[Lys⁴⁰(Ahx-DTPA-¹¹¹In)NH₂]-Exendin-4 Is a Highly Efficient Radiotherapeutic for Glucagon-Like Peptide-1 Receptor–Targeted Therapy for Insulinoma

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Abstract

Purpose: Although metabolic changes make diagnosis of insulinoma relatively easy, surgical removal is hampered by difficulties in locating it, and there is no efficient treatment for malignant insulinoma. We have previously shown that the high density of glucagon-like peptide-1 receptors (GLP-1R) in human insulinoma cells provides an attractive target for molecular imaging and internal radiotherapy. In this study, we investigated the therapeutic potential of [Lys⁴⁰(Ahx-DTPA-¹¹¹In)NH₂]-Exendin-4, an ¹¹¹In-labeled agonist of GLP-1, in a transgenic mouse model of human insulinoma.

Experimental Design: [Lys⁴⁰(Ahx-DTPA-¹¹¹In)NH₂]-Exendin-4 was assessed in the Rip1Tag2 mouse model of pancreatic β-cell carcinogenesis, which exhibits a GLP-1R expression comparable with human insulinoma. Mice were injected with 1.1, 5.6, or 28 MBq of the radiopeptide and sacrificed 7 days after injection. Tumor uptake and response, the mechanism of action of the radiopeptide, and therapy toxicity were investigated.

Results: Tumor uptake was >200% injected activity per gram, with a dose deposition of 3 Gy/MBq at 40 pmol [Lys⁴⁰(Ahx-DTPA-¹¹¹In)NH₂]-Exendin-4. Other GLP-1R⁺ positive organs showed ≥30 times lower dose deposition. A single injection of [Lys⁴⁰(Ahx-DTPA-¹¹¹In)NH₂]-Exendin-4 resulted in a reduction of the tumor volume by up to 94% in a dose-dependent manner without significant acute organ toxicity. The therapeutic effect was due to increased tumor cell apoptosis and necrosis and decreased proliferation.

Conclusions: The results suggest that [Lys⁴⁰(Ahx-DTPA-¹¹¹In)NH₂]-Exendin-4 is a promising radiopeptide capable of selectively targeting insulinoma. Furthermore, Auger-emitting radiopharmaceuticals such as ¹¹¹In are able to produce a marked therapeutic effect if a high tumor uptake is achieved.

During the last years, it has been shown that hormone and cytokine receptors that are expressed preferentially by cancer cells are suitable targets for both cancer diagnosis and therapy (1). Monoclonal antibodies were first used to target hormone or cytokine receptor–expressing cancer cells. To enhance the efficient clearance of peptide hormone receptor–expressing cancer cells, antibodies are also linked to radionuclides, thus combining the benefits of antibody-induced immunoresponses with the cytotoxic effects of radiation.

To overcome the limitations imposed by the large molecular weight of antibodies, small radiolabeled peptide ligands have been generated to specifically target suitable hormone or cytokine receptors on cancer cells. A prototype of this class of pharmaceuticals is the somatostatin receptor ligand octreotide, which is used for both diagnosis and therapy of a number of neuroendocrine tumors, including insulinoma (1). Another peptide receptor, glucagon-like peptide-1 receptor (GLP-1R), is also highly expressed on both benign and malignant human insulinomas (2). Insulinomas arise from pancreatic β cells and are the most frequent hormone-active tumors of the pancreas with an incidence of 0.4/100,000/y. Ninety percent of

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insulinomas are benign and present as small, encapsulated, solitary tumors. Multiple tumors occur in 13% of the cases, and ~10% of insulinomas progress to malignant cancer and the formation of tumor metastases. Morbidity and mortality of benign insulinomas are also due to the induction of profound hypoglycemia by the tumor. For benign insulinoma, 10-year survival is ~88% after complete surgical removal of the tumor. In case of malignant, metastasizing insulinoma, prognosis is poor with a median survival of 2 years and a 10-year survival rate of 29% (3). About 30% of the patients have a prolonged tumor-free survival due to the biology of the tumors rather than any treatment modality. Therapeutic options are limited to palliative therapies, including transarterial tumor embolization, chemotherapy, radical surgery, and radioablation (4). Alternative therapy options include Octreotide (Sandostatin) and the radiolabeled somatostatin modification [111In]DOTA-TOC, which are used for somatostatin-receptor-2 (SSTR-2) targeting in insulinomas. In SSTR-2–positive endocrine pancreatic tumors, including SSTR-2–positive insulinomas, the response rate of [111In]DOTA-TOC is 38% (partial and complete remission; ref. 5). Unfortunately, in ~40% of insulinomas, expression of SSTR-2 is too low to allow for efficient targeting (6). In contrast, the density of GLP-1R in insulinoma cells is considerably higher than that of other peptide hormone receptors, with an incidence of GLP-1R in 25 of 27 insulinomas, compared with SSTR-2 with 18 of 26 (2). Treatment of mice bearing a s.c. implanted rat insulinoma tumor (RINm5F) with [125I]-labeled GLP-1 [7-36] amide and [125I]-Exendin-3 has shown that the peptide is specifically internalized and that the implanted tumor cells can be detected by scintigraphy (7). However, the low peptide stability of [125I]-GLP-1 and the less effective radio-iodination of Exendin-3 have limited their clinical use. Therefore, [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4, a novel radiopeptide with an enhanced peptide stability and more effective radiolabeling, has been designed. Indeed, [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 is highly stable in human serum: analytical high-performance liquid chromatography analysis showed that after 4.5 h, 82.1% of the peptides were unaltered, whereas after 24 h 73.7% were still intact (8).

