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¹⁷⁷Lu labelled cyclic minigastrin analogues with therapeutic activity in CCK2R expressing tumours, preclinical evaluation of a kit formulation

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Abstract

Minigastrin (MG) analogues specifically target cholecystokinin-2 receptors (CCK2R) expressed in different tumours and enable targeted radiotherapy of advanced and disseminated disease when radiolabelled with a beta emitter such as ¹⁷⁷Lu. Especially truncated MG analogues missing the penta-Glu sequence are associated with low kidney retention and seem therefore most promising for therapeutic use. Based on [D-GluM¹,desGlu²⁻⁶]MG (MG11) we have designed the two cyclic MG analogues cyclo^{1,9}[γ-D-GluM¹,desGlu²⁻⁶,D-Lys⁹]MG (cyclo-MG1) and cyclo^{1,9}[γ-D-Glu¹,desGlu²⁻⁶,D-Lys⁹,Nle¹¹]MG (cyclo-MG2). In the present work we have developed and preclinically evaluated a pharmaceutical kit formulation for the labelling with ¹⁷⁷Lu of the two DOTA-conjugated cyclic MG analogues. The stability of the kits during storage as well as the stability of the radiolabelled peptides was investigated. A cell line stably transfected with human CCK2R and a control cell line without receptor expression were used for *in vitro* and *in vivo* studies with the radioligands prepared from kit formulations. In terms of stability ¹⁷⁷Lu-DOTA-cyclo-MG2 showed advantages over ¹⁷⁷Lu-DOTA-cyclo-MG1. Still, for both radioligands a high receptor-mediated cell uptake and favourable pharmacokinetic profile combining receptor-specific tumour uptake with low unspecific tissue uptake and low kidney retention were confirmed. Investigating the therapy efficacy and treatment toxicity in xenografted BALB/c nude mice a receptor-specific and comparable therapeutic effect could be demonstrated for both radioligands. A 1.7- to 2.6-fold increase in tumour volume doubling time was observed for receptor-positive tumours in treated versus untreated animals, which was 39-73% higher when compared to

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Author Contributions

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Supporting Information. Stability of the ¹⁷⁷Lu-labelled cyclic MG analogues in PBS and human serum; radiochromatograms showing the stability of the ¹⁷⁷Lu-labelled cyclic MG analogues prepared from kit formulations after 4 h storage; tumour volume doubling time calculated from mean specific growth rate of A431-CCK2R and A431-mock tumour xenografts of the different groups of animals in the therapy efficacy study.

receptor-negative tumours. The treatment was connected with transient bone marrow toxicity and minor signs of kidney toxicity. All together the obtained results support further studies for the clinical translation of this new therapeutic approach.

Keywords

minigastrin; cholecystokinin/gastrin receptor; peptide receptor radionuclide therapy; kit formulation; therapy efficacy; treatment toxicity

Introduction

Radiolabelled minigastrin (MG) analogues specifically targeting the cholecystokinin/gastrin subtype 2 receptor (CCK2R) overexpressed in various tumours open new diagnostic and therapeutic strategies in patients with advanced and disseminated disease [1,2]. CCK2R are expressed at high incidence in medullary thyroid carcinomas (MTC, 92%), small cell lung cancers (SCLC, 57%), astrocytomas (65%), and stromal ovarian cancers (100%) and are frequently expressed also in gastroenteropancreatic neuroendocrine tumours (22%) and other tumours [1,3–5]. Especially for MTC patients with advanced and disseminated state of the disease a high demand for more specific diagnostic biomarkers and efficient systemic adjuvant therapies exists [6,7]. Given the lower incidence and density of somatostatin receptors in MTC in comparison with other neuroendocrine tumours radiolabelled somatostatin analogues are of limited use [5]. An inverse relationship between somatostatin receptor expression and degree of tumour differentiation has been shown, whereas CCK2R expression seems to be preserved in patients suffering from metastatic disease associated with tumour dedifferentiation [8].

First diagnostic and therapeutic applications using radiolabelled MG analogues were based on human MG [9]. Gastrin receptor scintigraphy with ^{111}In -DTPA-MG0, a MG analogue obtained by replacing Leu in position 1 with D-Glu and N-terminal conjugation with the linear chelator DTPA, showed a higher tumour detection rate in comparison with ^{111}In -pentetreotide somatostatin receptor scintigraphy and 2- ^{18}F fluoro-2-deoxy-D-glucose positron emission tomography in MTC patients [8] and showed promising results also for the detection of SCLC and neuroendocrine tumours [10]. However, targeted radiotherapy of MTC with ^{90}Y -DTPA-MG0 had the drawback of severe nephrotoxic side effects connected with high kidney uptake [11]. The truncated MG analogue MG11 missing the penta-Glu sequence was developed in the aim to reduce kidney uptake [12–13]. ^{111}In -DOTA-MG11 and $^{99\text{m}}\text{Tc}$ -EDDA-HYNIC-MG11 both showed a clearly reduced kidney uptake in patients with MTC, but a very short half-life in blood impaired the imaging properties [14,15]. Different approaches have been explored to improve the tumour-to-kidney ratio of radiolabelled MG analogues. The tumour-to-kidney ratio of ^{111}In -DTPA-MG0 could be considerably reduced by co-infusion of the gelatin-based plasma expander Gelofusine or poly-glutamic acid reducing the kidney uptake by more than 40% in rats [16]. By co-injection of the enzyme inhibitor phosphoramidon ^{111}In -DTPA-MG11 could be metabolically stabilized *in vivo* resulting in a more than 7-fold higher tumour uptake in a preclinical mouse tumour model [17]. Still, alternative radioligands are needed to overcome

the issues of low metabolic stability or high kidney uptake and to enable the successful introduction of radiolabelled MG analogues into the clinical practice.

Based on truncated MG11 we have developed the two cyclic MG analogues cyclo^{1,9}[γ -D-Glu¹,desGlu²⁻⁶,D-Lys⁹]MG (cyclo-MG1) and cyclo^{1,9}[γ -D-Glu¹,desGlu²⁻⁶,D-Lys⁹,Nle¹¹]MG (cyclo-MG2) [18,19]. The peptides show specific substitutions with γ -D-Glu in position 1 and D-Lys in position 9 allowing the introduction of a cyclic constraint. Furthermore, Met in position 6 was replaced with Nle to avoid oxidative side products during radiolabelling and storage of the radiolabelled product. It has been shown previously that conversion of Met to the Met-sulfoxide leads to loss of receptor affinity [14], whereas by replacement with Nle the affinity is retained [18]. Within a collaborative study comparing twelve different CCK2R targeting peptide analogues DOTA-cyclo-MG1 showed a highly specific binding affinity for human CCK2R [20]. ¹¹¹In-DOTA-cyclo-MG1, besides showing promising tumour uptake and low kidney retention, displayed the lowest unspecific tissue uptake from all radioligands studied [21], and seems therefore most suited also for therapeutic use.

