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Cholecystokinin-2 receptor targeting with radiolabeled peptides: current status and future directions

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Abstract

A wide variety of radiolabeled peptide analogs for specific targeting of cholecystokinin-2 receptors (CCK2R) has been developed in the last decades. Peptide probes based on the natural ligands minigastrin (MG) and cholecystokinin (CCK) have high potential for molecular imaging and targeted radiotherapy of different human tumors, such as medullary thyroid carcinoma (MTC) and small cell lung cancer (SCLC). MG analogs with high persistent uptake in CCK2R expressing tumors have been preferably used for the development of radiolabeled peptide analogs. The clinical translation of CCK2R targeting has been prevented due to high kidney uptake or low metabolic stability of the different radiopeptides developed. Great efforts in the radiopharmaceutical development have been undertaken to overcome these limitations. Various modifications in the linear peptide sequence of MG have been introduced mainly with the aim to reduce kidney retention. Furthermore, improved tumor uptake could be obtained by *in situ* stabilization of the radiopeptide against enzymatic degradation through co-injection of peptidase inhibitors. Recent developments focusing on the stabilization of the C-terminal receptor binding sequence (Trp-Met-Asp-Phe-NH₂) have led to new radiolabeled MG analogues with highly improved tumor uptake and tumor-to-kidney ratio. In this review, all the different aspects in the radiopharmaceutical development of CCK2R targeting peptide probes are covered, giving also an overview on the clinical investigations performed so far. The recent development of radiolabeled MG analogs, which are highly stabilized against enzymatic degradation *in vivo*, promises to have a high impact on the clinical management of patients with CCK2R expressing tumors in the near future.

Keywords

cholecystokinin-2 receptor; molecular imaging; targeted radiotherapy; gastrin; cholecystokinin; radiometals

1 Introduction

Cellular structures like antigens, membrane receptors and enzymes are intensively studied in terms of their suitability for early cancer detection and their potential for different treatment strategies [1–3]. Especially G protein-coupled receptors (GPCR), involved in the cell signal

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

transduction, are outstanding targets for novel anti-cancer drugs because these proteins are often overexpressed on the cell membrane of different cancer cells [2,4]. Their natural ligands like regulatory peptides are promising lead structures to develop new targeting agents for molecular imaging and targeted radiotherapy [5,6]. Molecular-based investigations allowing to target structures overexpressed from such malignancies are highly interesting for nuclear medicine applications. Here ionizing radiation is used to visualize the tumor or damage the cancer cells through targeting with radiolabeled peptide analogs derived from the natural ligands [7,8].

More than three decades ago somatostatin based ^{123}I -iodinated [Tyr^3]octreotide was the first radiolabeled peptide analog clinically investigated in patients suffering from neuroendocrine tumors (NETs) known to overexpress somatostatin receptors [9]. The introduction of the radionuclide iodine-123 into the structure of this somatostatin analog was achieved by a direct radiolabeling approach in which the radionuclide is covalently bound to a functional group of the molecule. This relatively easy labeling strategy has the major disadvantage that the formed covalent bond shows a high tendency of *in vivo* dehalogenation limiting the intended targeting effect [10,11]. Nowadays regulatory peptides are mainly labeled *via* an indirect approach, in which a bifunctional chelator is conjugated to the peptide sequence which can form complexes with different radiometals [11]. Such bifunctional chelators are divided into acyclic and macrocyclic chelators. The acyclic chelator diethylenetriaminepentaacetic acid (DTPA) and especially the macrocyclic chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) are two examples of bifunctional chelators, which have found their way into routine clinical use. In the last decades, special interest has arisen on combining the ability of a diagnostic and a therapeutic application in one radiopharmaceutical after labeling with a corresponding radiometal [12]. Such theranostic investigations had a high impact especially on the clinical management of NET patients. The clinical use of different somatostatin analogs, such as DTPA-octreotide (DTPA-OCT), DOTA-Tyr³-octreotide (DOTA-TOC) and DOTA-Tyr³-octreotate (DOTA-TATE), is well established in nuclear medicine. Radiolabeling of the peptide analogs with the β^+ emitter gallium-68 allows for tumor staging using high sensitive positron emission tomography (PET) [13,14]. The same peptide analog complexed with β^- emitting lutetium-177 or yttrium-90 [8,15] is used for peptide receptor radionuclide therapy (PRRT). This theranostic approach provides a significant advantage compared to other cancer treatments such as chemotherapy or radiation therapy as a preselection of patients who will benefit from PRRT is possible [12]. Great efforts have been undertaken to translate the successful clinical use of somatostatin analogs also to other regulatory peptides targeting alternative GPCRs. One of these intensively investigated GPCRs is the cholecystokinin-2 receptor (CCK2R). CCK2R are expressed at high incidence in various tumors like medullary thyroid carcinoma (MTC), small cell lung cancer (SCLC), astrocytoma, stromal ovarian and gastrointestinal stromal tumors (GIST). CCK2R are further expressed in about 20% of gastroenteropancreatic (GEP) tumors, in particular insulinomas, and less frequently in other tumors of neuroendocrine origin and with different histology [4,16–18].