Here, we have used the Rip1Tag2 transgenic mouse model of multistage insulinoma development to investigate the therapeutic efficacy of [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4. Rip1Tag2 transgenic mice express the SV40 large T antigen under the control of the rat insulin promoter in β cells of islets of Langerhans (9). These mice reproducibly develop tumors in a well-defined multistage tumorigenesis pathway, whereby carcinomas begin to form at 9 to 10 weeks of age and represent 2% to 4% of all islets by 14 weeks of age. Recently, we have shown that the GLP-1R density in β tumor cells of Rip1Tag2 mice is 17,269 ± 1,906 disintegrations/min/mg and thus comparable with that of human insulinomas with 8,133 ± 1,829 disintegrations/min/mg (8). In addition, a remarkably high tumor uptake of 287% injected activity per gram (IA/g) tissue at 4 h after injection of 2 pmol [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 can be achieved (8). [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 is also selectively and efficiently internalized in cell lines established from tumors of Rip1Tag2 mice. Hence, these mice offer a suitable model for testing the therapeutic targeting of GLP-1R by [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4.

The decay of the radioisotope 111Indium (111In) results in the release of γ-rays; therefore, 111In-coupled peptides have predominantly been used for tumor imaging. However, 111In is also an Auger and conversion electron emitter. Auger and conversion electrons have a tissue penetration of only 0.02 to 10 μm and 200 to 500 μm, respectively. Because of the short range of these electrons, the biological effects of Auger emitters are highly dependent on the subcellular distribution of the radionuclide (10). Auger electrons have a high linear energy transfer, which is comparable with that of α-emitters (11). It has been shown that internalization and translocation of Auger emitters to the cell nucleus results in cell death (12). In addition, accumulation of 111In in the cytoplasm or in the cell nucleus increases the cellular absorbed dose fraction (13). A number of chemotherapeutic compounds (e.g., bleomycin) and antibodies (e.g., anti–epidermal growth factor receptor, anti-CD74, and anti-HER2) have been labeled with 111In and tested in both cellular and animal xenograft models with moderate therapeutic effects (14–17). A higher antitumor efficacy of Auger electron emitters compared with β-emitting radiometals or 131I have also been detected when conjugated to internalizing antibodies (18). A clinical study investigating the effect of 111In-DTPAoctreotide in patients with neuroendocrine tumors has revealed a low incidence of clinical side effects, yet only a moderate therapeutic response to the radiopptide (19). In all these studies, an efficient therapeutic effect of 111In has been mainly hampered by a rather low uptake of the radiopeptide into the tumor cells. Hence, a significant improvement of tumor cell uptake would markedly enhance the therapeutic effect of 111In-labeled compounds. Here, we show that GLP-1R is a suitable target for the efficient therapy of insulinoma and that the Auger electron emitting [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 is a potent radiotherapeutic.

Materials and Methods

Reagents and instrumentation. All chemicals were obtained from commercial sources and used without further purification. 111InCl3 was purchased from Mallinkrodt. Analytic high-performance liquid chromatography was done on a Metrohm high-performance liquid chromatography system LC-CaDi 22-14 and a Benhold LB 509 flow-through γ-detector with a Macherey-Nagel Nucleosil 120-3 C-18 column. Quantitative γ-counting was done on a COBRA 5003 γ-system well counter (Packard Instrument Company). Fasting blood glucose levels were measured with the Akku-Check system (Roche).

Peptide synthesis and radiolabeling. [Lys40(Ahx-DTPA)NH2]-Exendin-4 was custom synthesized by Peptide Specialty Laboratories. The peptide conjugate was characterized by matrix-assisted laser desorption ionization-mass spectrometry measurements that were done on a Voyager sSTR equipped with a Nd:YAG laser (355 nm, Applied Biosystems). An aliquot of 4 μg [Lys40(Ahx-DTPA)NH2]-Exendin-4 was dissolved in sodium acetate buffer (0.4 mol/L/pH 5.0), incubated with 555 MBq high specific 111InCl3 at room temperature for 30 min, and then subjected to quality control by analytic high-performance liquid chromatography (Supplementary Fig. S1; eluents: A, 0.1% trifluoroacetic acid in water; B, acetonitrile; flow, 0.75 mL/min; 0 min 95% A, 30 min 55% A, 31 min 0% A, 34 min 0% A, 37 min 95% A).

