done	Not done	TP	Done	Benign insulinoma	Head of pancreas	15 mm
Patient 8	FN	Not done	TP	TP	Done	Benign insulinoma	Head of pancreas	14 mm
Patient 9	Not done	TP	TP	TP	Done	Benign insulinoma	Tail of pancreas	30 mm
Patient 10	TN	FP	FP	FP	Done	Islet-cell hyperplasia	Body and tail of pancreas	N/A
Patient 11	Not done	TN	Not done	FP	Done	Islet-cell hyperplasia	Head of pancreas	N/A
Patient 12	FN	Not done	Not done	TP	Done	Benign insulinoma	Head of pancreas	14 mm
Patient 13	TP	Not done	Not done	TP	Done	Benign insulinoma	Body of pancreas	15 mm
Patient 14	FN	Not done	TP	TP	Done	Benign insulinoma	Head of pancreas	9 mm
Patient 15	_	_	_	+	Not done	No diagnosis, refused surgery	Head of pancreas	N/A
Patient 16	ТР	TP	Not done	ТР	Done	Multiple insulinomas; MEN1	Tail of pancreas	15 mm (two additional lesion <2 mm were not detected)
Patient 17	FN	Not done	TP	TP	Done	Benign insulinoma	Uncinate process of pancreas	25 mm
Patient 18	-	-	-	-	Not done	No diagnosis	N/A	N/A
Patient 19	TN	TN	FP	FP	Done	Histology negative for tumours	N/A	N/A
Patient 20	+	Not done	Not done	+	Not done	No diagnosis refused surgery	Head of pancreas	N/A
Patient 21	FN	FN	Not done	TP	Done	Benign insulinoma	Body of pancreas	10 mm
Patient 22	FN	ТР	Not done	TP	Done	Benign insulinoma	Head of pancreas	14 mm
Patient 23	TP	Not done	Not done	TP	Done	Benign insulinoma	Body of pancreas	15 mm
Patient 24	TP	TP	Not done	TP	Done	Benign insulinoma	Head of pancreas	19 mm
Patient 25	TP	Not done	Not done	TP	Done	Benign insulinoma	Tail of pancreas	11 mm
Patient 26	FN	Not done	Not done	TP	Done	Benign insulinoma	Body tail transition	9 mm
Patient 27	FN	ТР	Not done	TP	Done	Benign insulinoma	Head of pancreas	9 mm
Patient 28	Not done	-	-	-	Not done	No diagnosis	N/A	N/A
Patient 20	Hordone				Hordone	i to ulugitosis		
Tumour 1	ТР	ТР	Not done	ТР	Done	Multiple insulinomas, MEN1	Head (two lesions) and body of pancreas	17 mm, and 33 mm (one additional lesion of 4 mm was not detected)
Tumour 2	-	-	Not done	-	Done	Multiple gastrinomas, MEN1	Duodenum (two lesions) and one local lymph-node metastasis	6 mm and 9 mm (lymph nod metastases not measured)
Tumour 3	-	-	Not done	-	Done	Glucagon-producing neuroendocrine tumour, MEN1	Tail of pancreas	25 mm
Patient 30	FN	Not done	Not done	TP	Done	Benign insulinoma	Body of pancreas	7 mm

EUS=endoscopic ultrasound. ASVS=selective intra-arterial calcium stimulation and venous sampling. GLP-1R=glucagon-like peptide-1 receptor. TP=true positive. -=negative. +=positive. FN=false negative. FP=false positive. TN=true negative. N/A=not applicable. MEN1=multiple endocrine neoplasia type 1.

Table 3: Comparison of imaging, surgical, and histological results in 30 patients with suspected insulinoma

(table 3). Two patients (patients 15 and 20) had a positive GLP-1R scan but declined surgical intervention. All five patients have been treated medically and followed up.

Seven (23%) of 30 patients (patients 7, 12, 21, 26, and 30 with true-positive results and patients 5 and 11 with false-positive results) showed evidence of an insulinoma only

on ¹¹¹In-DTPA-exendin-4 SPECT/CT. For these seven patients, ¹¹¹In-DTPA-exendin-4 SPECT/CT changed the clinical management by reinforcing the recommendation for surgery. Five of these patients with a proven insulinoma showed a normalisation of blood glucose levels after surgery. In the remaining two patients, only biopsies of the pancreas were done, which on histology showed islet-cell hyperplasia in one patient and no islet pathology in the second patient.

Discussion

To our knowledge, our prospective multicentre imaging study shows for the first time that GLP-1R imaging is a more sensitive technique than conventional imaging for preoperative localisation of small insulinomas (panel). In our study, seven patients were operated on because GLP-1R imaging was the only method that showed a suspicious lesion in the pancreas. Five of these patients had a confirmed insulinoma with normalisation of hyperinsulinism after surgery, supporting the clinical value of GLP-1R imaging. However, conventional imaging was weakly associated with an increased PPV (100%) compared with GLP-1R imaging (86%; p=0.069). This

Panel: Research in context

Systematic review

We searched PubMed for studies published in English before Feb 28, 2013, with the terms "insulinoma" and the conventional non-invasive imaging modalities ("computed tomography" and "magnetic resonance imaging"), the invasive modalities ("selective arterial stimulation and venous sampling" and "endoscopic ultrasonography"), and the available methods that use radioisotopes ("Octreoscan", "18F-DOPA-PET", and "GLP-1 receptor imaging"). After exclusion of case reports and narrative reviews, we identified eight relevant prospective studies and 17 relevant retrospective studies. Only three studies reported use of glucagon-like peptide-1 receptors (GLP-1R) imaging in insulinomas, one study was our own proof-of-principle study of six patients, the second study assessed GLP-1R imaging only in malignant insulinoma, and the third study summarised the experience in 11 patients with a different radiotracer to our study (99mTc-HYNIC-exendin-4). The prospective). The prospective studies included 6–27 patients with endogenous hyperinsulinaemic hypoglycaemia. Sensitivities of CT/MRI for detection of insulinoma ranged from 37% to 59%, selective arterial calcium stimulation and venous sampling (ASVS) from 96% to 100%, and endoscopic ultrasound from 65% to 93%. The sensitivity of GLP-1R imaging was 90% and 100% in the two small studies. The retrospective studies, assessing between six and 237 patients, reported sensitivities of 39-75% with conventional non-invasive imaging, 65–92% for endoscopic ultrasound, and 87–100% for ASVS. The sensitivity of somatostatin receptor 2 imaging using Octreoscan (Covidien, Hazelwood, MO, USA) is usually low (33–50%) and inconsistent for ¹⁸F-DOPA-PET (90% in a prospective study and 20% in a retrospective analysis).

Interpretation

Different imaging modalities show a wide range of sensitivity in the detection of insulinomas. Contributors to this difference (beyond the nature of the imaging technology itself) include local availability and competence and experience of the operators. Present data confirm that conventional non-invasive imaging and Octreoscan are less effective than are invasive methods (endoscopic ultrasound and ASVS) for detection of insulinomas. However, this evidence is mainly based on retrospective analysis as few studies have assessed non-invasive techniques with a prospective design. In our prospective study of 30 patients, GLP-1R imaging was superior to conventional imaging in the detection of insulinoma. Furthermore, our data suggest that the sensitivity of GLP-1R imaging is much the same as the performance of the established invasive methods with their inherent risks and costs. Therefore, GLP-1R imaging could become a key component of the preoperative management of patients with endogenous hyperinsulinaemic hypoglycaemia.

effect was attributable to an increased rate of false positive results with GLP-1R imaging compared with conventional techniques. Invasive investigations such as endoscopic ultrasound and ASVS also showed false positive results.