Within this study we have evaluated DOTA-cyclo-MG1 and DOTA-cyclo-MG2 for the radiolabelling with ¹⁷⁷Lu, a beta-particle emitting radionuclide with concomitant gamma emission. The *in vitro* characterization included receptor affinity studies of the peptide conjugates and cell uptake studies with the ¹⁷⁷Lu-labelled conjugates in a human cell line with and without expression of human CCK2R, as well as stability studies in different media. We have developed and preclinically evaluated a freeze-dried kit formulation for the straightforward preparation of the radioligands in the clinical setting. Additionally, biodistribution studies and an experimental radionuclide therapy study in a double-tumour xenograft mouse model were carried out with ¹⁷⁷Lu-DOTA-cyclo-MG1 and ¹⁷⁷Lu-DOTA-cyclo-MG2 prepared from kit formulations to obtain first insights in the therapy efficacy and treatment toxicity of this therapeutic approach.

Materials and Methods

All commercially obtained chemicals were of analytical or pharmaceutical grade and used without further purification unless otherwise stated.

DOTA-cyclo-MG1 and DOTA-cyclo-MG2 were synthesized by CS Bio, Inc. (Menlo Park, USA) with a purity of >95% as analyzed by MALDI-TOF mass spectrometry and reversed-phase high-performance liquid chromatography (RP-HPLC). DOTA-cyclo-Met(O)-MG1 containing the Met-sulfoxide was obtained by incubation of the peptide analogue in 10% hydrogen peroxide solution at 37°C for 10 min followed by HPLC purification and solid phase extraction on a Sep-Pak Light tC18 cartridge (Waters Corporation, Milford, MA, USA). A >95% purity was confirmed by HPLC analysis and the identity was confirmed by MALDI-TOF mass spectrometry. CP04, a MG analogue with D-Glu substitution in position 1-6 currently under clinical investigation [22], was kindly provided by POLATOM (Otwock, Poland). The detailed amino acid sequences of human MG and different MG analogues are summarized in Table 1. Radioiodination of human gastrin-I (H-3085, Bachem, Weil am Rhein, Switzerland) used as radioligand in receptor binding studies was carried out using the

chloramine-T method and carrier free ^{125}I (Perkin Elmer, Boston, MA, USA). Non-carrier-added [^{125}I -Tyr 12]gastrin-I was obtained by HPLC purification, stored in aliquots at -20°C and used within one week after preparation.

$^{177}\text{LuCl}_3$ was obtained from two different suppliers, non-carrier-added $^{177}\text{LuCl}_3$ (3000 GBq/mg) from ITG Isotope Technologies Garching GmbH (Garching, Germany) or carrier-added $^{177}\text{LuCl}_3$ (570-740 GBq/mg) from NCBJ Radioisotope Centre POLATOM (Otwock, Poland). Both sources of $^{177}\text{LuCl}_3$ were used for radiolabelling purposes and *in vitro* characterization of the cyclic DOTA-peptides. Stability studies on the radioligands prepared from kit formulations and *in vivo* animal studies were performed only with carrier-added $^{177}\text{LuCl}_3$.

A431 human epidermoid carcinoma cells stably transfected with human CCK2R (A431-CCK2R) and mock transfected cells lacking receptor expression (A431-mock) were kindly provided by Dr. Luigi Aloj. A number of 4.7×10^6 binding sites per cell have been determined for A431-CCK2R cells in binding affinity studies [23]. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and a solution containing penicillin, streptomycin and L-glutamine at 37°C in a humidified 95% air/5% CO_2 atmosphere.

***In vitro* characterization of the cyclic DOTA-peptides**

Next to DOTA-cyclo-MG1 and DOTA-cyclo-MG2, also the binding affinity of DOTA-cyclo-Met(O)-MG1 containing the Met-sulfoxide was tested in a competition assay against [^{125}I]Tyr 12 -gastrin-I on A431-CCK2R cells. The assay was performed also with the DOTA-peptides chelated with $^{\text{nat}}\text{Lu}$ (3-times molar excess) by incubation in 0.4 M sodium acetate solution adjusted to pH 5 at 80°C for 15-25 min. Binding assays were carried using in 96-well filter plates (MultiScreenHTS-FB, Merck Group, Darmstadt, Germany) pretreated with 10 mM TRIS/139 mM NaCl buffer, pH 7.4 ($2 \times 250 \mu\text{L}$). For the assay a number of 400,000 A431-CCK2R cells per well were prepared in 35 mM HEPES buffer, pH 7.4, containing 10 mM MgCl_2 , 14 μM bacitracin and 0.5% bovine serum albumin (BSA), a hypotonic solution disturbing the integrity of the cell membranes. The cells were incubated in triplicates with increasing concentrations of the peptide conjugates (0.0003-1,000 nM) and [^{125}I -Tyr 12]gastrin-I (50,000 cpm) for 1 h at room temperature (RT). Incubation was interrupted by filtration of the medium and rapid rinsing with ice-cold 10 mM TRIS/139 mM NaCl buffer, pH 7.4 ($2 \times 200 \mu\text{L}$), and the filters were counted in a 2480 Wizard² automatic gamma-counter (Perkin Elmer Life Sciences and Analytical Sciences, Turku, Finland). Half maximal inhibitory concentration (IC₅₀) values were calculated following nonlinear regression with Origin software (MicroCal Origin 6.1, Northampton, MA).

Cell uptake studies were performed as previously described [24] using both ^{177}Lu -labelled cyclic DOTA-peptides, as well as ^{177}Lu -DOTA-cyclo-Met(O)-MG1 and ^{177}Lu -CP04. The DOTA-peptides were labelled with $^{177}\text{LuCl}_3$ in 0.05 N HCl ($\sim 50 \text{ GBq}/\mu\text{mol}$) using an equivalent volume of a 0.28 M sodium ascorbate buffer adjusted to pH 5 and incubation at 80°C for 5-25 min. To increase the radiochemical purity (RCP) also gentisic acid (0.007 M) reducing radiolysis and L-Met (0.07 M) limiting the oxidation of the Met residue in DOTA-cyclo-MG1 and CP04 were added to the buffer. For the assay A431-CCK2R and A431-mock

cells were seeded at a density of 1.0×10^6 cells per well in 6-well plates (Greiner Bio-One, Kremsmünster, Austria) and grown to confluency for 48 h. The cells were incubated in triplicates with $\sim 100,000$ cpm of the ^{177}Lu -labelled peptides at a concentration of 0.4 nM (~ 600 fmol of total peptide) and 2 h after incubation the cells were processed to obtain the internalized radioligand fraction. All fractions were counted in the gamma counter together with a standard and mean values were calculated. The internalized fraction was expressed in relation to the total activity added (% of total).

Stability in human serum and phosphate buffered saline

The chemical stability of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 (120 pmol/mL) was tested in phosphate buffered saline (PBS) at RT. This medium was used for dilution steps of the radioligands in the different assays. Additionally, the radioligands were incubated in human serum at 37°C. This experiment was performed to gain first insights on the stability against enzymatic degradation even though a higher degree of proteolytic digestion has to be expected *in vivo* [18]. At different time points up to 24 h after incubation a 100 μL sample was taken and analyzed by HPLC. Serum samples were treated with acetonitrile (1:1.5) to precipitate proteins, centrifuged (centrifuge 5424, Eppendorf AG, Germany) and diluted with water (1:1) before HPLC analysis. The degradation of the radioligands was evaluated based on the RCP after radiolabelling and the percentage of intact radiopeptide during incubation in the different media. The half-life in human serum was calculated using a first-order decay model (exponential regression using Microsoft Excel).