Mice. Phenotypic and genotypic analyses of Rip1Tag2 transgenic mice in a C57Bl/6 background have been described previously (9). Rip1Tag2 and wild-type C57Bl/6J mice were used for organ toxicity studies. Animals were maintained and treated in compliance with the guidelines of the Swiss Veterinary Office and the Cantonal Veterinary
office of Basel-Stadt (approval 2085 and 789). Rip1Tag2 mice were injected into the tail vein with single doses of 1.1 MBq (2 pmol), 5.6 MBq (8 pmol), and 28 MBq (40 pmol) [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 or with 40 pmol nonradioactive [Lys40(Ahx-DTPA-42In)NH2]-Exendin-4 as control. At indicated times, mice were sacrificed, tumor diameters were measured with a grid (for small tumors) or with a caliper (for large tumors), and tumor volumes were calculated assuming a spherical shape of the tumors. Mice were stratified according to their age and randomly assigned to the different cohorts. Mouse age at time of sacrifice was, for all cohorts, 11.4 ± 0.3 weeks.

**Biodistribution in Rip1Tag2 mice.** Male and female Rip1Tag2 mice, 18 to 27 g and 10 to 14 weeks old, were injected with 40 pmol (192 ng total peptide mass/28 MBq) [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 in 100 µL NaCl solution 0.9% into the tail vein. At 1, 12, 36, 84, 168, and 264 h after injection, mice (n = 5 per cohort) were sacrificed. Organs, blood, and tumors were collected and the activity concentration C(tissue) (%IA/g) was determined. To determine radiopeptide uptake in organs and tumors, mice were injected with 2, 10, 50, and 250 pmol (0.1-14 MBq) [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 as described above and sacrificed 4 h after injection. The measured γ emission of 111In is proportional to the emission of Auger electrons.

**Dosimetry.** Determination of the absorbed dose in the tumor and normal tissue from 111In was done according to the MIRD formalism (20). The biodistribution data (40 pmol/28 MBq [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4) were used to generate the time-activity curve between 1 and 264 h. The mean cumulated activity (A) in selected organs was obtained by analytic integration over a biexponential fit to the time-activity curve:

\[
A(t) = \int_0^\infty A_{tissue}(t) dt = \frac{A_1}{\lambda_1} + \frac{A_2}{\lambda_2}
\]

Fitting was done using the GNU implementation of the Levenberg-Marquard algorithm (21, 22). The mean absorbed dose D(tissue) to a tissue with mass m(tissue) was estimated for various tissues as follows: D(tissue) was estimated by dividing the energy (E) released per nuclear transformation and the absorbed fraction (Φ) by the energy deposition of γ-rays in small animals such as mice (23). The absorbed fraction Φ for all measured organs was set as 1 because of the very short path length of Auger electrons in tissues. Tissue mass/mass was calculated as TNPD = D(tumor)/D(tissue).

**Histologic analysis.** Tissue was fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. For frozen sections, tissue was immersed overnight in 20% sucrose in HBS-Ca2+ (HEPES-buffered saline with 1 mmol/L CaCl2), and embedded and snap-frozen in optimum cutting temperature compound (Tissue-Tek). Immunohistology on paraffin and frozen sections was done by quenching the slides in 3% H2O2 in PBS for 10 min, blocking in 5% goat serum for 1 h, incubation with primary antibody overnight at 4°C, incubation with secondary antibody for 30 min, and development with the ABC-kit of Vectastain (Vector Laboratories) and Sigma Fast 3,3′Diaminobenzidine tetrahydrochloride with metal enhancer (Sigma) or AEC-kit (Vector Laboratories). For immunofluorescence stainings, frozen sections were permeabilized in 0.2% Triton X-100 in PBS, blocked in 5% goat serum for 1 h, and incubated with rabbit anti-cyclin D2 (Santa Cruz Biotechnology) or primary antibody for 1 h and with a fluorescent secondary antibody for 30 min.

**Proliferation and apoptosis assay.** For bromodeoxyuridine (BrdUrd) staining (proliferation assay), BrdUrd (5 mg/mL in 10 mmol/L Tris-HCl [pH 7.4]; 0.8% NaCl; 0.25 mmol/L EDTA) was injected at 100 µg BrdUrd per gram body weight i.p. 2 h before sacrifice. After antigen retrieval (2 N HCl for 1 h and trypsinization), pancreatic sections were incubated in a 1:100 solution of biotinylated mouse anti-BrdUrd antibody (Zymed). Incorporated BrdUrd was visualized with the ABC-kit of Vectastain (Vector Laboratories) and Sigma Fast 3,3′Diaminobenzidine tetrahydrochloride with metal enhancer (Sigma).

For terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL; apoptosis) assays, antigen retrieval on slides was done by digestion with proteinase K (Fluka). Slides were incubated for 1 h in TUNEL reagents (Roche) at 37°C and apoptotic cells were visualized with the ABC-kit (Vector Laboratories).

**Microscopy.** Immunohistochemical stainings were analyzed on a AxioVert microscope (Zeiss). Immunofluorescence stainings were analyzed on a LSM510 META confocal microscope using LSM510 software (Zeiss). Histologic grading of tumors as hyperplastic islets, adenomas, or carcinomas was done following the WHO International Classification of Rodent Tumors, Part II, The Mouse. Histologic staging and grading were done in a blindfold manner and were repeated twice.

**Statistical analysis.** Statistical analysis was done using the GraphPad Prism software (GraphPad Software, Inc.). Tumor volume and mass were calculated using nonparametric statistical analysis (Kruskal-Wallis test) with Dunn’s posttest. Proliferation, vessel density, number of immune cells, and blood glucose levels and animal weight were analyzed by parametric testing (one-way ANOVA and Newman-Keuls posttest).

**Results**

**Biodistribution and dosimetry.** Previously, we have shown by autoradiography that GIP-1R is ~10,000-fold overexpressed in insulinoma cells of Rip1Tag2 transgenic mice compared with normal β-cells of the islets of Langerhans. Moreover, in vitro internalization studies showed that the synthetic ligand [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 is linearly internalized into β-cells, resulting in an uptake of 9.8 ± 0.9% 4 h after incubation. Eighty-five percent to 97% of the radioligand was internalized specifically (8). Here, we set out to use [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 for therapeutic intervention studies of insulinoma development in Rip1Tag2 transgenic mice. By using highly specific 111InCl3, [Lys40(Ahx-DTPA)NH2]-Exendin-4 was labeled with yields of 97.5% and a high specific activity of 700 GBq/µmol. The radiochemical purity of the compound was 97.6% (Supplementary Fig. S1).

To investigate the uptake of [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 into the tumor cells in vivo, varying concentrations of the radiopeptide were injected i.v. into Rip1Tag2 mice and the biodistribution after different time intervals was determined. Uptake of the radiopeptide was concentration dependent, which is in accordance with similar experiments measuring the uptake of radiolabeled octreotide derivatives in a SSTR-2–expressing tumor model (24). Injected peptide concentrations from 2 to 50 pmol yielded a tumor uptake of >200% IA/g, with the highest relative tumor uptake (307 ± 38% IA/g) after injection of 10 pmol (5.6 MBq) [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 (Fig. 1A). However, a 25 times higher peptide concentration resulted in a tumor uptake of only 59% IA/g, whereas the kidney uptake was still 190% IA/g. Based on these results, a maximum dose of 40 pmol (28 MBq) [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 was injected to assess the kinetics of its tumor-specific uptake. Eighty-four hours after injection of the radiopeptide, tumor uptake was ~60% IA/g, whereas 168 and 264 h after injection, tumor uptake was 180 ± 107% IA/g and 203 ± 125% IA/g, respectively. At these later time points,
all other organs showed a rapid decline in the levels of detectable radiopeptide (data not shown).

After a single injection of 28 MBq [Lys⁴⁰(Ahx-DTPA-¹¹¹In)]-Exendin-4, the highest dose deposition (3 Gy/MBq) was found in tumors of Rip1Tag2 mice. The absorbed dose per mega-Becquerel in kidney, liver, spleen, bone marrow, and blood and GLP-1R–positive organs, including lung, pancreas, small intestine, and tumor, is shown in Table 1. The absorbed dose in kidney was 2 Gy/MBq resulting in a TND<sub>kidney</sub> (tumor-to-kidney absorbed dose ratio) of 1.5. All other organs, including pancreas and blood, showed a TND<sub>tissue</sub> ≥ 30. The high and specific uptake of [Lys⁴⁰(Ahx-DTPA-¹¹¹In)]-Exendin-4 into GLP-1R–expressing tumor cells as well as the observed high dose deposition into the tumor are critical for a therapeutic application of the radioligand. These dosimetry results indicate that the tumors of Rip1Tag2 transgenic mice can be specifically targeted with [Lys⁴⁰(Ahx-DTPA-¹¹¹In)]-Exendin-4 at high specific activities and that the kidney is expected to be the dose-limiting organ.