Despite our inclusion criteria that required patients to have only one or no suspicious lesion on conventional imaging, two patients had malignant insulinoma, as defined by suspicious lymph nodes identified intraoperatively and confirmed by histological assessment. One patient was positive on GLP-1R imaging and the second patient had a false negative result (the only false negative result on GLP-1R imaging in our study). These findings corroborate a recent report that showed a low detection rate of malignant insulinomas with ¹¹¹In-DTPAexendin-4 SPECT/CT.²⁵ Conventional imaging, by contrast, detected malignant insulinomas in all patients in that study and our own study. Overall, these findings suggest that conventional imaging should be done first to exclude malignant disease whereas GLP-1R imaging could be used after negative conventional imaging.

In a proof-of-principle study,18 six patients with endogenous hyperinsulinaemic hypoglycaemia were successfully studied with ¹¹¹In-DOTA-exendin-4. By contrast, our study used DTPA as a chelator, mainly because of the straightforward labelling procedure and high specific activity (90 GBq/µmol for ¹¹¹In-DTPAexendin-4 vs 20 GBq/µmol for ¹¹¹In-DOTA-exendin-4), resulting in a smaller peptide load^{13,18} and decreasing potential side-effects (eg, nausea, hypoglycaemia). We noted no clinically significant differences in the decrease in glucose concentrations between 111In-DTPA-exendin-4 (median decrease 1.3 mmol/L, IQR 0.8-2.1) and ¹¹¹In-DOTA-exendin-4 (1.4 mmol/L, IQR 1.1-1.6).18 In our study, regular monitoring of glucose concentrations after injection led to no serious episodes of hypoglycaemia. Notably, nausea was only reported with the chelator DOTA and not with DTPA. Whether this side-effect is related to the different chelators or to the lower concentration of exendin-4 required when DTPA is used as a chelator has to be proven in future studies. More importantly, different chelators do not seem to affect the sensitivity of GLP-1R imaging.

For four patients, GLP-1R imaging detected false positive lesions. In two of these patients, intraoperative evaluation (palpation, ultrasound, and pancreatic biopsy sampling) did not reveal an insulinoma or islet-cell hyperplasia. The underlying reason for these findings remains unclear. Because one of these lesions was located in the region of the pancreatic head, Brunner's gland of the duodenum (which homogeneously expresses GLP-1R at high density²⁶) might have interfered. GLP-1R imaging of islet-cell hyperplasia yielded conflicting findings with two confirmed positive and one negative result. Recently, in-vitro GLP-1R autoradiography of pancreatic tissue of patients with post-bariatric nesidioblastosis²⁷ showed much the same density of GLP-1R in islet-cell hyperplasia as normal β cells. This finding corresponds to a five-to-six-times lower density of GLP-1R than is noted in insulinomas,²⁷ thereby potentially explaining the negative GLP-1R scan in the present study. Nesidioblastosis in adults (without previous bariatric surgery) might vary between patients with respect to the number of islets, size of islets, and surface receptors; this variation might explain why GLP-1R imaging of islet-cell hyperplasia could result in positive and negative scans.

Five patients did not undergo a surgical procedure in our study and a final diagnosis could not be established by histology: two had positive GLP-1R scans but declined surgery, two patients had no positive findings on imaging, and one patient had a negative result on GLP-1R imaging, but a positive finding with ASVS and ¹⁸F-DOPA PET/CT suggestive of adult nesidioblastosis. Assuming the worst case scenario, all three patients with negative scans might have had a benign insulinoma that was not detectable with the present imaging modalities. Therefore, overestimation of the calculated sensitivity is possible.

Epidemiological data suggest that about 6% of insulinomas are genetically linked to MEN1.6 In keeping with previous data, two (8%) of our 25 patients who underwent histological assessment had a confirmed germline mutation of MEN1.6 In both patients, GLP-1R imaging was positive with one lesion in one patient and two positive lesions in a second patient. Histological assessment of both patients revealed additional insulinstaining microadenomas not detected by GLP-1R or other imaging with diameters of 2-4 mm. By contrast, insulinomas of 7 mm and more were detected by GLP-1R imaging, suggesting that the minimum size for detection with this technique is about 7 mm. Notably, gastrinstaining and glucagon-staining tumours were not detected by GLP-1R imaging, underscoring the specificity of the method.

This study has limitations. First, because of differences in local availability, preoperative choice of investigations could not be standardised. However, consensus does not exist about use of an established MRI protocol or a preference for use of CT/MRI in the detection of insulinoma.28,29 Therefore, comparison between GLP-1R imaging and conventional imaging (CT/MRI) seems reasonable. Second, the specificity of GLP-1R imaging was low (20%), because of a small number of true negative results. This feature, in turn, is related to the high sensitivity of the biochemical assessment done before imaging procedures and underscores the fact that careful biochemical assessment is mandatory to benefit from the high sensitivity of GLP-1R imaging and to avoid false positive results. Third, in our study, conventional imaging had a tendency to underperform compared with the published literature.^{2,3,29} This discrepancy might be explained by the fact that many of the patients in the study were referred following negative conventional imaging. Finally the study was slightly underpowered because the protocol suggested 30 patients with a dropout of 10% (ie, 27 patients) but only 23 were included in the MRT/CT versus GLP-1R imaging analysis. Nevertheless, the difference between the imaging modalities was significant.

Overall, our study suggests that GLP-1R imaging is a more sensitive method for detection of insulinomas than is CT/MRI and changes clinical management in a substantial percentage of patients with endogenous hyperinsulinaemic hypoglycaemia. Our limited experience with insulinoma in the context of MEN1 suggests that GLP-1R imaging can detect lesions in these patients. The detection of islet-cell hyperplasia by GLP-1R imaging is inconsistent.

Contributors

EC, DW, PJE, JCR, and FF participated in the design and concept of the study. EC, MEC, MBr, TC, SF, CS, JS, and BG recruited patients. DW and MBé prepared the radiotracer. DW, SE, GN, and FF did the imaging and collected data. TC and BG did the surgeries and AP did the pathological review. EC, DW, SE, and FF participated in the data analysis and in the interpretation of results. All authors participated in drafting and finalising the report.

Conflicts of interest

MBé declares that he is an inventor and holder of the following patent: Invention affecting GLP-1 and exendin (Philipps-Universität Marburg, June 17, 2009). All other authors declare that they have no conflicts of interest.

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Concomitant expression of several peptide receptors in neuroendocrine tumours: molecular basis for in vivo multireceptor tumour targeting