Kit formulation and radiolabelling with ^{177}Lu

A freeze-dried kit formulation was developed for both cyclic peptide conjugates to provide ready to use kits for the labelling with ^{177}Lu . The kit formulation was based on an ascorbic acid buffer (Sigma Aldrich, St. Louis, MO, catalogue no. 95212,) for pH adjustment [25]. Gentisic acid (Sigma Aldrich, catalogue no. 85707) and L-Met (Sigma Aldrich, catalogue no. M8439) were added to the kit composition to increase the RCP and decrease Met-oxidation [26]. The lyophilized kits were composed of 50 μg DOTA-cyclo-MG1 or DOTA-cyclo-MG2, 25 mg ascorbic acid, 5.2 mg L-Met and 0.53 mg gentisic acid. Radiolabelling was performed by addition of ~ 2 GBq $^{177}\text{LuCl}_3$ in 1 mL of diluted HCl and heating at 80°C for 15-25 min. RCP was monitored by HPLC. The stability of the kits during storage for up to 6 months, as well as the stability of the radiolabelled peptides for up to 48 h after preparation, was evaluated at different storage conditions. For isocratic HPLC analysis a Phenomenex Kinetex 5 μm C18 100 Å column (4.6×150 mm) with mobile phase of 28% acetonitrile/0.1% trifluoroacetic acid/water, flow rate of 1 mL/min, UV-detection at 215 nm and radiodetection was used.

Biodistribution and experimental radionuclide therapy

All animal studies were approved by the Hungarian authorities (NÉBIH, PEI/001/2073-6/2014) and carried out in compliance with the relevant European, national and institutional regulations.

The biodistribution profile of the ^{177}Lu -labelled cyclic MG analogues was evaluated in 5-6 week old BALB/c nude mice of both sexes xenografted with A431-CCK2R and A431-mock cells (left and right flank). For the induction of the tumour xenografts mice were injected with 2×10^6 A431-CCK2R and A431-mock cells/mouse and the tumours were allowed to grow for ~14 days reaching a tumour volume of ~0.2 g. The biodistribution of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 prepared from kit formulations was evaluated for different time points post injection (p.i.). For each time point groups of five animals were injected intravenously into the tail vein with 1 MBq of the ^{177}Lu -labelled cyclic DOTA-peptides corresponding to 20 pmol of peptide (50 GBq/ μmol). The different groups of animals were euthanized at 30 min, 4 h, 1 d, 2 d, 3 d and 7 d p.i. Different organs and tissues were dissected to measure the radioactivity in a gamma-counter and the injected activity per gram tissue (% IA/g) was calculated. Based on linear scaling of the % IA/g in different tissues between animals and humans a dose extrapolation to humans was calculated using OLINDA/EXM software to evaluate the activity for first patient studies.

The same animal model was used for an experimental radionuclide therapy study. We assumed that a 30% reduction of the tumour growth rate would be clinically significant and performed a power analysis for the study. Considering a standard deviation of 20% in the control group five animals per group were included in the study to detect a 30% reduction in the treated groups with a power of 80%. BALB/c nude mice from both sexes were subcutaneously injected with 1×10^6 A431-CCK2R and A431-mock cells (both flanks) at an age of 5-6 weeks and 17.5 ± 0.8 g body weight ($n=25$). After two weeks when the tumours had reached a tumour volume of ~70 mm³ (70.3 ± 9.0 mm³ for A431-CCK2R xenografts and 72.5 ± 8.6 mm³ for A431-mock xenografts) a single treatment of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 was administered using two different levels of intravenously injected radioactivity. The injected radioactivity was calculated based on dose extrapolation to humans and FDA guidelines for allometric scaling. For the experimental radionuclide therapy study a lower radioactivity amount of $^{177}\text{LuCl}_3$ was available and radiolabelling was carried out at a specific activity of 24 GBq/ μmol . Before starting the treatment mice were randomized to form three groups: (1) *control group* of five mice receiving no treatment (intravenous injection of physiological saline into the tail vein), (2) *15 MBq group* of five mice each injected with 15 MBq of ^{177}Lu -labelled DOTA-cyclo-MG1 or DOTA-cyclo-MG2 corresponding to 0.6 nmol of peptide and (3) *30 MBq group* of five mice each injected with 30 MBq of ^{177}Lu -labelled DOTA-cyclo-MG1 or DOTA-cyclo-MG2 corresponding to 1.2 nmol of peptide. Over a period of up to 5 weeks after treatment body weight and tumour volume were evaluated weekly. The tumour volume was calculated by measuring two dimensions with a digital calliper and calculated according to the equation $[\pi/6 \times (L \times W^2)]$, where L is the greatest dimension of the tumour and W is the dimension of the tumour in the perpendicular direction [27]. The specific growth rate (SGR) and tumour volume doubling time (TVDT) was calculated using the formulas $\text{SGR} = \ln(V_2/V_1)/(t_2-t_1)$ and $\text{TVDT} = \ln(2)/\text{mean SGR}$, where V_1 and V_2 are the tumour volumes on t_1 and t_2 , respectively (t_1 : week of treatment; t_2 : week when tumour volume was measured for the last time) [28]. Animals were eliminated from the study when tumours reached a size $>1,500$ mm³.

Toxicity evaluation

The toxicity of the treatment was evaluated in double-xenografted BALB/c nude mice. The animals were treated as described for the experimental radionuclide therapy study. In each week of the study period a venous blood sample of 0.1 mL was drawn from the superficial temporal vein, pricked by a lancet (up to eight samples per animal). Variations in the numbers of white blood cells (WBC) and platelets (PLT) were analyzed to evaluate medullary toxicity. Blood cell concentrations were determined by a BC-2800Vet automatic haematology analyzer (Mindray Medical International, Shenzhen, China). At different time points the ratio of the cell counts in relation to the basal values at the beginning of the study period was calculated.

Determination of creatinine and urea concentrations in serum was used to evaluate the renal toxicity. Kidney parameters were determined by a Lab-Analyse Practical biochemical semiautomatic analyzer (Orvostechnika Kft., Budapest, Hungary) using specific kits for creatinine and urea (Norma Diagnosztika Kft., Budapest, Hungary). Renal toxicity was evaluated comparing the values in the treated group with the control group.

Statistical analysis

Statistical analysis was based on the unpaired two-tailed Student's *t*-test at a significance level of $P=0.05$ and calculated using Origin software. A *P* value of less than 0.05 was considered as statistically significant.