![Fig. 1.](image-url) [Lys⁴⁰(Ahx-DTPA-¹¹¹In)]-Exendin-4 represses insulinoma growth. A, biodistribution 4 h after injection of 2, 10, 50, and 250 pmol [Lys⁴⁰(Ahx-DTPA-¹¹¹In)]-Exendin-4 in tumor-bearing Rip1Tag2 mice. Points, result of two independent experiments with a total of six animals in each group; bars, SD. B, analysis of H&E stainings of pancreas sections 8 d after injection of [Lys⁴⁰(Ahx-DTPA-¹¹¹In)]-Exendin-4 and nonradioactive In¹¹¹-Exendin-4. Tumor cell death and formation of hemorrhagic lacunae in the tumors of the interventional group (top) are apparent, whereas morphologically normal islets are not affected by the treatment (bottom). Bar, 100 µm. C, dose-dependent reduction of tumor volumes 8 d after injection of [Lys⁴⁰(Ahx-DTPA-¹¹¹In)]-Exendin-4. The percentages of tumor volume reduction in treated compared with control mice (% treated versus control) are 27.9%, 13.7%, and 5.6% for the 1.1, 5.6, and the 28 MBq groups (P = 0.0003, Kruskal-Wallis test). D, kinetic of tumor mass reduction in the 28 MBq group. A first decrease in tumor mass is detectable 36 h after injection of the radiopeptide and a progressive reduction in tumor mass is apparent until 10 d after treatment.
Reduced tumor volume. To investigate the therapeutic effect of [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4, cohorts of seven Rip1Tag2 mice each were injected with single doses of 1.1, 5.6, and 28 MBq [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4. A control group of eight Rip1Tag2 mice was injected with nonradioactive [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4. Mice were sacrificed 8 days after the single injections. Histologic analysis revealed dramatic morphologic changes in tumor sections of radiopeptide-injected mice. With an increasing dose of radiopeptide, tumors disaggregated and developed large hemorrhagic lacunae (Fig. 1B). In mice treated with a single injection of 28 MBq (equal to an average absorbed tumor dose of 84 Gy), \pm 50\% of the tumors were completely dissolved with only cellular debris and hemorrhagic lacunae remaining. In the other 50\% of tumors, a thin tumor rim of morphologically intact cancer cells remained detectable (Fig. 1B, top right). No morphologic changes were evident in normal islets, indicating that exclusively cells of \(\beta\) adenoma or carcinoma were targeted, whereas untransformed islet cells were not significantly affected by the treatment (Fig. 1B, bottom).

The observed reduction in tumor volumes was dependent on the dose of injected radiopeptide (Fig. 1C). Eight days after injection of the radiopeptide, the percentage of tumor volume in mice treated with 28 MBq compared with control mice (n treated versus control) was 5.6\% (\(P < 0.001\), Dunn’s posttest). To investigate the kinetics of tumor growth and decrease, we also examined the total tumor mass per mouse at different time points after the injection of 28 MBq [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4 (Fig. 1D). The first therapeutic effect was detectable as soon as 12 h after injection, and a continued decrease in tumor mass could be observed until 11 days after the single injection of the radiopeptide. In a cohort of untreated Rip1Tag2 mice (n = 4), within the same timeframe, the tumor volume increased by \pm 50\% (data not shown).

Taken together, these results show that a single injection of 28 MBq [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4 is sufficient to induce a dramatic and prolonged tumor response (i.e., a 94\% decrease of tumor volume within 8 days after application of the radiopeptide). At the same time, the residual hemorrhagic tumor mass still exhibited an uptake of 180\% IA/g. This finding underlines the prolonged therapeutic effect of [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4, which is due to its persistence in GLP-1R–expressing cells.

Reduced tumor cell proliferation. To determine the mechanism of tumor regression exerted by [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4 in Rip1Tag2 mice, the rate of tumor cell proliferation was determined by labeling mice with BrdUrd 2 h before sacrifice and visualizing cells in S-phase by immunohistochemical stainings of tumor sections with antibodies against BrdUrd. In radiopeptide-treated mice, the percentage of BrdUrd-positive tumor cells declined markedly with increasing radioactivity (Fig. 2A, top). In cases where the tumor was not completely ablated, single proliferating tumor cells could still be detected in the tumor rim (Fig. 2A, top right).

The reduction of proliferating tumor cells in treated compared with control mice (%T/C) was 29\% for the 28 MBq group (Fig. 2C). Analysis of morphologically unaltered normal islets and small hyperplasias revealed few proliferating cells, independent from the injected activity (Fig. 2A, bottom). The reduction in the rate of BrdUrd-positive tumor cells argues in favor of a cell cycle arrest in the G\(_1\)-S phase. The transition of cells from the G\(_1\) to the S phase is dependent on the activity of specific cyclin-dependent kinases and their corresponding cyclins. In \(\beta\) tumor cells of Rip1Tag2 transgenic mice, cyclin D\(_2\) is a critical regulator of the G\(_1\)-S phase transition (25). Injection of escalating doses of the radiopeptide revealed a significant, dose-dependent loss of cyclin D\(_2\) expression in the tumor cells (Fig. 2B and D). These results indicate that treatment with [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4 strongly represses insulinoma cell proliferation, mediated, at least in part, by a loss of cyclin D\(_2\) expression in tumor cells.