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Abstract. Peptide receptors have been found to represent excellent targets for in vivo cancer diagnosis and therapy. Recent in vitro studies have shown that many cancers can overexpress not only one but several peptide receptors concomitantly. One of the challenges for nuclear medicine in this field in the coming decade will be to take advantage of the co-expression of peptide receptors for multireceptor tumour targeting. In vitro receptor studies can reveal which peptide receptor is overexpressed in which tumour and which receptors are coexpressed in an individual tumour; such knowledge is a prerequisite for successful in vivo development. One group of tumours of particular interest in this respect is the neuroendocrine tumours, which have previously been shown often to express peptide receptors. This review summarises our investigations of the concomitant expression of 13 different peptide receptors, in more than 100 neuroendocrine tumours of the human intestine, pancreas and lung, using in vitro receptor autoradiography with subtype-selective ligands. The incidence and density of the somatostatin receptors sst_1-sst_5 , the VIP receptors VPAC₁ and VPAC₂, the CCK₁ and CCK₂ receptors, the three bombesin receptor subtypes BB₁ (NMB receptor), BB₂ (GRP receptor) and BB₃, and GLP-1 receptors were evaluated. While the presence of VPAC₁ and sst₂ was detected in the majority of these neuroendocrine tumours, the other receptors, more differentially expressed, revealed a characteristic receptor pattern in several tumour types. Ileal carcinoids expressed sst₂ and VPAC₁ receptors in virtually all cases and had CCK₁, CCK₂, sst₁ or sst₅ in approximately half of the cases; they were the only tumours of this series to express NMB receptors. Insulinomas were characterised by a very high incidence of GLP-1, CCK₂ and VPAC₁ receptors, with the GLP-1 receptors expressed in a particularly high density; they expressed sst₂ in two-thirds and sst_1 in approximately half of the cases and lacked CCK1 and NMB receptors. All gastrinomas had sst2 and GLP-1 receptors; they expressed GRP receptors in three-quarters of the cases and CCK₁ or VPAC₁ in approximately half of the cases. Most bronchial carcinoids had VPAC₁, while sst₁, sst₂ and CCK₂ were found in two-thirds of the cases and BB3 in one-third of the cases. These data provide evidence for the vast biological diversity of these neuroendocrine tumours. Moreover, the results represent a basis for starting and/or optimising the in vivo targeting of these tumours by selecting the suitable radiopeptides for tumour diagnosis and/or therapy. Finally, the data strongly encourage concomitant application of several radiopeptides to permit more efficient targeting of these tumours.

Keywords: Peptide receptors – Neuroendocrine tumours – Tumour targeting – Somatostatin

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Introduction

The presence of somatostatin receptors in neuroendocrine tumours of the intestine, pancreas and lung has led to development of the field of somatostatin receptor targeting in oncology, at both the diagnostic [1] and the therapeutic level [2]. The success of this novel approach has also triggered interest in studying the in vitro expression of other peptide receptors, e.g. vasoactive intestinal peptide (VIP) receptors, cholecystokinin (CCK) receptors and bombesin receptors [3, 4, 5], and in evaluating their potential for peptide receptor targeting in vivo [6, 7, 8]. Specifically, neuroendocrine tumours can express

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various peptide receptors [3, 9, 10], apart from somatostatin receptors [11].

Up to now, however, the peptide receptor most frequently targeted in vivo has been the somatostatin receptor. Various somatostatin radioligands have been used for this purpose, with different levels of success. Octreoscan has been considered the gold standard for detection of somatostatin receptors in many neuroendocrine tumours [12, 13]; other radiotracers, such as ¹¹¹In-DOTA-lanreotide or ^{99m}Tc-P829, are being used less frequently, due in part to a lower sensitivity and higher background [14]. However, even Octreoscan, which binds primarily to sst₂ receptors, does not allow the detection of every neuroendocrine tumour: while virtually all gastrinomas and their metastases can be precisely visualised [12], a much lower percentage of insulinomas can be identified with this method [13]. It has been argued that this may be due to the lower frequency of sst₂ receptor expression in insulinomas [12]. These data indicate that the success of in vivo somatostatin receptor targeting is very much dependent on the presence in the tumour of the appropriate receptor subtype in a sufficient amount and on the particular receptor affinity profile of the used radioligand.

Only a very small number of studies have tried to visualise neuroendocrine tumours through peptide receptors other than somatostatin receptors. It has been shown that VIP receptor scintigraphy is able to detect gut neuroendocrine tumours [6]. However, the high background over many VIP receptor-positive tissues, such as lung, and the very unstable radioligand are likely to prevent successful development of this technique. In addition, in vivo CCK and bombesin receptor scintigraphy, although not yet evaluated in gut neuroendocrine tumours, have successfully been used to target medullary thyroid cancers [7] and prostate and breast cancers [8], respectively.

For each of these peptide receptors, the proof of principle has been established that their respective radioligands can be used, separately, to successfully target tumours. As a further step, it is tempting to speculate that the tracers may also be used as a cocktail to target several co-expressed peptide receptors in a single tumour, in order to obtain a much more efficient and powerful means of diagnosis and therapy. A prerequisite for such successful in vivo development is knowledge of which receptor is expressed in which tumour and which receptors are co-expressed in an individual tumour. Recently, taking breast cancers as example, in vitro studies have reported the concomitant expression of several of these peptide receptors [15], in particular gastrin-releasing peptide (GRP) receptors and neuropeptide Y (NPY) Y_1 receptors, in individual tumours. As neuroendocrine tumours are known to express various peptide receptors, it may be of particular interest to know the extent of peptide receptor co-expression in these types of tumour.

The present review summarises the data obtained in a large number of neuroendocrine tumours of the intestine, pancreas and lung in which we evaluated the concomitant expression of various peptide receptors that are of established or potential interest in nuclear medicine and oncology, namely somatostatin, VIP, CCK, bombesin and glucagon-like peptide (GLP) receptors. Because most of these peptide receptors exist as multiple subtypes [16, 17, 18, 19], it is crucial to evaluate as many of the subtypes as possible; for this study, these are the five somatostatin receptor subtypes sst_1-sst_5 [11], the three bombesin receptors, namely BB₁ [or neuromedin B (NMB) receptors], BB₂ (or GRP receptors) and BB₃ receptors [10], the CCK₁ and CCK₂ receptors [3], the VIP receptor subtypes VPAC1 and VPAC2 [9] and, finally, the GLP-1 receptors [20]. The choice of a series of more than 100 gastroenteropancreatic and lung neuroendocrine tumours, including bronchial carcinoids, ileal carcinoids and functioning neuroendocrine pancreatic tumours consisting of insulinomas, gastrinomas, glucagonomas and vipomas, was made on the basis that these tumours have previously been shown often to express, individually, various somatostatin receptor subtypes [11], as well as VIP receptors [9] or CCK receptors [3]. Moreover, the bombesin receptor subtypes have recently been found to be expressed differently in these types of tumour, with GRP receptors preferentially found in gastrinomas, NMB receptors in gut carcinoids and BB3 in lung carcinoids [10]. Furthermore, GLP-1 receptors, although never investigated in human cancers, have previously been shown to be expressed in rat insulinomas. In vitro information on concomitant receptor expression in these tumours not only should allow the nuclear physician to choose the appropriate radiopeptides for optimal targeting of the respective tumours, but also may give a better insight into the pathobiological behaviour of these different neuroendocrine tumours.

Methodological aspects

Which in vitro methodology and which parameters are best able to yield the required receptor information? It is likely that a method detecting proteins is more relevant than one detecting mRNA. A method that can quantify the number of receptors is also of prime importance. Further, the method should be sensitive enough to detect small amounts of receptors. Finally, the method should preferably identify the receptor binding sites. Among the available techniques, the first choice is likely to be in vitro receptor autoradiography, a highly sensitive method that has the advantage of identifying and quantifying peptide receptor proteins rather than the mRNA [21]. Moreover, it recognises the binding sites of the receptor protein that correspond precisely to the molecular targets reached by the radioligands, as used in vivo by nuclear physicians both for diagnosis and for therapy of tumours. It is also possible and advantageous to use subtypeselective receptor autoradiography to identify the various peptide receptor subtypes [3, 9, 10, 11].