Results

Receptor affinity and cell uptake

In the receptor binding studies a high affinity for CCK2R could be confirmed for DOTA-cyclo-MG1 and DOTA-cyclo-MG2 with IC₅₀ values of 2.54 ± 0.30 and 3.23 ± 0.91 nM, respectively. Similar values were found also for ^{nat}Lu-DOTA-cyclo-MG1 (2.22 ± 0.32 nM) and ^{nat}Lu-DOTA-cyclo-MG2 (2.85 ± 0.63 nM). DOTA-cyclo-Met(O)-MG1 containing the Met-sulfoxide showed a much lower affinity. An IC₅₀ value of 114 ± 49 nM was calculated for DOTA-cyclo-Met(O)-MG1 and of 163 ± 26 nM for ^{nat}Lu-DOTA-cyclo-Met(O)-MG1.

Both ¹⁷⁷Lu-DOTA-cyclo-MG1 and ¹⁷⁷Lu-DOTA-cyclo-MG2 showed a high receptor-specific internalization into A431-CCK2R cells. The internalized radioligand fraction after 2 h incubation was $15.2 \pm 2.6\%$ for ¹⁷⁷Lu-DOTA-cyclo-MG1 and $16.1 \pm 1.9\%$ for ¹⁷⁷Lu-DOTA-cyclo-MG2. With the control peptide ¹⁷⁷Lu-CP04 a somewhat higher cell uptake of $20.4 \pm 1.0\%$ was found, whereas ¹⁷⁷Lu-DOTA-cyclo-Met(O)-MG1 showed a very low uptake of $0.44 \pm 0.03\%$. The unspecific uptake in A431-mock cells remained well below 0.3% for all radioligands confirming the high receptor mediated uptake in the A431-CCK2R cell line expressing the human CCK2R. A summary of the cell uptake in A431-CCK2R and A431-mock cells of the different radioligands is shown in Fig. 1.

Radiolabelling and stability studies

Radiolabelling of both cyclic DOTA-peptides using the described labelling buffer containing sodium ascorbate, gentisic acid and L-Met resulted in high labelling yield and RCP. For

labelling of Met-containing DOTA-cyclo-MG1 the addition of L-Met to the labelling buffer and reduction of the incubation time at 80°C from 25 to 15 min allowed to reduce the formation of oxidized side products from 10-20% to <5%. Labelling of DOTA-cyclo-MG2 using an incubation time of 25 min resulted in a RCP of >99% and no difference could be observed using labelling buffers with and without addition of L-Met. Also ^{177}Lu -CP04 and ^{177}Lu -DOTA-cyclo-Met(O)-MG1 used for comparative internalization studies were obtained at high labelling yield and RCP.

The stability studies of the ^{177}Lu -labelled DOTA-peptides in PBS showed a higher chemical stability for ^{177}Lu -DOTA-cyclo-MG2. The RCP of ^{177}Lu -DOTA-cyclo-MG2 remained above 98% for up to 4 h, whereas ^{177}Lu -DOTA-cyclo-MG1 showed increasing Met-oxidation over time, resulting in a RCP of 86% after 4 h incubation. The enzymatic degradation in human serum showed a higher half-life for ^{177}Lu -DOTA-cyclo-MG1 (30.1 h), whereas ^{177}Lu -DOTA-cyclo-MG2 showed a somewhat lower half-life of 17.8 h. These half-lives are, however, not representative for the biological half-life *in vivo*. The stability in PBS and human serum of both ^{177}Lu -labelled cyclic DOTA-peptides over time is summarized in Suppl. Fig. S1.

Kit formulation and radiolabelling

^{177}Lu labelling of the kit formulations prepared with both cyclic MG compounds at specific activity of 58-63 GBq/ μmol resulted in labelling yields >99%. For standard radiolabelling with ^{177}Lu an incubation time of 80°C for 15 min was defined for DOTA-cyclo-MG1 kits, whereas for DOTA-cyclo-MG2 kits a longer incubation time of 25 min was used. Evaluation of long term stability during storage revealed a higher stability for DOTA-cyclo-MG2 kits as compared to DOTA-cyclo-MG1 kits (Fig. 2A). The mean RCP of ^{177}Lu -DOTA-cyclo-MG2 for the different time points after storage tested was $97.8 \pm 1.3\%$ (n=4). For ^{177}Lu -DOTA-cyclo-MG1 a much lower RCP ($89.4 \pm 7.9\%$; n=4) was observed. The degree of Met-oxidation reached values of 3.8-6.1%. After 6 months storage a RCP of less than 80% with 15.1% free ^{177}Lu and 4.3% Met-oxidation was found for DOTA-cyclo-MG1 kits. For all other storage time points the values of free ^{177}Lu remained well below 0.5% for both kit formulations. Also the stability after preparation was tested for ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 at different storage conditions (Fig. 2B). When stored at RT the RCP of ^{177}Lu -DOTA-cyclo-MG2 decreased from $97.8 \pm 1.3\%$ after preparation to $96.3 \pm 1.4\%$ at 24 h after preparation (n=4). For ^{177}Lu -DOTA-cyclo-MG1 a lower stability was observed with a RCP decreasing from $93.3 \pm 1.6\%$ after preparation to $89.8 \pm 2.8\%$ after 24 h (n=3). Only up to 4 h after preparation a RCP of >92% was reached with this radioligand. Freezer storage (-20°C) improved the RCP of ^{177}Lu -DOTA-cyclo-MG1 by ~10%. Consequently, a shelf-life of 6 months and a limit for the RCP of >95% together with an expiry of the labelled product 24 h after labelling was defined for DOTA-cyclo-MG2 kits. For DOTA-cyclo-MG1 kits a lower shelf-life of 3 months and a limit for the RCP of >92% together with an expiry of the labelled product of 4 hours after preparation was defined. Representative radiochromatograms at 4 h after preparation are shown in Suppl. Fig. S2.

Pharmacokinetic profile and therapy efficacy

The results of the biodistribution study performed with ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 are summarized in Fig. 3 showing the uptake in selected tissues (A431-CCK2R-xenograft, A431-mock xenograft, liver, small intestine, stomach, pancreas, spleen, kidneys, bone, lung) over time. In the other tissues analyzed (brain, heart, lung, lymph node, muscle, pancreas) the uptake of both radioligands was comparable to the very low uptake shown for blood and muscle. In most organs the uptake remained well below the uptake in the receptor-positive A431-CCK2R xenograft during the whole observation period. At early time points p.i. a somewhat higher uptake was observed for liver and intestine, especially for ^{177}Lu -DOTA-cyclo-MG2. The uptake in kidneys of both ^{177}Lu -labelled cyclic MG analogues was very low with mean tumour-to-kidney ratios in the order of 2.4-3.1 up to 1 d p.i.. The uptake in receptor expressing organs, stomach and pancreas, started from mean values of 1.09-1.11% IA/g and 0.51-0.52% IA/g at 30 min p.i. and slowly decreased over time. The uptake in bone showed a temporary increase to mean values of 0.41-0.82% IA/g at 1 d p.i.. The mean uptake in A431-CCK2R xenografts remained above the uptake in A431-mock xenografts up to 2 days and reached comparable values 3 days p.i.. At 30 min and 4 h p.i. a tumour uptake of $3.74 \pm 1.27\%$ and $3.50 \pm 0.78\%$ IA/g was found for ^{177}Lu -DOTA-cyclo-MG1 in A431-CCK2R xenografts. The values for ^{177}Lu -DOTA-cyclo-MG2 resulted to be $3.91 \pm 1.59\%$ and $3.72 \pm 1.23\%$ IA/g for the same time points. The uptake in A431-mock tumours was highest at 1 d p.i. showing values of $1.10 \pm 0.32\%$ IA/g for ^{177}Lu -DOTA-cyclo-MG1 and $1.05 \pm 0.12\%$ IA/g for ^{177}Lu -DOTA-cyclo-MG2. At this time point the uptake in A431-CCK2R xenografts was 2.40 ± 1.06 for ^{177}Lu -DOTA-cyclo-MG1 and 2.12 ± 0.97 for ^{177}Lu -DOTA-cyclo-MG2. Based on the dose extrapolation to humans four repeated administrations of 3,200-4,300 MBq were calculated for initial patient treatments allowing to observe a limit of $<27\text{Gy}$ for the cumulative kidney dose. By patient individual dosimetry the activity could however be increased accordingly.