Induction of necrosis and apoptosis. Quantification of tumor cell apoptosis by TUNEL assay on histologic tumor sections revealed an increase of tumor cell apoptosis in a dose-dependent manner. No significant difference could be detected when comparing the control and the 1.1 MBq group, whereas a significant increase of apoptotic cells was observed in the 5.6 and 28 MBq treatment groups (Fig. 3A and B). Quantification of microvessel density by staining of pancreatic sections of mice from all treatment groups with antibodies against CD31 did not reveal any significant differences, suggesting that tumor cell apoptosis was not due to a lack of tumor oxygenation and nutrition (data not shown).

The hemorrhagic and necrotic appearance of treated tumors (Fig. 1B) led us to investigate whether the injection of [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4 induced an inflammatory response. Infiltration of leukocytes into tumors was analyzed by staining of pancreatic sections of mice from all study groups with anti-CD45 antibodies 8 days after injection of the radiopeptide (Fig. 3C). The number of intratumoral CD45-positive cells significantly increased with treatment in a dose-dependent manner, whereas no change was evident in the exocrine pancreas or in small noncancerous islets. Immunofluorescence stainings for different types of immune cells, including CD4+, CD8+, Gr-1+, and F8/40-positive cells, revealed that the numbers of T cells or granulocytes did not change in the tumors of treated mice (data not shown).

### Table 1. Absorbed dose per mega-Becquerel injected activity and tumor-to-normal tissue absorbed dose of [Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4 in tumor-bearing Rip1Tag2 mice

<table>
<thead>
<tr>
<th>Organ/tissue</th>
<th>Absorbed dose per MBq (Gy/MBq)*</th>
<th>Tumor-to-normal tissue absorbed dose ratio (TND\textsubscript{tissue}) (^{1})</th>
</tr>
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<tbody>
<tr>
<td>Lung</td>
<td>0.08</td>
<td>37</td>
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<tr>
<td>Pancreas</td>
<td>0.10</td>
<td>30</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.06</td>
<td>50</td>
</tr>
<tr>
<td>Tumor</td>
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<td>—</td>
</tr>
<tr>
<td>Kidneys</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Liver</td>
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<td>300</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.027</td>
<td>111</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0.013</td>
<td>230</td>
</tr>
<tr>
<td>Blood</td>
<td>0.003</td>
<td>1,000</td>
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*Peptide uptake was measured at 1, 12, 36, 84, 168, and 264 h after injection (n = 5 per cohort). The absorbed dose was calculated from the serial biodistribution measurements using the algorithm described in Materials and Methods.

\(^{1}\) TND\textsubscript{tissue} was calculated using the absorbed dose values.
not shown). In contrast, the numbers of F8/40-positive cells increased significantly upon treatment in a dose-dependent manner (Fig. 3D; Supplementary Fig. S2B). A concomitant increase of macrophages was not apparent in the surrounding exocrine tissue and in histologically normal islets.

Restoration of glucose homeostasis and body weight. Clinically, human insulinomas present with the symptoms of hypoglycemia. Hence, blood glucose level is an important surrogate marker for the blood insulin concentration of insulinoma patients. A second, more general marker for
health is body weight, which is reduced in patients with tumor cachexia. These two functional readouts can be used to monitor the efficacy of therapies directed against insulinoma. Indeed, in mice treated with 28 MBq $[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]$-Exendin-4, a stabilization of glucose levels was observed within 8 days after treatment, whereas in the control group glucose levels significantly declined with insulinoma progression (Fig. 4A). In addition, a significant decrease in body weight could be detected in mice of the control group, whereas the weight of animals treated with 28 MBq $[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]$-Exendin-4 was stabilized (Fig. 4B). These results show that the antitumoral therapy with $[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]$-Exendin-4 exerts a significant effect on both functional control of blood glucose levels and tumor cachexia.

**Therapy toxicity.** As shown in the dosimetry experiments described above, a significant uptake of $[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]$-Exendin-4 was observed in kidney, lung, bowel, total pancreas, and tumor. All other organs did not significantly incorporate the radiopeptide. We therefore assessed the general toxicity of $[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]$-Exendin-4.

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**Fig. 3.** Tumor cell death induced by $[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]$-Exendin-4 occurs by both apoptosis and necrosis. A, TUNEL staining of tumor sections. More apoptotic cells are visible in the 5.6 and 28 MBq groups than in the $\text{In}^{111}$ and 11 MBq groups. Brown, TUNEL-positive cells. Bar, 50 μm. B, quantification of TUNEL-positive cells in tumors of treated and control-treated RipTag2 mice, as indicated. The number of apoptotic tumor cells per 0.1 mm$^2$ is 0.85 in the control group, 0.92 in the 11 MBq group, 1.30 in the 5.6 MBq group, and 1.64 in the 28 MBq group ($P = 0.019$, one-way ANOVA; Newman-Keuls test: $\ast$, $P < 0.05$ for the 5.6 and 28 MBq group, and $P > 0.05$ for the 11 MBq group). C, quantification of infiltrating CD45-positive cells in tumors, normal islets, and exocrine pancreas of control and treated mice, as indicated. In control mice, the number of CD45-positive cells per 0.05 mm$^2$ was 2.8 in the 11 MBq group 3.6, in the 5.6 MBq group 4.7, and in the 28 MBq group 4.6. No increase in CD45-positive cells could be detected in morphologically normal islets or exocrine pancreas ($P < 0.0001$, one-way ANOVA; Newman-Keuls test: $\ast$, $P < 0.05$, $\ast\ast$, $P < 0.01$). D, quantification of F4/80-positive macrophages in tumors, normal islets, and exocrine pancreas of RipTag2 mice treated with $[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]$-Exendin-4 or nonradioactive $[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]$-Exendin-4. The increase of tumor-infiltrating immune cells was almost exclusively due to an increase in macrophages. No increase in macrophage numbers could be detected in morphologically normal islets or exocrine pancreas ($P < 0.0001$, one-way ANOVA; Newman-Keuls test: $\ast$, $P < 0.05$, $\ast\ast$, $P < 0.01$).