In this study, frozen neuroendocrine tumours of the intestine, pancreas and lung, including 27 ileal carcinoids (most of them metastatic to lymph nodes and/or liver), 29 bronchial carcinoids, 27 insulinomas, 10 gastrinomas, 4 glucagonomas and 4 vipomas, were cut into 20um-thick successive cryostat sections and prepared to be used for in vitro receptor autoradiography of the various peptide receptors, as described below. Subtype-selective somatostatin receptor autoradiography was performed as described recently [11] using ¹²⁵I-[Leu⁸, D-Trp²², Tyr²⁵]somatostatin-28 (125I-LTT-SS-28; 2,000 Ci/mmol; Anawa, Wangen, Switzerland) as radioligand and the following sst-selective analogues: the sst₁-selective CH288, the sst₂-selective L-779-976, the sst₃-selective sst₃-ODN-8, the sst_4 -selective L-803,087 and the sst_5 -selective L-817,818 [11]. Also subtype-selective VIP receptor autoradiography was performed as described previously [9] using ¹²⁵I-VIP (2,000 Ci/mmol; Anawa, Wangen, Switzerland) as radioligand with the VPAC₁-selective [K¹⁵, R¹⁶, L²⁷]VIP(1-7)/GRF(8-27) and the VPAC₂selective Ro25-1553. Subtype-selective CCK receptor autoradiography was performed as described previously [3] using ¹²⁵I-[D-Tyr-Gly, Nle^{28,31}]-CCK26-33 (¹²⁵I-CCK; 2,000 Ci/mmol; Anawa, Wangen, Switzerland) as radioligand, displaced with CCK-8 and/or gastrin to discriminate between CCK1 and CCK2 receptors. Subtypeselective bombesin receptor autoradiography was performed using ¹²⁵I-[D-Tyr⁶, β-Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) (2,000 Ci/mmol; Anawa, Wangen, Switzerland) as radioligand [10] and unlabelled GRP, NMB and [D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6–14) to discriminate between GRP, NMB and BB₃ receptors. GLP-1 receptor autoradiography is briefly summarised below, as it has not been published previously. Twenty-micrometre-thick sections were incubated for 2 h at ambient temperature in the presence of 32 pM 125 I-GLP-1 (2,000 Ci/mmol; Anawa, Wangen, Switzerland). The incubation solution was 170 mM Tris-HCI buffer (pH 8.2) containing 1% bovine serum albumin, bacitracin (40 μ g/ml) and MgCl₂ (10 mM) to inhibit endogenous proteases. Non-specific binding was determined by adding 100 nM solution of unlabelled GLP-1. Incubated sections were washed twice for 5 min in cold incubation buffer containing 0.25% bovine serum albumin, then in buffer alone, and dried quickly. Finally, the sections were apposed to Biomax MR films (Kodak) and exposed for 1 week in X-ray cassettes. In selected cases, displacement experiments were performed in successive tissue sections using increasing concentrations of GLP-1, GLP-2, exendin 4 and glucagon 1-29 (Bachem, Bubendorf, Switzerland), in order to identify the GLP-1 receptor subtype.

In all experiments, the autoradiograms were quantified using a computer-assisted image processing system, as described previously [9, 22]. Tissue standards for iodinated compounds (Amersham, Aylesbury, UK) were used for this purpose. A tissue was defined as receptorpositive when the absorbance measured in the total binding section was at least twice that of the non-specific binding section. When multiple peptide receptor subtypes of a single family were detected in a tumour, only those present in a density equal to or higher than 10% of the density of the most abundantly expressed receptor subtype in that tumour were considered positive. Moreover, in tumours expressing sst₁ and sst₅ simultaneously, it was necessary to take into account the cross-reactivity of the sst₅-selective L-817,818 with sst₁ and to correct the sst₅ value measured at 10 nM L-817,818 by subtracting 15% of the sst₁ density value measured in that tumour [11]. Finally, it should be remembered that it cannot be completely excluded, by using subtype-selective receptor autoradiography with universal radioligands, that a receptor subtype expressed in very low amounts may be masked by another subtype expressed in very high density in the same tumour.

Incidence and density of peptide receptors in neuroendocrine tumours

Tables 1, 2 and 3 report the incidence and density of the 13 peptide receptors investigated in each individual tumour tested in this study, i.e. in ileal carcinoids (Table 1), functioning pancreatic neuroendocrine tumours (Table 2) and bronchial carcinoids (Table 3). We did not find a single neuroendocrine tumour that did not express at least one of these peptide receptors. In most cases, several peptide receptors were concomitantly expressed. While the great majority of the tested tumours expressed $VPAC_1$ and sst₂, the more selective expression pattern of the other peptide receptors may allow pathobiochemical distinction between various tumour types. For a better overview, Fig. 1 shows the incidence and mean receptor density for the four main groups of tumours tested, namely ileal carcinoids, insulinomas, gastrinomas and bronchial carcinoids.

Virtually all ileal carcinoids expressed VPAC₁, while VPAC₂ was absent (Table 1, Fig. 1). They all expressed sst₂, but in half of the cases sst_1 and/or sst_5 was also present. They rarely expressed sst₃ and sst₄. The highest receptor densities were found for sst₂, followed by sst₁. Characteristic for ileal carcinoids was the expression of NMB receptors, as seen in 11/27 of the cases. Such receptors were virtually not expressed by any of the other tested neuroendocrine tumour types (Table 1, Fig. 1). The ileal carcinoids also expressed GLP-1 receptors in one-third of the cases and CCK₁ and CCK₂ in half and two-thirds of the cases, respectively, with the density of CCK₁ receptors being several times higher than that of the CCK₂ receptors (Table 1, Fig. 1). Figure 2 shows a typical example of the heterogeneous CCK receptor expression in an ileal carcinoid. Very high expression of CCK_1 was seen in one area of the tumour, while another area had CCK₂ receptors in low density. Histopathologi-

Case	VIP-R	VIP-R		Somatostatin-R						Bombesin-R			GLP-1-R
	VPAC ₁	VPAC ₂	sst ₁	sst ₂	sst ₃	sst ₄	sst ₅	NMB	GRP	BB ₃	CCK ₁	CCK ₂	
1 CL	7,353	_	2,487	9,477	1,752	_	2,437	_	_	_	_	847	831
2 MK	6,322	_	3,619	5,014	_	_	1,909	751	_	_	_	1,099	_
3 MT	6,517	_	4,698	_	1,666	_	7,476	1,993	_	_	_	_	_
4 JM	602	_	652	822	_	_	_	_	_	_	_	_	_
5 JS	3,907	_	5,056	8,963	_	_	2,895	_	_	_	_	436	_
6 DV	1,145	_	4,511	8,121	1,954	_	2,231	_	_	_	_	_	_
7 ST	6,014	_	8,413	591	_	_	2,782	_	_	_	_	295	1,260
8 OS	4,528	_	4,641	3,200	_	_	_	1,034	_	_	_	_	1,058
9 PB	2,151	_	2,027	1,469	_	_	1,526	_	_	_	_	_	_
10 KF	1,682	_	2,145	7,586	_	_	_	_	_	_	1,159 ^b	1,419 ^b	_
11 BZ	4,089	_	_	1,559	_	_	_	632	_	_	_	_	_
12 PM	2,699	_	_	5,962	_	_	896	968	_	_	1,054 ^b	194 ^b	_
13 MS	650	_	_	6,506	_	_	_	_	_	_	5,917 ^b	_	_
14 HM	1,102	_	_	10,759	_	_	_	_	_	_	2,430 ^b	686 ^b	_
15 WS	1,740	_	_	6,106	_	_	_	_	_	_	_	_	_
16 BS	11,081	_	_	5,129	_	1,459	1,803	1,456	_	_	_	918	_
17 EM	4,828	_	1,219	7,633	_	_	_	_	_	_	_	945	303
18 HZ	1,127	_	_	4,750	_	_	_	1,038	_	_	4,325 ^b	_	384
19 GV	1,688	_	_	3,563	_	_	1,307	1,746	_	_	_	1,584	_
20 GN	174	_	_	10,710	_	_	_	_	_	_	4,096 ^b	1,026 ^b	2,955ª
21 JZ	7,380	_	5,485	3,499	_	_	4,229	341	_	_	1,403 ^b	1,385 ^b	1,029
22 LW	547	_	_	16,915	_	_	_	2,860	_	_	_	458	_
23 SV	2,341	_	_	1,318	_	_	365	_	_	_	1,831 ^b	1,555 ^b	_
24 HM	_	_	_	2,570	_	_	_	_	_	_	5,576 ^b	_	_
25 SK	7,491	_	940	1,459	_	_	1,805	4,583	_	_	_	_	_
26 AN	1,718	_	_	6,393	_	_	_	_	_	_	3,338 ^b	895 ^b	395
27 FB	2,484	-	1,015	1,173	1,051	-	-	-	_	_	3,025 ^b	834 ^b	-
Incidence	26/27	0/27	14/27	26/27	4/27	1/27	13/27	11/27	0/27	0/27	11/27	16/27	8/27
Mean density	3,516		3,351	5,433	1,606	1,459	2,435	1,582			3,105	911	1,027