Therefore, in the experimental radionuclide therapy study two different activity levels of 15 and 30 MBq were evaluated in double-xenografted BALB/c nude mice. By increasing the injected activity also the injected peptide amount resulted to be higher (0.6 and 1.2 nmol, respectively). From the tumour volumes measured at different time points after therapy the SGR and TVDT was calculated to evaluate the therapy efficacy of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2. In Fig. 4 treatment scheme, body weight and tumour growth curves of the receptor-positive and receptor-negative tumour xenografts are shown for the different treatment groups. A comparison of the calculated TVDT can be found in Suppl. Fig. S3. No statistically significant difference in tumour volume of A431-CCK2R and A431-mock tumours was found at the beginning of the treatment when considering all the different treatment groups ($P=0.3754$). The mean SGR in the control group and in the groups treated with ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 at the two different activity levels of 15 MBq and 30 MBq are summarized in Table 2. In the control group injected with physiological saline a very similar SGR was found for receptor-positive and receptor-negative xenografts. In comparison to the control group the mean SGR of A431-CCK2R xenografts of animals treated with 15 and 30 MBq of both radioligands was reduced by 41-44% and 60-62%, indicating a clinically significant therapeutic effect. The respective reduction in SGR in A431-mock xenografts was only 13-17% and 35-46%. The reduction of

tumour growth of A431-CCK2R xenografts in comparison to the control group was statistically significant in both activity levels of 15 MBq ($P<0.0001$ for ^{177}Lu -DOTA-cyclo-MG1; $P=0.0005$ for ^{177}Lu -DOTA-cyclo-MG2) and 30 MBq ($P<0.0001$ for ^{177}Lu -DOTA-cyclo-MG1; $P<0.0001$ for ^{177}Lu -DOTA-cyclo-MG2). The mean SGR of CCK2R-negative A431-mock xenografts was reduced to a lesser extent. Still the delay in tumour growth in comparison to the control group was statistically significant for both activity levels of 15 MBq ($P=0.0125$ for ^{177}Lu -DOTA-cyclo-MG1; $P=0.0311$ for ^{177}Lu -DOTA-cyclo-MG2) or 30 MBq ($P=0.0002$ for ^{177}Lu -DOTA-cyclo-MG1; $P<0.0001$ for ^{177}Lu -DOTA-cyclo-MG2). A statistically significant difference in tumour growth delay was also observed when comparing A431-CCK2R xenografts with A431-mock xenografts for animals treated with 15 MBq ($P=0.0002$ for ^{177}Lu -DOTA-cyclo-MG1; $P=0.0078$ for ^{177}Lu -DOTA-cyclo-MG2) or 30 MBq ($P=0.0005$ for ^{177}Lu -DOTA-cyclo-MG1; $P=0.0015$ for ^{177}Lu -DOTA-cyclo-MG2). This difference reflects the receptor-specific therapeutic effect. The TVDT of animals treated with ^{177}Lu -DOTA-cyclo-MG1 in comparison to the control group showed an increase by a factor of 1.8 in the low activity group and of 2.6 in the high activity group. The corresponding values for ^{177}Lu -DOTA-cyclo-MG2 were 1.7 and 2.5. In A431-mock tumours only an increase by a factor of 1.2 in comparison to the control group was found for both radioligands in the low activity group. In the high activity group this factor was 1.5 for ^{177}Lu -DOTA-cyclo-MG1 and 1.8 for ^{177}Lu -DOTA-cyclo-MG2. Starting from two weeks after treatment also a decrease in body weight was observed. At the end of the observation time the mean body weight of animals treated with 15 MBq of both radioligands was reduced by 8-10% in comparison to the control group, whereas in animals treated with 30 MBq this reduction was more pronounced (19-21%).

Treatment toxicity

In the weeks after cell inoculation and before starting the treatment the cell numbers remained within the reference values for WBC ($3.11\text{-}13.04\times 10^9/\text{L}$) and PLT ($558\text{-}1,454\times 10^9/\text{L}$). In the control group in line with repeated blood sampling a constant decline of the cell numbers over time was observed. At the end of the study period a reduction of 42.0-43.8% in WBC and of 53.8-73.1% in PLT compared to the basal concentrations before cell inoculation (week -2) was observed. In both control groups only in week 5 after treatment a PLT value below the lower reference limit ($334\text{-}549\times 10^9/\text{L}$) was found. Detailed graphs showing the variation in cell numbers during the study period are displayed in Fig. 5.

In the groups treated with 15 MBq of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 one week after treatment an evident drop in WBC and PLT counts corresponding to a reduction of 37.8-40.8% and 51.2-52.8% compared to the basal value was observed. The values remained, however, within the reference values. Only for PLT a value slightly below the lower limit of the reference value was observed in week 2 after treatment for ^{177}Lu -DOTA-cyclo-MG1 ($531\pm 26\times 10^9/\text{L}$) and in week 1 after treatment for ^{177}Lu -DOTA-cyclo-MG2 ($531\pm 45\times 10^9/\text{L}$). The corresponding decline in the control group one week after treatment was 9.6-11.6% for WBC and 12.8-20.1% for PLT.

In the groups treated with 30 MBq of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 a more drastic drop in WBC and PLT counts was observed. In week 1 after treatment the WBC value was reduced by 67.2-70.9% compared to the basal value and remained below the lower reference limit also in week 2 ($2.17\text{-}2.94 \times 10^9/\text{L}$). The PLT value showed a decline of 44.8-58.0% compared to the basal value and also remained below the reference values in week 1 and 2 after treatment for ^{177}Lu -DOTA-cyclo-MG1 ($486\text{-}530 \times 10^9/\text{L}$) and in week 2 and 3 for ^{177}Lu -DOTA-cyclo-MG2 ($524\text{-}549 \times 10^9/\text{L}$).