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Fig. 4. Blood glucose levels, body weight, and organ toxicity after treatment with [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4. A, blood glucose levels in RipTag2 mice treated with [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4, nonradioactive Innat-Exendin-4, or not treated at all. Blood glucose levels of the 28 MBq group were restored to normal and comparable with healthy control mice, whereas blood glucose levels of untreated control mice continued to deteriorate during an 8-d observational period after injection of the radiopeptide (P < 0.05, Newman-Keuls posttest). B, body weights of RipTag2 mice treated with [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4, nonradioactive [Lys40(Ahx-DTPA-natIn)NH2]-Exendin-4, or not treated at all. In the control group, tumor cachexia led to a decrease in body weights during the observational period (P < 0.05, Newman-Keuls posttest). In contrast, body weights were stable in the animals treated with [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 and slightly increased in healthy control animals. C, histopathologic examination of various organs of C57Bl/6 mice, 6 mo after injection of 28 MBq [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 or saline (control), as indicated. Small intestine, lung, and endocrine and exocrine pancreas show no morphologic signs of toxicity (H&E staining). In contrast, periodic acid-Schiff staining of kidney sections reveals signs of chronic radiotoxicity, which manifests itself by glomerular shrinking and compensatory hypertrophy, tubular necrosis, interstitial fibrosis, and narrowing of the cortex. Bar, 100 μm. D, fasting blood glucose levels of C57Bl/6 mice injected with either 28 MBq [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 or saline are not significantly different, indicating that treatment with [Lys40(Ahx-DTPA-111In)NH2]-Exendin-4 does not affect normal islet physiology and function (P = 0.21, two-sided t test).
the therapy as well as the dose-limiting organ toxicity by investigating morphologic changes in these organs after the injection of radiopeptide. To this end, Rip1Tag2 mice and healthy C57Bl/6 mice were injected with 1.1, 5.6, or 28 MBq of [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 and sacrificed after 3, 10, and 30 days. Due to the continuing expression of the transgenic SV40 large T oncogene, Rip1Tag2 mice start to form new tumors ~2 weeks after termination of the therapy, thus impeding further analysis of Rip1Tag2 mice at later time points. Therefore, wild-type C57Bl/6 mice were used for the analysis at 60 and 180 days, respectively, after injection of [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4. Control animals were injected with saline. All animals survived until their sacrifice and did not exhibit any apparent pathophysiologic symptoms. Histologic analysis of bowel, pancreas, and lung was without pathologic findings, independently from the injected dose and the time point of sacrifice (Fig. 4C). In particular, the morphology of pancreatic islets was unaltered between treated and control mice, and no significant differences in blood glucose levels were found (Fig. 4D). These results indicate the absence of any significant toxicity of [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 on normal β-cells.

However, consistent with the high uptake of radiopeptide in kidney, analysis of kidney sections 180 days after injection of 28 MBq [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 revealed signs of radiation damage. In particular, tubular necrosis, glomerular sclerosis, and minor interstitial fibrosis accompanied by an atrophy of the cortex of the kidneys was evident (Fig. 4C). Electron microscopy imaging of these kidneys showed a marked enlargement of the glomerular mesangium, with concomitant signs of glomerular endothelial damage and regeneration. In glomerular capillaries, the lamina rara interna was enlarged considerably, although the other parts of the basal membrane were unaltered (Supplementary Fig. S3). The tubular epithelium showed signs of cell death and partial denudation of some tubuli, whereas others were without pathologic findings (Supplementary Fig. S4). In contrast, light microscopy of kidneys after 3, 10, 30, and 60 days and electron microscopy after 30 and 60 days did not reveal any signs of drug toxicity. These findings are therefore consistent with a chronic radiation damage of the kidneys after injection of 28 MBq [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 with a total dose deposition of 56 Gy in the kidneys.