Numbers represent receptor density measured as dpm/mg tissue; – signifies absence of receptors; NA, not assessed ; R, receptors ^a GLP-1 receptors were heterogeneously distributed in the tumour samples

 b CCK₁ and/or CCK₂ receptors were heterogeneously distributed in the tumour samples

Table 2. Peptide receptor expression in functioning pancreatic neuroendocrine tumours

Case	VIP-R	VIP-R		Somatostatin-R						Bombesin-R			GLP-1-R
	VPAC ₁	VPAC ₂	sst ₁	sst ₂	sst ₃	sst ₄	sst ₅	NMB	GRP	BB ₃	CCK ₁	CCK ₂	
Insulinoma	s												
1 A9	1,706	NA	11,673	_	2,763	_	8,125	_	_	2,746	_	396	10,146
2 BS	293	_	1,975	2,582	_	_	_	_	956	_	_	2,705	7,779
3 1 2	2,682	_	1,757	_	408	_	524	_	_	_	_	462	9,665
4 12	635	_	_	5,775	_	_	_	_	_	_	_	1,467	6,005
5 AS	650	_	_	6,541	1,095	_	_	_	_	_	_	_	9,555
6 WM	1,192	_	_	4,579	_	_	_	_	_	_	_	586	3,604
7 A3	253	_	2,767	755	1,547	_	_	_	727	_	_	1,051	7,710
8 34	387	_	_	6,521	_	_	_	_	_	_	_	6,696	5,807
9 34	1,576	_	_	4,398	_	_	_	_	_	_	_	5,428	8,490
10 AF	385	_	727	4,534	_	_	_	_	_	_	_	1,154	5,384
11 32	_	_	_	1,261	_	_	_	_	_	_	_	1,490	7,440
12 HF	1,859	_	NA	NA	NA	NA	NA	_	_	_	_	4,108	10,236
13 GY	3,761	_	-	1,057	405	_	—	-	-	-	-	6,432	7,129

Case	VIP-R		Somato	ostatin-R				Bombe	sin-R		CCK-R		GLP-1-R
	VPAC ₁	VPAC ₂	sst ₁	sst ₂	sst ₃	sst ₄	sst ₅	NMB	GRP	BB ₃	CCK ₁	CCK ₂	
14 SC	662	_	419	_	191	_	_	_	_	_	_	3,446	9,580
15 BD	1,783	_	_	6,268	_	_	NA	_	_	_	_	2,389	6,142
16 P4	7,764	_	1,379	2,359	_	_	_	_	_	_	_	1,145	9,854
17 47	156	_	_	374	303	_	391	_	_	_	_	325	8,424
18 48	1,619	_	_	_	_	_	_	_	_	_	_	1,227	9,567
19 48	242	_	1,300	8,167	_	1,038	_	_	_	_	_	1,742	6,575
20 49	261	_	994	_	_	_	_	_	_	_	_	6,800	6,804
21 P5	1,145	_	5,395	_	_	_	_	_	_	_	_	836	10,205
22 P5	4.395	_	3.292	1.661	3.131	_	1.483	_	_	_	_	543	9,944
23 PL	366	_	2.262	435	_	_	_	_	320	_	_	575	8.658
24 GI	3.748	_	1.821	_	_	_	_	_	_	_	_	377	10.184
25 AN	4.365	_	638	_	_	_	_	_	_	_	_	3.508	8.430
26 EM	1.615	_	1.284	6.477	988	_	2.080	_	_	_	NA	NA	_
27 33	2.772	_	1.858	4.778	_	_	_,000	_	_	4.680	_	287	_
I 1	2,107	0/26	1(10)	19/06	0/06	1/07	5105	0/07	2/07	2/07	0/06	25/06	25/27
Mean density	1,780	0/26	2,471	18/26 3,807	9/26 1,203	1,038	5725 2,521	0/27	3727 668	3,713	0/26	2,207	8,133
Gastrinoma	lS												
1 39	_	_	_	11,305	_	_	_	_	4,116	_	1,032	_	1,718 ^a
2 40	2,125	_	_	1,881	1,453	_	NA	_	2,909	_	_	_	4,426 ^a
3 43	_	_	2,345	12,679	_	_	_	_	5,401	3,106	2,037	_	1,532
4 MT	3,111	_	_	6,204	_	_	_	_	_	_	204	_	412
5 P4	4,619	_	_	7,431	_	_	_	_	_	_	_	_	428
6 WT	_	_	_	9,118	_	_	2,512	_	2,644	_	2,266	_	641
7 DM	2,050	_	_	11,543	_	_	_	_	6,627	_	_	_	4,712
8 P3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	_	_	4,504
9 HI	3,551	_	_	5,106	_	_	_	_	5,301	_	_	_	487
10 JI	_	_	_	7,853	_	_	5,017	_	2,093	_	152	_	5,745ª
11 MT	2.883	_	_	8.811	1.429	_	2.071	_	_	3.053	_	_	NA
Incidence	6/10	0/10	1/10	10/10	2/10	0/10	3/9	0/10	7/10	2/10	5/11	0/11	10/10
density	3,057		2,345	8,195	1,441		3,200		4,130	3,080	1,138		2,401
Glucagonor	nas												
1 GT	2,550	-	4,669	8,043	_	-	-	_	_	1,313	-	-	-
2 LH	1,290	-	6,152	3,664	1,712	-	-	_	_	3,312	-	162	899 ^a
3 JS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	941
4 KP	2,333	_	_	_	_	_	_	_	_	1,166	_	171	_
Incidence	3/3	0/3	2/3	2/3	1/3	0/3	0/3	0/3	0/3	3/3	0/3	2/3	2/4
Mean	2.058	0,0	5.411	5.854	1.712	0,2	0,0	0/2	0,0	1.930	0,0	167	920
density	2,000		0,111	0,001	1,712					1,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,		10,	20
Vipomas													
1 KT	825	_	9,324	6,380	3,436	_	2,499	-	618	_	-	403	-
2 PM	393	_	_	20,166	_	_	_	-	-	3,045	1,050	2,527	-
3 MT	636	-	-	18,013	_	-	-	-	_	3,924	-	6,603	-
4 WM	4,299	_	_	20,207	_	_	-	-	311	_	-	603	3,028
Incidence	4/4	0/4	1/4	4/4	1/4	0/4	1/4	0/4	2/4	2/4	1/4	4/4	1/4
Mean density	1,538		9,324	16,192	3,436		2,499		465	3,485	1,050	2,534	3,028