The biochemical parameters for kidney function are summarized in Table 3. The values remained well within the reference values of 0.1-0.5 mg/dL for creatinine and 17-38 mmol/L for urea for all study groups and for both radioligands until week 2 after treatment. In one of the control groups a slight increase above the upper limit of the reference value for creatinine (0.6 mg/dL) and urea (40 mmol/L) was observed in week 5 after treatment. A somewhat higher increase was observed in the groups treated with 15 MBq of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 with values above the upper limit of the reference value for creatinine in week 5 after treatment (0.7 mg/dL) for both radioligands and for urea beginning from week 3 for ^{177}Lu -DOTA-cyclo-MG1 (39-48 mmol/L) and from week 4 for ^{177}Lu -DOTA-cyclo-MG2 (40-44 mmol/L). Treatment at the higher activity level of 30 MBq influenced the kidney parameters to a higher extent. For the animals treated with ^{177}Lu -DOTA-cyclo-MG1 the creatinine levels with values of 0.6-0.8 mg/dL remained above the reference limit beginning from week 4 after treatment whereas the urea levels with values of 39-52 mmol/L were above the reference limit already beginning from week 2. For animals treated with ^{177}Lu -DOTA-cyclo-MG2 a similar increase in creatinine levels with values of 0.6-0.8 mg/dL was observed already in week 3 after treatment and a higher increase in urea levels (39-63 mmol/L) beginning from week 2. For both 30 MBq groups the values did not recover to levels within the reference values during the observed study period.

Discussion

Radiolabelled peptides for nuclear medicine applications are typically prepared on-site for individual patients. The preparation of the radiopharmaceutical shortly before administration is required especially when dealing with short-lived radionuclides and products with low stability. The development of freeze-dried kits containing the peptide analogue and suitable excipients for radiolabelling is considered a practical solution allowing an efficient, fast and reproducible radiolabelling procedure. In this work we have developed such a kit formulation for two DOTA-conjugated cyclic MG analogues. The addition of L-Met avoiding Met-oxidation and gentisic acid preventing radiolytic side reactions enabled ^{177}Lu -labelling with specific activities of $>55 \text{ GBq}/\mu\text{mol}$ and with high RCP ($>95\%$ for DOTA-cyclo-MG2 and $>92\%$ for DOTA-cyclo-MG1) suitable for clinical use. Monitoring of the stability of the kit formulations during storage and of the final product after radiolabelling revealed a better labelling performance for kits based on DOTA-cyclo-MG2 - mainly because of the substitution of Met with Nle. Kits with DOTA-cyclo-MG1 showed a higher degree of degradation over time as indicated by the drop in the labelling yield after 6 months storage. When considering the kit production of both peptides DOTA-cyclo-MG2 shows advantages in terms of stability and seems to be more promising for a future clinical

application. Still, both formulations were included in the preclinical evaluation *in vitro* and *in vivo* to evaluate possible differences between the two MG analogues.

To investigate if the cyclic constraint in the peptide sequence influences the receptor interaction we have performed competitive binding studies on A431-CCK2R cells. We could confirm a high receptor affinity in the nanomolar range for both cyclic DOTA-peptides and also for the respective complexes with ^{nat}Lu . A much lower affinity of >100 nM was found for DOTA-cyclo-Met(O)-MG1. Using an oxidative side product of ^{111}In -DOTA-MG11 for *in vitro* autoradiography on CCK2R-positive tumour sections a similar loss of receptor affinity has been reported previously [14]. Cell uptake studies performed with both ^{177}Lu -labelled cyclic DOTA-peptides on A431-CCK2R and A431-mock cells revealed a slightly lower receptor-specific cell uptake in comparison with the linear peptide analogue ^{177}Lu -CP04. This lower cell uptake is possibly related to the cyclic constraint introduced in the peptide sequence of DOTA-cyclo-MG1 and DOTA-cyclo-MG2 influencing the receptor interaction and cell uptake. With oxidized ^{177}Lu -DOTA-cyclo-Met(O)-MG1 almost no specific cell uptake ($<0.5\%$) was detectable.

The analysis of the chemical stability of the complex in PBS revealed a lower stability for ^{177}Lu -DOTA-cyclo-MG1 mainly due to oxidation of Met over time. We could show that Met-oxidation clearly reduces receptor affinity and abolishes cell uptake. Similar stability issues related to Met-oxidation were observed in the kit formulation. By substitution with Nle the formation of oxidative side products in the radiolabelling process was avoided and receptor affinity and cell uptake was retained. When incubating the ^{177}Lu -labelled cyclic MG analogues in human serum *in vitro* we found a somewhat higher stability for ^{177}Lu -DOTA-cyclo-MG1 in comparison with ^{177}Lu -DOTA-cyclo-MG2. Metabolic studies *in vivo*, taking into account the exposure to a broad variety of proteolytic enzymes during circulation and transit through different organs [17], have been shown to be more suitable for evaluating enzymatic degradation [18,22,29]. Recently, by investigating Nle-substitution for ^{177}Lu -CP04 *in vivo* a higher metabolic stability was observed for the Nle-containing peptide analogue [30]. For the above mentioned reasons, a kit formulation containing DOTA-cyclo-MG2 showing higher stability during storage and lower formation of side products in the radiolabelling process is clearly advantageous for further radiopharmaceutical development.

The biodistribution profile of the two radioligands in double-xenografted BALB/c nude mice was very similar. ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 showed a comparable and low unspecific uptake in most tissues. The obtained results are in agreement with previous studies performed with the ^{111}In -labelled cyclic MG analogues and confirm the promising pharmacokinetic properties [19,21]. Possibly due to its higher lipophilicity ^{177}Lu -DOTA-cyclo-MG2 showed a somewhat higher uptake in blood and liver at earlier time points p.i. when compared to ^{177}Lu -DOTA-cyclo-MG1. For the time point of 30 min p.i. a maximal tumour uptake of 5.18% IA/g and 5.99% IA/g was observed for ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2, whereas kidney uptake showed maximal values of 1.75 and 2.64% IA/g, respectively. The tumour-to-kidney ratio of the ^{177}Lu -labelled cyclic MG analogues with values of 2.9-3.1 at 4 h p.i. resulted to be clearly improved in comparison with ^{177}Lu -CP04 showing a tumour-to-kidney ratio of 1.6 in the same tumour model at this time point [30].

In the present work we report on a first experimental radionuclide therapy study in BALB/c nude mice bearing A431-CCK2R (CCK2R-positive) and A431-mock (CCK2R-negative) tumour xenografts. Our aim was to investigate the therapeutic effect and toxicological side effects of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 at two different activity levels. As a model of a CCK2R expressing tumours we have chosen A431-CCK2R cells stably transfected with human CCK2R, allowing direct comparison with the same cell line transfected with the empty vector alone. A high CCK2R expression has been confirmed for different tumour tissues and A431-CCK2R cells using real-time PCR, whereas A431-mock cells lack receptor expression [31,32]. Recently, a new xenograft model based on a human MTC cell line (MZ-CRC-1) showing a clearly improved tumour uptake has been first used for preclinical evaluation [30]. Still, the A431-CCK2R xenograft is a representative model for CCK2R expressing tumours. An important aspect of our study is that besides investigating the therapeutic effect in comparison with a control group treated with physiological saline also the receptor-specific effect was evaluated by direct comparison of tumour xenografts with and without CCK2R expression. Using a double-tumour xenograft model we were able to evaluate the receptor-mediated therapeutic effect in the same animal without the use of additional groups of animals for blocking experiments (co-injection of an excess of unlabelled peptide).