The development of CCK2R targeting radiopeptides was driven mainly by the need to improve the medical care of MTC patients. The clinical management of thyroid cancer is of particularly high historical significance for nuclear medicine. Already more than 70 years

ago nuclear medicine images were acquired in patients suffering from thyroid disorders [12]. Since then molecular imaging played an important role in diagnosing and managing thyroid cancer. Scintigraphic imaging and radiotherapy based on the uptake of ^{99m}Tc -pertechnetate or radioiodine (iodine-123 and iodine-131) *via* the sodium iodide symporter is only possible in thyroid cancer subtypes originating from the follicular cells of the thyroid [19]. MTC originates from the parafollicular C cells, not presenting this symporter on the cell surface and therefore unable to uptake iodine and pertechnetate. Beside histopathology, calcitonin and carcinoembryonic antigen produced by MTC cells are used as serum markers for diagnosis and follow-up [20,21]. Due to the high frequency of local lymph node metastases and the risk to overlook them in preoperative neck ultrasonography, total thyroidectomy coupled with central lymph node dissection is often recommended as primary treatment [21–23]. However, about 50% of the patients show persistent or recurrent disease and in more than 10% of the patients distant metastases are present already at the time of diagnosis [20,24]. Chemotherapy and external beam radiation showed limited response rates in patients with advanced disease [23]. Two systemic therapy options approved by the European Medicines Agency and the Food and Drug Administration are currently available. Due to the significant toxicity associated with vandetanib and cabozantinib, the use of these tyrosine kinase inhibitors is indicated only in patients with progressive and symptomatic MTC with unresectable locally advanced or metastatic disease [20,21]. A lack of sensitive imaging modalities and adjuvant therapies exists for patients with persistent or recurrent disease. The combination of ultrasonography, computed tomography (CT), magnetic resonance imaging and bone scintigraphy allows the detection of tumor lesions in less than 50% of the patients with elevated postoperative calcitonin levels. PET/CT with 6- ^{18}F fluorolevodopa (^{18}F -DOPA) showing a detection rate of 70% is more useful to detect persistent or recurrent lesions and provides better results when compared to ^{18}F fluoro-2-deoxy-D-glucose (^{18}F -FDG) and ^{68}Ga -labeled somatostatin analogs [20,24]. Radiopeptides targeting CCK2R have a high potential to improve the diagnosis of patients suffering from MTC, but more importantly would enable targeted therapy in patients with advanced disease.

The CCK2R is a GPCR belonging to the cholecystokinin receptor (CCKR) family. CCKR are classified into two receptors subtypes, CCK1R and CCK2R. CCK1R are mainly expressed in the gallbladder and the pancreas and are involved in the stimulation of the pancreatic secretion and gallbladder contraction. CCK2R are found in the central nervous system and the stomach and mediate anxiety, gastric acid secretion and other physiological actions [25,26]. The endogenous ligands for CCKR are members of the regulatory peptide families of cholecystokinin (CCK) and gastrin. Both share the C-terminal sequence Trp-Met-Asp-Phe-NH₂, proven essential for receptor binding. The endogenous ligands differ in the position of a tyrosyl residue, which can be sulfated or nonsulfated. CCK1R mainly recognizes sulfated CCK, whereas CCK2R binds both CCK and gastrin and distinguishes less between nonsulfated and sulfated forms [26–29]. After ligand binding, the peptide-receptor complex is rapidly internalized therefore allowing specific targeting of CCK2R expressing tumors [30].

Over the last decades an intense radiopharmaceutical development has been undertaken to provide radiolabeled CCK or gastrin analogs with optimal properties for diagnostic and

therapeutic use in nuclear medicine [5,11, 30–32]. However, the clinical translation of this promising nuclear medicine application has been hampered by either high kidney uptake or low metabolic stability of the radiopeptides developed so far. Thus, the development of radiolabeled CCK2R targeting ligands and their preclinical and clinical evaluation is still ongoing. This review focuses on the previous success, major drawbacks and the recent progresses in the development of radiolabeled peptide probes suitable for diagnostic and therapeutic application in CCK2R expressing diseases.