Numbers represent receptor density measured as dpm/mg tissue; – signifies absence of receptors; NA, not assessed; R, receptors ^a GLP-1 receptors were heterogeneously distributed in the tumour samples

Table 2. (continued)

Case	VIP-R		Somato	statin-R				Bombe	esin-R		CCK-R		GLP-1-R
	VPAC ₁	VPAC ₂	sst ₁	sst ₂	sst ₃	sst ₄	sst ₅	NMB	GRP	BB ₃	CCK ₁	CCK ₂	
1 WT	7.979	_	_	8.395	_	_	_	_	_	_	_	1.787	2,333ª
2 DF	3.311	_	_	_	_	_	177	_	_	_	_	933	_
3 TW	841	_	11,036	2,497	_	_	_	_	2,281	2,064	_	817	7,179 ^a
4 BX	2,361	_	_	8,849	_	_	NA	_	_	_	_	_	4,339ª
5 HL	_	_	1,699	2,590	_	_	_	_	_	1,191	_	380	_
6 SH	58	_	1,771	2,475	_	_	_	508	_	_	_	8,779	_
7 DT	2,764	_	511	_	_	_	_	_	_	288	_	3,418	_
8 HL	836	_	897	7,582	_	_	_	_	_	2,202	_	720 ^b	_
9 KN	4,997	_	4,780	3,468	_	_	1077	_	_	3,368	_	1,238	_
10 BR	_	_	2,998	4,738	_	_	_	_	_	_	_	2,904	_
11 VM	6,021	NA	_	6,914	_	_	_	_	_	_	_	2,775	_
12 SH	4,555	_	327	_	_	_	_	_	_	_	_	_	2,725 ^a
13 WP	3,226	_	221	_	_	_	282	_	_	_	_	_	_
14 KH	5,945	_	NA	NA	NA	NA	NA	_	_	_	1,408	_	_
15 TK	1,880	_	465	_	_	_	_	_	_	_	_	_	_
16 BT	1,600	_	_	7,630	_	_	_	_	_	2,394	424	_	_
17 RS	3,618	_	1,472	4,359	_	_	_	_	_	_	_	2,040	253
18 WS	6,524	_	3,756	_	_	_	_	_	_	_	1,382	_	_
19 CM	3,545	_	4,002	891	_	_	_	_	_	6,337	_	561	3,699ª
20 JT	4,382	_	_	3,837	_	_	_	_	_	_	_	181 ^b	_
21 KM	NA	NA	_	_	_	_	_	_	_	_	_	2,127	456
22 SM	5,745	_	4,002	5,904	1,743	_	2,939	_	_	4,732	_	_	_
23 MN	2,393	_	5,184	_	_	_	_	_	_	4,650	_	616	3,089
24 SD	4,752	_	1,264	1,366	_	_	_	_	_	_	_	43	589 ^a
25 01	6,773	_	6,701	_	_	_	1,740	_	_	1,476	_	324	_
26 KG	2,549	_	8,185	3,128	_	_	_	_	_	_	_	_	1,901ª
27 LR	4,943	_	2,934	709	_	_	_	_	_	_	929	_	_
28 KC	6,161	_	5,630	2,844	_	_	1,396	_	_	_	_	_	118
29 SD	986	_	_	7,412	_	_	NA	-	_	_	_	1,451	_
Incidence	26/28	0/27	20/28	19/28	1/28	0/28	6/26	1/29	1/29	10/29	4/29	18/29	11/29
Mean density	3,796		3,392	4,505	1,743		1,269	509	2,281	2,870	1,036	1,727	2,456

Numbers represent receptor density measured as dpm/mg tissue; – signifies absence of receptors; NA, not assessed; R, receptors

 b CCK₁ and/or CCK₂ receptors were heterogeneously distributed in the tumour samples

^a GLP-1 receptors were heterogeneously distributed in the tumour samples

cal evaluation revealed that the CCK_1 receptor-expressing tumour area consisted of a more differentiated, cribriform, tubulo-acinar carcinoma, compared with the more solid and less differentiated CCK_2 -expressing part. Similar histopathological observations were made in several other ileal carcinoids with a heterogeneous CCK receptor distribution. Furthermore, the whole tumour sample in Fig. 2 also expressed a high density of sst₂ and a moderate density of VPAC₁ receptors.

Insulinomas were characterised by the expression of VPAC₁, CCK₂ and GLP-1 receptors in almost all cases, whereas they were devoid of CCK₁ and VPAC₂ (Table 2, Fig. 1). Of 26 insulinomas, 18 expressed sst₂, an incidence which is considerably lower than that found in ileal carcinoids (26/27). However, another somatostatin receptor subtype, sst₁, was found in more than half of the insulinoma cases, often in high density. Interestingly, sst_1 was expressed in all but one of the sst_2 -lacking insulinomas, often in high amounts. An extremely high receptor density was found for GLP-1 receptors, followed, in a subgroup of patients only, by sst_2 and CCK_2 receptors. Conversely, bombesin receptors were extremely rarely expressed in insulinomas (Table 2, Fig. 1). Figure 3 is a typical example of an insulinoma expressing multiple peptide receptors. Figure 4 shows a typical displacement curve characterising GLP-1 receptors in an insulinoma with high-affinity displacement of the radioligand by GLP-1 or exendin 4 but not by GLP-2 or glucagon 1–29.

Gastrinomas were characterised by a high expression of sst_2 receptors in all cases, whereas the other somatostatin receptors were rarely detected (Table 2, Fig. 1).

Fig. 1. Histograms summarising the incidence and the mean density of each of the 13 peptide receptors tested in ileal carcinoids, insulinomas, gastrinomas and bronchial carcinoids. The mean density (dpm/mg tissue) is visualised as relative darkness ranging from 0 to 5,000 dpm/mg tissue. Those cases with density values above 5,000 dpm/mg are represented by dark bars in which the numbers of the mean density values have been inserted (see sst₂ and GLP-1 receptors). n, Number of tumours tested



GLP-1 receptors were also expressed in all cases. VPAC₁ receptors were detected in two-thirds of the cases while VPAC₂ receptors were absent. Perhaps most characteristic for gastrinomas was the frequent expression of GRP receptors, as compared with the rare expression of the other bombesin receptor subtypes, and that of CCK₁ receptors, while CCK₂ are undetectable (Table 2, Fig. 1). Figure 5 shows a gastrinoma expressing sst₂, CCK₁ and GRP receptors.

Since the number of tested vipomas and glucagonomas was limited, only a trend towards a pattern can be proposed, with vipomas expressing VPAC₁, sst₂, CCK₂ and at least one of the bombesin receptor subtypes in all cases, whereas glucagonomas contained VPAC₁ and BB₃ in all cases but also a very high density of sst₁ and sst₂ in two of three cases (Table 2).