Kidney sections of mice treated with 1.1 and 5.6 MBq (corresponding to a dose-deposition of 2.2 and 11.2 Gy, respectively) did not show signs of radiotoxicity at any time points, although an efficient therapeutic effect on tumor growth was detectable. Together, the results indicate that [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 has a favorable toxicity profile, which is mainly due to its highly specific internalization into tumor cells and its short range of action sparing the healthy tissue surrounding the tumor.

**Discussion**

Insulinoma, a hormone-active neuroendocrine tumor, is diagnosed by metabolic disturbances affecting patients. Yet, although benign insulinoma can be usually cured by surgical resection of the tumor mass, in many cases the usually small tumors are difficult to detect in the pancreas. Moreover, malignant insulinoma with metastatic spread is difficult to treat. The high and specific expression of the GLP-1R on insulinoma cells make this surface molecule an attractive target for imaging of the tumors and for novel therapeutic approaches. Recently, we have shown that various radio-labeled versions of a synthetic peptide ligand to GPL-1R, Exendin-4, can be efficiently used to visualize insulinoma in the Rip1Tag2 transgenic mouse model of malignant insulinoma (8).

Here, we show that the radi peptide [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 efficiently represses insulinoma growth in Rip1Tag2 mice. A single dose of [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 results in a dramatic reduction of tumor burden within a few days without any apparent side effects. Of the many treatment regimens tested in Rip1Tag2 mice, including antiangiogenic therapies, genetic ablation of tumor-promoting factors, and classic chemotherapy, the current treatment seems to be one of the most efficient, with up to 94% reduction in tumor volume, compared with 67% to 89% in other studies (26–31).

Cell biological and biochemical analyses reveal that upon treatment, tumor cells fail to enter the cell cycle and cease to proliferate. At the same time, tumor cells undergo massive apoptosis and inflammation-mediated necrosis. The development of an inflammatory reaction and subsequent migration of macrophages into the damaged tissue is a typical feature of tumor necrosis. Tumor regression after therapeutic intervention with [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 enables the organism to regain control over the regulation of blood glucose and to normalize metabolism, also evidenced by stabilization of the treated mice body weights.

The high density and high specificity of GLP-1R expression in insulinoma cells of Rip1Tag2 mice allow an efficient and highly specific targeting of the tumor cells. The observed increased uptake of radiopeptide per tumor mass at later stages of treatment, when the radiopeptide has already been cleared from the circulation, can be explained by the significant reduction of the tumor volume and phagocytic uptake of apoptotic neighboring cells, whereas no significant externalization of the peptide from GLP-1R–expressing tumor cells occurs during this time period (8). Other variables that influence the uptake of the radiopeptide include the cell surface density of GLP-1R, radionuclide sequestration, and changes in tumor blood flow mediated by radiation. A similar phenomenon of tumor-specific uptake of a radiolabeled peptide has been previously reported for a therapeutic dose of 30 MBq [\(^{177}\)Lu-DOTA\(^{9}\), Tyr\(^3\),Thr\(^8\)]-octreotide in nude mice bearing a human midgut carcinoid GOT I xenograft (24).

Treatment of mice with the highest dose of 28 MBq, corresponding to a dose deposition of 56 Gy, resulted in detectable morphologic changes and in toxic side effects only in kidneys, but not in any other organ analyzed. Preinjection or coinjection of lysine or triglutamic acid (H-Glu-Glu-Glu-OH) had no significant effect on the kidney uptake of [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 (data not shown). In the long run, it will be necessary to uncover the molecular aspects of the kidney uptake to design drugs with a reduced kidney toxicity. However, at lower doses, where an efficient therapeutic effect is already apparent, no toxic effects on kidney morphology are detected. Hence, treatment with [Lys\(^{40}\)(Ahx-DTPA-\(^{111}\)In)NH\(_2\)]-Exendin-4 offers a useful therapeutic window for efficient treatment protocols. External radiation of the kidneys with 23 to 27 Gy results in kidney failure in 5% to 50% of treated
patients over 5 years (32). Our results are consistent with these findings and underline that kidney toxicity is the dose-limiting factor in the treatment of insulinomas with [Lys$^{40}$ (Ahx-DTPA)$^{[111}$In$]_{2}$]-Exendin-4.

Together with our earlier results (8), this study shows that GLP-1R is both a suitable target for molecular imaging as well as for insulinoma therapy. Based on the high and specific expression of GLP-1R in human insulinoma, this hypothesis can now be tested in a clinical setting. It also underlines that Auger emitters, in particular $^{111}$In, are potent therapeutic agents if an adequate delivery of the radiopeptide into cancer cells can be achieved. Moreover, targeting an Auger emitter–tagged radiopeptide to cancer cells may contribute to implementing a therapy with a minimum of associated toxicity.

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**References**


[Lys\textsuperscript{40}(Ahx-DTPA-\textsuperscript{111}In)NH\textsubscript{2}]-Exendin-4 Is a Highly Efficient Radiotherapeutic for Glucagon-Like Peptide-1 Receptor–Targeted Therapy for Insulinoma

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