Based on the biodistribution studies and the dose extrapolation to humans we suggest to perform first therapy studies in humans using four repeated injections starting with an initial activity of ~3,700 MBq and to increase the injected activity in the subsequent treatments after patient-individual dosimetry. Following the FDA guidelines for allometric scaling we have calculated two activity levels of 15 and 30 MBq for the experimental radionuclide therapy study which correspond to a human equivalent activity of 3,700 and 7,400 MBq, respectively [33]. Also for peptide receptor radionuclide therapy (PRRT) with radiolabelled somatostatin analogues four therapy cycles with an injected activity of 7,400 MBq and intervals of several weeks between treatments are usually administered to patients [34]. According to the higher activity levels in comparison with biodistribution studies also the injected peptide mass used for the experimental radionuclide therapy study showed an increase by a factor of 30-60. Due to this limitation partial receptor blocking effects reducing the radiation dose delivered to the tumour xenografts cannot be totally excluded. For ^{111}In -CP04 studied in the same tumour model about 40% reduction in tumour uptake was observed when increasing the injected peptide mass by a factor of 10 [35]. A similar effect might have occurred in our therapy study and the results of the biodistribution study do not exactly depict the distribution of the radioligands in the therapy study.

In the control group injected with physiological saline a very similar SGR was found for receptor-positive and receptor-negative xenografts. In the treated groups a statistically significant reduction of tumour growth in comparison to the control group was observed for A431-CCK2R and A431-mock xenografts. However, a reduction of >30% in SGR which we defined as clinically meaningful was reached only in A431-CCK2R xenografts at both activity levels. The reduction in tumour growth of A431-CCK2R tumours was statistically significant when compared to A431-mock tumours. This difference reflects the receptor-specific therapeutic effect. The reduction of tumour growth observed for receptor-negative tumours could possibly be explained by unspecific effects such as tumour xenograft

perfusion, cross-irradiation from other organs and tissues and whole body irradiation. These effects specifically affect the results in preclinical models based on small animals. By only comparing the therapeutic effect with a control group injected with physiological saline the therapeutic effect would therefore possibly be overestimated. More importantly, partial receptor blocking effects connected with the higher peptide mass injected explain the lower difference found in the reduction of tumour volume between A431-CCK2R and A431-mock tumours, especially in the high activity group. When considering these effects, in the present study, still a statistically significant receptor-mediated therapeutic effect could be observed for the animals treated with 15 MBq of both radioligands. The prolongation of the TVDT of receptor-positive tumours was increased by 42-50% in relation to receptor-negative tumours. In animals treated with 30 MBq this increase was somewhat higher for ^{177}Lu -DOTA-cyclo-MG1 (73%) than for ^{177}Lu -DOTA-cyclo-MG2 (39%). In the groups of animals treated at higher radioactivity levels also a higher decrease in body weight was observed.

A transient medullary toxicity was observed in treated versus untreated animals. The decline in WBC and PLT counts in the 30 MBq group falling below the limit of the reference values for about two weeks was more evident in comparison with the 15 MBq group. However, both groups recovered to acceptable blood count levels within the end of the study period. A transient mild haematological toxicity is reported for about 50% of patients treated with ^{177}Lu -labelled somatostatin analogues. A similar toxicity could be expected also for the ^{177}Lu -labelled cyclic DOTA-peptides. The patients usually recover spontaneously from these side effects and do not need further supportive treatment [34]. The study period of up to five weeks after treatment was too short to analyze long-term renal toxicity. Still some minor effects on the biochemical parameters creatinine and urea in serum were measurable in the last weeks of the study period and were more pronounced in the high activity group. These effects beside to the radioactivity injected could be attributed also to the progressing tumour load of the animals. Toxicological studies in mice analyzing long-term renal toxicity related to renal accumulation of radioactivity are usually carried out in animals without tumours for a time period of several months and report a considerable elevation of serum creatinine and serum urea levels, especially in animals treated with high activity levels [36,37]. To reduce the renal accumulation of radiolabelled peptide analogues beside amino acid infusions also the infusion of gelatin-based plasma expanders has been suggested [16]. For the ^{111}In -labelled MG analogue CP04 by co-injection of Gelofusine in mice a 50% reduction of the renal uptake could be achieved [22]. For PRRT with radiolabelled somatostatin analogues fractionation of therapy is usually used to reduce renal toxicity. During the interval between treatments the kidney function and bone marrow are allowed to recover. Multiple injections are administered aiming at a cumulative absorbed curative dose of 60 Gy in the tumour, while maintaining the cumulative kidney dose to <27 Gy [35]. Given the high radiation doses which are generally accepted for stomach in radiation therapy, the receptor-specific uptake in stomach seems not to be a limiting factor for therapeutic application, as it decreases over time, and may therefore cause only minor unwanted side effects [38]. Still, a highly individualized treatment strategy needs to be applied in patients. Administration of radiolabelled MG analogues has to be performed by slow infusion over time and monitoring vital signs to limit pharmacological side effects such as transient episodes of hypertension, flushes, nausea and vomiting [39]. Besides accurate

staging of the disease including the evaluation of the receptor status of the tumour lesions and dosimetry assessing the tumour and organ doses, also other parameters such as pre-treatments, organ function and kidney protection have to be considered. When using a similar approach also for PRRT with ^{177}Lu -labelled cyclic MG analogues in CCK2R expressing tumours, a response to therapy or palliative effects on the tumour related symptoms can be expected in most of the patients without major signs of toxicity.

Conclusions

The freeze-dried kit formulation developed within this study allows the straightforward preparation of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 with high RCP acceptable for clinical use. Regarding shelf-life of the kits and expiry of the radiopharmaceutical after preparation ^{177}Lu -DOTA-cyclo-MG2 shows advantages over ^{177}Lu -DOTA-cyclo-MG1. In the preclinical evaluation both radioligands showed a high receptor-specific cell uptake in CCK2R expressing cells and very favourable biodistribution with low unspecific organ uptake. In the experimental radionuclide therapy study a clear receptor-specific therapeutic effect could be demonstrated for both radioligands. Therefore, also in patients a therapeutic response in terms of stabilization of the disease or partial remission could be expected after repetitive treatment cycles. All together the results confirm the promising properties of ^{177}Lu -labelled cyclic MG analogues for clinical translation and support further studies to introduce this new therapeutic approach for patients with advanced and disseminated CCK2R related tumours.

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Abbreviations

DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DTPA	diethylenetriaminepentaacetic acid
CCK2R	cholecystokinin/gastrin subtype 2 receptor
HPLC	high performance liquid chromatography
IA/g	injected activity per gram tissue
MTC	medullary thyroid carcinoma

MG	minigastrin
PLT	platelets
PRRT	peptide receptor radionuclide therapy
RCP	radiochemical purity
SCLC	small cell lung cancer
SGR	specific growth rate
TVDT	tumour volume doubling time
WBC	white blood cells

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Figure 1.