Bronchial carcinomas also expressed several peptide receptors in high amounts. Most of them had VPAC₁ and



 $CCK_1 + CCK_2$

Fig. 2A-H. Receptor autoradiography of an ileal carcinoid expressing CCK_1 and CCK_2 receptors (A–D) simultaneously with sst_2 (**E**, **F**) and VPAC₁ (**G**, **H**). A Haematoxylin-eosin stained section showing the tumour. Bar = 1 mm. B Autoradiogram showing total binding of ¹²⁵I-CCK in the tumour tissue. The right part is more intensively labelled than the left one. C Autoradiogram showing ¹²⁵I-CCK binding in the presence of 50 nM cold CCK-8. All the labelling is displaced. D Autoradiogram showing ¹²⁵I-CCK binding in the presence of 50 nM of gastrin. Gastrin displaces the radioligand in the left part of the tumour, but not in the right part, indicating that the left part expresses CCK₂ while the right part has CCK₁. E, F Autoradiograms showing total binding of ¹²⁵I-LTT-SS-28 (E) displaced by 100 nM of the sst₂-selective L-779,976 (F), indicating a very strong expression of sst₂. In F, the left side of the tumour shows a significant residual non-specific binding. G, H Autoradiograms showing total binding of ¹²⁵I-VIP (G) displaced by 20 nM of the VPAC₁-selective [K¹⁵, R^{16} , L²⁷]VIP(1–7)/GRF(8–27) (KRL; H), indicating moderate expression of VPAC₁

somatostatin receptors of either the sst_1 or the sst_2 type while VPAC₂, sst_3 and sst_4 were virtually not detected. More than one-third of the cases had GLP-1 receptors, which were, however, often heterogeneously distributed. Most characteristic for bronchial carcinoids was the preferential expression of the bombesin receptor subtype BB₃ and of CCK₂ receptors (Table 3, Fig. 1). Figure 6 shows the multiple receptor expression seen in one bronchial carcinoid with a high density of sst_1 , BB₃ and CCK₂ receptors, and in another with BB₃, GLP-1 and VPAC₁ receptor expression.



In vitro receptor profile as a predictor for in vivo tumour targeting

The above-mentioned in vitro data clearly demonstrate that neuroendocrine tumours of the small intestine, pancreas and lung can concomitantly express multiple peptide receptors, often in high density, and that the various types of tumour appear to have rather characteristic receptor profiles often distinct from each other. This knowledge may be used by nuclear physicians to select a radioligand, or a mixture of radioligands, suitable for each individual case, in order to achieve efficient and optimal in vivo tumour targeting.

Somatostatin receptors

The high incidence and high density of the sst₂ protein reported for the various tumours in Tables 1, 2 and 3 and Fig. 1 can be seen as one of the main keys to the success of Octreoscan in diagnosing the majority of neuroendocrine tumours of the small intestine, pancreas and lung, since Octreoscan has a preferential affinity for sst₂. The particularly high incidence and density of sst₂ in gastrinomas may be the explanation for the extremely good results found with in vivo Octreoscan imaging of these tumours [12]. The same may be true for ileal carcinoids. Conversely, the lower incidence and density of sst₂ in insulinomas may explain the lower rate of detection with Octreoscan in vivo. One can also foresee that precisely those tumours in Fig. 1 with the highest sst₂ density will be particularly amenable to successful radiotherapy with ¹¹¹Y-labelled DOTATOC or ¹⁷⁷Lu-labelled DOTATATE [2, 23]. In



Fig. 3A–I. Insulinoma expressing concomitantly CCK₂ receptors (**B**, **C**), GLP-1 receptors (**D**, **E**), sst₂ receptors (**F**, **G**) and VPAC₁ receptors (**H**, **I**). **A** Haematoxylin-eosin stained section showing the tumour tissue. *Bar* =1 mm. **B**, **C** Autoradiograms showing total binding of ¹²⁵I-CCK (**B**) completely displaced by 50 n*M* of gastrin (**C**), thus indicating the presence of CCK₂ receptors. **D**, **E** Autoradiograms showing total binding of ¹²⁵I-CCK (**B**) completely displaced by 100 n*M* of GLP-1 (**E**). **F**, **G** Autoradiograms showing total binding of ¹²⁵I-LTT-SS-28 (**F**) displaced by 100 n*M* of the sst₂-selective L-779,976 (**G**); this indicates the presence of sst₂ receptors. **H**, **I** Autoradiograms showing total binding of ¹²⁵I-VIP (**H**) displaced by 20 n*M* of the VPAC₁-selective [K¹⁵, R¹⁶, L²⁷]VIP(1–7)/GRF(8–27) (**I**), indicating the presence of VPAC₁ receptors. Note the very high density of CCK₂, GLP-1 and sst₂ receptors, compared with VPAC₁ receptors

many of these tumours, sst_2 may even be targeted concomitantly with other peptide receptors (see below).

Whereas the present study confirmed the predominance of sst₂ protein expression in neuroendocrine tumours [11, 24, 25, 26], it also revealed that sst₁ is the second most abundant somatostatin receptor subtype after sst₂ in many gut and lung neuroendocrine tumours, and in particular in bronchial carcinoids. In insulinomas, it was even more abundant than sst₂ and was most often expressed in tumours lacking sst₂. Commercially available somatostatin analogues for scintigraphy, including Octreoscan, are unable to bind to sst₁ receptors [19]. However, either sst₁-selective compounds, such as CH-288 [27], or pan-somatostatins, such as KE108 [28], that would be coupled to chelators, may be developed for this indication. Compared with other sst₁-expressing tumours such as prostate cancers or sarcomas [29, 30], the neuroendocrine tumours of the present study often had a much higher density of sst_1 receptors; it is probable, therefore, that the sst_1 targeting in vivo of these particular tumours may be successful.

VIP receptors

The great majority of the tested neuroendocrine tumours expressed $VPAC_1$. In theory, it can be predicted that most neuroendocrine tumours should be targeted with radiolabelled VIP analogues. This has at least been shown previously for a group of intestinal neuroendocrine tumours [6]. However, high expression of VIP receptors is found in a large number of normal tissues and organs [9], and it is unlikely that VIP receptor scintigraphy will be of great help in detecting distant metastases of neuroendocrine tumours, i.e. lymph node or liver metastases, owing to high background activity. Moreover, neuroendocrine lung tumours would be difficult to visualise, as their receptors would be masked by the high VIP binding to the lungs [9]. Also, combination of VIP radioligands with other peptide ligands, with the aim of achieving increased sensitivity for tumour detection, may not be an advantage owing to the high VIP background in healthy tissues.

Bombesin receptors

This study confirms and extends the results of an earlier investigation showing that bombesin receptor subtypes 790

GLP-1 receptors in insulinoma



Fig. 4. GLP-1 receptors in an insulinoma. Competition experiment using successive tissue sections incubated with ¹²⁵I-GLP-1 and increasing concentrations of unlabelled GLP-1 (*circles*), GLP-2 (*squares*), glucagon 1–29 (*triangles*) or exendin 4 (*diamonds*). The high affinity for exendin 4 or GLP-1 and the low affinity for GLP-2 or glucagon 1–29 clearly indicates the presence of GLP-1 in this insulinoma

are differentially overexpressed in neuroendocrine tumours. BB₃ is frequently found in bronchial carcinomas, glucagonomas and vipomas, but is absent in ileal carcinoids and insulinomas. Conversely, NMB receptors are expressed in ileal carcinoids but are absent in other neuroendocrine tumours, whereas the high incidence and density of GRP receptors found in gastrinomas and some vipomas should be particularly stressed. These results point towards different biological characteristics of these tumours. They also indicate that it will be of great utility to know the bombesin receptor subtype affinity profile of newly developed bombesin radioligands foreseen for in vivo tumour targeting [31]. Up to now, only radioligands with strong GRP receptor affinity have been developed for in vivo targeting [8, 31, 32].