Cell uptake studies using A431-CCK2R and A431-mock cells after 2 h incubation of ^{177}Lu -DOTA-cyclo-MG1, ^{177}Lu -DOTA-cyclo-MG2 and ^{177}Lu -DOTA-cyclo-Met(O)-MG1 in comparison with ^{177}Lu -CP04.

Figure 2.

Stability of kit formulations containing DOTA-cyclo-MG1 and DOTA-cyclo-MG2 as analyzed by RP-HPLC: Radiochemical purity A) during storage of the kit formulations and B) after radiolabelling with storage at room temperature (RT) or in the freezer (-20°C).

Figure 3. Biodistribution of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 in A431-CCK2R and A431-mock tumour xenografted BALB/c nude mice over time. Values are expressed as % IA/g (means $\pm$ SD, n=5): A) A431-CCK2R-xenograft, B) A431-mock xenograft, C) liver, D) small intestine, E) stomach, F) spleen, G) kidneys, H) bone, I) blood and J) muscle; dotted line demarking the uptake in A431-CCK2R-xenografts.

Figure 4. Therapy efficacy of ^{177}Lu -DOTA-cyclo-MG1 and ^{177}Lu -DOTA-cyclo-MG2 in double-xenografted BALB/c nude mice: A) treatment scheme, B) body weight over time, tumour volume of A431-CCK2R and A431-mock xenografts in treated versus control groups over time: C) 15 MBq group and D) 30 MBq group.

Figure 5.

Blood analysis of A431-CCK2R and A431-mock tumour xenografted BALB/c nude mice treated with the ¹⁷⁷Lu-labelled cyclic MG analogues: white blood cell counts (WBC) for A) ¹⁷⁷Lu-DOTA-cyclo-MG1 and B) ¹⁷⁷Lu-DOTA-cyclo-MG2; platelet counts (PLT) for C) ¹⁷⁷Lu-DOTA-cyclo-MG1 and D) ¹⁷⁷Lu-DOTA-cyclo-MG2. Values are expressed as means $\pm$ SD (n=5); week -2 = cell inoculation; week 0 = week of treatment; dotted line demarcating the lower limit of the reference value.

Table 1

Amino acid sequences of human minigastrin (MG) and different MG analogues.

MG analogue	Amino acid sequence
human minigastrin	Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
MG0	D-Glu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
MG11	D-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
cyclo-MG1	$ \begin{array}{c} \gamma\text{-D-Glu} \text{-----} \text{D-Lys-Trp-Met-Asp-Phe-NH}_2 \\ \hspace{10em} \\ \text{Ala} \text{-----} \text{Tyr} \end{array} $
cyclo-MG2	$ \begin{array}{c} \gamma\text{-D-Glu} \text{-----} \text{D-Lys-Trp-Nle-Asp-Phe-NH}_2 \\ \hspace{10em} \\ \text{Ala} \text{-----} \text{Tyr} \end{array} $

Table 2

Mean specific growth rate (SGR) of A431-CCK2R and A431-mock tumour xenografts of mice in the control group and mice treated with two different activity levels of the ^{177}Lu -labelled cyclic MG analogues.

Group	Treatment	mean SGR	
		A431-CCK2R	A431-mock
Control group	Physiological saline	0.082±0.007	0.078±0.007
15 MBq group	^{177}Lu -DOTA-cyclo-MG1	0.046±0.002	0.065±0.006
	^{177}Lu -DOTA-cyclo-MG2	0.048±0.011	0.068±0.005
30 MBq group	^{177}Lu -DOTA-cyclo-MG1	0.031±0.006	0.051±0.006
	^{177}Lu -DOTA-cyclo-MG2	0.033±0.002	0.042±0.004

Table 3

Biochemical parameters for kidney function of A431-CCK2R and A431-mock tumour xenografted BALB/c nude mice treated with two levels of injected activity of the ^{177}Lu -labelled cyclic MG analogues in comparison with control groups. Values are expressed as means $\pm$ SD (n=5); week -2 = cell inoculation; week 0 = week of treatment.

^{177}Lu -DOTA-cyclo-MG1						
time [week]	Creatinine [mg/dL] (reference value 0.1-0.5)			Urea [mmol/L] (reference value 17-38)		
	control group	15 MBq group	30 MBq group	control group	15 MBq group	30 MBq group
-2	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	25 $\pm$ 2	31 $\pm$ 3	27 $\pm$ 5
-1	0.1 $\pm$ 0.05	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	27 $\pm$ 5	33 $\pm$ 4	28 $\pm$ 3
0	0.3 $\pm$ 0.1	0.3 $\pm$ 0.2	0.3 $\pm$ 0.2	37 $\pm$ 2	26 $\pm$ 2	32 $\pm$ 5
1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	32 $\pm$ 4	32 $\pm$ 3	29 $\pm$ 4
2	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	29 $\pm$ 2	33 $\pm$ 2	39 $^*\pm$ 2
3	0.3 $\pm$ 0.2	0.3 $\pm$ 0.2	0.5 $\pm$ 0.2	33 $\pm$ 2	39 $^*\pm$ 4	41 $^*\pm$ 3
4	0.4 $\pm$ 0.1	0.5 $\pm$ 0.1	0.6 $^*\pm$ 0.1	35 $\pm$ 3	45 $^*\pm$ 6	44 $^*\pm$ 3
5	0.6 $^*\pm$ 0.2	0.7 $^*\pm$ 0.3	0.8 $^*\pm$ 0.1	40 $^*\pm$ 2	48 $^*\pm$ 8	52 $^*\pm$ 5

^{177}Lu -DOTA-cyclo-MG2						
time [week]	Creatinine [mg/dL] (reference value 0.1-0.5)			Urea [mmol/L] (reference value 17-38)		
	control group	15 MBq group	30 MBq group	control group	15 MBq group	30 MBq group
-2	0.1 $\pm$ 0.05	0.1 $\pm$ 0.02	0.2 $\pm$ 0.1	27 $\pm$ 3	30 $\pm$ 4	26 $\pm$ 3
-1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2	25 $\pm$ 5	32 $\pm$ 2	29 $\pm$ 4
0	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	28 $\pm$ 7	25 $\pm$ 7	27 $\pm$ 2
1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	25 $\pm$ 4	27 $\pm$ 6	37 $\pm$ 3
2	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2	0.3 $\pm$ 0.2	33 $\pm$ 3	34 $\pm$ 3	39 $^*\pm$ 5
3	0.3 $\pm$ 0.2	0.2 $\pm$ 0.1	0.6 $^*\pm$ 0.1	28 $\pm$ 6	34 $\pm$ 5	46 $^*\pm$ 3
4	0.4 $\pm$ 0.1	0.5 $\pm$ 0.2	0.7 $^*\pm$ 0.3	32 $\pm$ 3	40 $^*\pm$ 4	59 $^*\pm$ 4
5	0.5 $\pm$ 0.2	0.7 $^*\pm$ 0.1	0.8 $^*\pm$ 0.2	35 $\pm$ 4	44 $^*\pm$ 7	63 $^*\pm$ 9

* Value above the reference limit of creatinine or urea