CCK receptors

The results of this study with respect to CCK receptors suggest that selected tumour types may become potential targets for CCK_2 receptor labelling in vivo. Insulinomas and vipomas appear to be highly promising CCK_2 targets



Fig. 5A–K. Gastrinoma (**A**) expressing concomitantly CCK₁ receptors (**B–D**), GRP receptors (**E–G**) and sst₂ receptors (**H–K**). A Haematoxylin-eosin stained section. *Bar* =1 mm. **B–D** Autoradiograms showing total binding of ¹²⁵I-CCK (**B**) displaced by 50 n*M* of CCK-8 (**C**) but not by 50 n*M* of gastrin (**D**), indicating the presence of CCK₁ receptors. **E–G** Autoradiograms showing total bind-

ing of ¹²⁵I-[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin 6–14 (**E**) displaced by 50 n*M* of GRP (**F**) but not by 50 n*M* NMB (**G**), indicating the presence of GRP receptors. **H–K** Autoradiograms showing total binding of ¹²⁵I-LTT-SS-28 (**H**) displaced by 100 n*M* of the sst₂-selective L-779,976 (**I**) but not by the sst₁-selective CH-288 (**K**), indicating the predominance of sst₂ receptors

Bronchial carcinoids



sst1 sel.+NMB+ GastrinSST1BB3CCK2Fig. 6. Peptide receptor pattern in two bronchial carcinoids. Upper figure: Bronchial carcinoid expressing BB3 (A-D), GLP-1 (E,
F) and VPAC1 receptors (G, H). A-D Autoradiograms showing total binding of ¹²⁵I-[D-Tyr⁶, β-Ala¹¹, Phe¹³, Nle¹⁴]-bombesin 6–14(A) displaced completely by 50 nM of [D-Tyr⁶, β-Ala¹¹, Phe¹³, Nle¹⁴]-bombesin 6–14 (univ.; B) but not displaced by 50 nM of

GRP (C) or NMB (D), indicating the presence of BB₃ receptors. E, F Autoradiograms showing total binding of ¹²⁵I-GLP-1 (E) completely displaced by 100 n*M* of GLP-1 (F). G, H Autoradiograms showing total binding of ¹²⁵I-VIP (G) displaced by 20 n*M* of the VPAC₁-selective [K¹⁵, R¹⁶, L²⁷]VIP(1–7)/GRF(8–27) (H), indicating the presence of VPAC₁ receptors. *Lower figure:* Bronin most cases, as do some bronchial and ileal carcinoids. CCK_2 receptor scintigraphy may even be preferable to Octreoscan in those neuroendocrine tumours with few or no sst₂ receptors.

GLP-1 receptors

The GLP-1 receptor, which is massively overexpressed in virtually all insulinomas and gastrinomas and in a large number of intestinal and bronchial carcinoids, is a novel peptide receptor with a high potential for tumour targeting. The present in vitro study describes for the first time the overexpression of this receptor in human cancer. It is reasonable to expect successful in vivo targeting of these tumours with radiolabelled GLP-1 receptor-selective analogues; indeed, a GLP-1 receptor-containing rat insulinoma could be visualised recently with the radiolabelled GLP-1-selective ¹²³I-exendin 4 [33]. The present in vitro results predict that the use of GLP-1 receptor targeting in vivo should permit not only the efficient visualisation of all insulinomas, but also, because of the extraordinarily high receptor density, their successful radiotherapy; it may represent a considerable improvement over Octreoscan in these tumours.

The present data therefore strongly indicate that there may be several options for the targeting of neuroendocrine tumours, aside from somatostatin receptor scintigraphy. For insulinomas, the first choice should be not Octreoscan but GLP-1 receptor scintigraphy, since the incidence and density of these receptors are very close to those of sst_2 in gastrinomas, the gold standard indication for Octreoscan. Another alternative to Octreoscan in insulinomas may be CCK₂ receptor scintigraphy. However, in those insulinomas expressing sst_2 , GLP-1 (and CCK₂) receptor targeting may be used advantageously together with Octreoscan (see below).

Another interesting aspect of the very high expression of the GLP-1 receptor in insulinomas and other tumours is related to its biological role. Knowing the potent effect of GLP-1 in stimulating insulin release from normal pancreatic beta cells [20], it is probable that GLP-1 will also massively affect insulin release from insulinoma tissue

chial carcinoid (A) expressing sst₁ receptors (**B**, **C**), BB₃ receptors (**D–F**) and CCK₂ receptors (**G–I**). A Haematoxylin-eosin stained section. *Bar* =1 mm. **B**, **C** Autoradiograms showing total binding of ¹²⁵I-LTT-SS-28 (**B**) displaced by 100 n*M* of the sst₁-selective analogue CH288, indicating the presence of sst₁. **D–F** Autoradiograms showing total binding of ¹²⁵I-[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin 6–14 (**D**) displaced by 50 n*M* of [D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin 6–14 (univ.; **E**) but not by 50 n*M* of NMB (**F**) or GRP (not shown), indicating the presence of BB₃ receptors. **G–I** Autoradiograms showing total binding of ¹²⁵I-CCK (**G**) displaced by 50 n*M* of CCK-8 (**H**) and gastrin (**I**), indicating the presence of CCK₂ receptors

through the numerous GLP-1 receptors. On the one hand, such release may play a significant pathophysiological role in this disease. On the other hand, it may be used as a potent diagnostic strategy: a GLP-1 stimulation test using a single injection of GLP-1 would trigger a release of large amounts of insulin from the insulinoma that could be detected in the circulation. This might offer a useful and easy test for the detection of insulinomas in the early stage of the disease, in analogy with the pentagastrin test, which stimulates calcitonin release from medullary thyroid cancers through CCK₂ receptors [3, 7].

Receptor co-expression as a basis for in vivo multireceptor targeting

The co-expression of multiple receptors in human tumours may be a ubiquitous feature of peptide receptors, as it is not confined to various neuroendocrine tumours but has been shown previously in other cancers, such as breast cancers [15]. Its in vivo application may be extremely attractive as a means to improve the efficacy of peptide targeting in tumours; the concomitant application of multiple radioligands will selectively increase the radioactivity accumulation in tumours, an advantage not only for diagnostic but especially for radiotherapeutic purposes. Specifically, the present data predict the combination of GLP-1 and CCK₂ receptors to be highly efficient targets in all insulinomas, and indicate that the use of a mixture of sst₂, GLP-1 and GRP radioligands would offer optimal targeting of gastrinomas. As some of the receptors are non-homogeneously expressed by tumours, such as CCK₁ and CCK₂ in ileal carcinoids, a combination of the corresponding receptor-selective radiopeptides may further improve the targeting efficacy during radiotherapy by destroying more than one receptor-expressing tumour area. Furthermore, a cocktail of different peptides may reduce the risk of a loss of efficacy during peptide radiotherapy, which may be due to tumour dedifferentiation with a resulting loss of some but not all peptide receptors. Finally, an advantage of using a cocktail of radioligands is the possibility of labelling each of them with different isotopes, namely with β emitters of different ranges, in order to achieve optimal radiotherapy for large and small tumoural lesions [34]. One could conceive that the use of ¹⁷⁷Lu-labelled DOTATATE [23] together with ¹⁸⁸Re- or ⁹⁰Y-labelled GRP analogues [8] may be of benefit in gastrinoma patients with multiple, large and small metastases. Whenever possible, prior to the concomitant use of several radiopeptide ligands in vivo, it may be worth determining the individual peptide receptor affinity profile of the tumour under consideration by in vitro receptor determination using the described methodology in a surgically resected biopsy sample.

A prerequisite for development of multireceptor tumour targeting in vivo is, however, the availability of adequate radioligands. During the past few years, novel and more potent somatostatin radioligands such as ¹⁷⁷Lulabelled DOTATATE [23] or ⁹⁰Y-DOTANOC [35] have been reported. In addition, analogues with affinity for the GRP receptor, such as Demobesin [31] or RP527 [8], NPY(Y₁)-selective analogues such as the one reported by Soll et al. [36] and more potent CCK₂-selective analogues [37] have recently been developed, which may be used for more efficient and powerful in vivo multireceptor targeting of tumours.

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