2 Development of Radiolabeled Peptide Analogs Targeting the CCK2R

For *in vitro* evaluations, exploring the binding affinity of a new radioligand to its biological target, as well as *in vivo* biodistribution studies, investigating the tumor uptake in animal xenograft models, a suitable cell model is substantial. This cell model should express the corresponding receptor at a sufficient level ($>10^6$ binding sites/cell) and without high variations over time.

Additionally, rapid and endless growth of the cell line is of particular importance as well as the possibility to generate subcutaneous tumor xenografts in immunodeficient mice or rats [33]. In earlier studies investigating CCK2R targeting peptide analogs the human medullary thyroid carcinoma cell line TT, with relatively slow growth of tumor xenografts after subcutaneous injection in mice [34,35], or the rat pancreatic cell lines AR42J [36] and CA20948 [37], known to naturally express the rat CCK2R, were used. To avoid variable receptor expression, several groups started to work with transfected cell lines artificially overexpressing the CCK2R, such as human embryonic kidney 293 cells (HEK293) [38] or Chinese hamster ovary cells (CHO) [39] stably transfected with human CCK2R. Using the human epidermoid carcinoma cell line A431, yielding subcutaneous tumors with high efficiency, Aloj *et al.* developed a novel cell model for the evaluation of CCK2R targeting peptides. The cell line was transfected with the plasmid pCR3.1 containing the full coding sequence of human CCK2R (A431-CCK2R) as well as with the empty vector alone for negative control (A431-mock) [33,40].

The A431-CCK2R/A431-mock cell model seems to be a more adequate model for evaluating CCK2R binding peptide analogs, as divergent findings in AR42J versus A431-CCK2R cells with a cyclic minigastrin (MG) analog were related to possible differences in the binding domain of rat versus human CCK2R [41]. Recently, the human medullary carcinoma cell line MZ-CRC-1 has been used for the preclinical evaluation of radiolabeled CCK2R targeting peptides [42]. This human cell line was established from a metastatic site of a 43-year old female MTC patient. The experience with this cell line is very limited and comparative data performed by different research groups are still missing. For this reasons the preliminary results obtained so far are not included in this review.

Starting from the natural ligands displayed in table 1, a variety of CCK2R targeting radiopeptides was developed and preclinically evaluated. The amino acid sequences of the different peptide analogs developed are given in table 2, whereas in table 3 all clinical relevant radioligands are summarized. For successful application in molecular imaging and targeted radiotherapy radiolabeled peptides need to meet important prerequisites, such as

high receptor affinity, sufficient metabolic stability, as well as high and persistent tumor uptake together with low uptake in non-target tissue and preferential renal excretion [32].

The feasibility of CCK2R targeting was first shown using the 17 amino acid long peptide derivative gastrin-I labeled with iodine-131. In TT-xenografted female nude mice a tumor uptake of up to 9% injected activity per gram tissue (% IA/g) at 1 h post injection (p.i.) was reported for ^{131}I -gastrin-I giving high promise for the diagnostic and therapeutic use in patients [34]. Soon thereafter, the 13 amino acid long sequence of human MG as well as a peptide analog derived from the 8 amino acid long sequence of CCK-8, conjugated to the acyclic chelator DTPA or to the macrocyclic chelator DOTA suitable for labeling with trivalent radiometals, were developed [37,43]. It was expected that the use of a “residualizing radiolabel” leading to “trapping” of the radiometal-chelate in the cell after internalization may allow to enhance the retention of radioactivity in the target tissue as well as the tumor-to-background ratio [37,43]. When comparing ^{111}In -DTPA-MG and ^{111}In -DTPA-sCCK8 in TT-xenografted nude mice a similar tumor uptake of 5% IA/g at 1 h p.i. was observed. Due to the higher background activity of ^{111}In -DTPA-sCCK8 tumor visualization was superior for ^{111}In -DTPA-MG [33]. To increase the kinetic stability of DTPA complexes, leucine (Leu) in position 1 of the peptide sequence of DTPA-MG was substituted with D-glutamic acid (Glu) leading to DTPA-MG0. For both, ^{90}Y -DTPA-MG0 and ^{111}In -DTPA-MG0, a higher resistance towards transchelation was found in DTPA solution and human serum, suggesting an involvement of Glu in the complexation of the radiometal. Substitution with DGlu was connected with lower blood levels as well as decreased bone and liver uptake, whereas no influence on the tumor uptake could be observed [44]. The biodistribution of ^{111}In -DOTA-MG0 was further compared to sulfated ^{111}In -DOTA-sCCK8, known to show a higher tumor uptake when compared to the nonsulfated peptide. In female BALB/c nude mice xenografted with HEK293 cells stably transfected with CCK2R as well as the same cell line transfected with a splice variant of the receptor (CCK2i4svR), the radiopeptides showed a comparable tumor uptake (~3% IA/g at 24 h p.i.). However, the kidney retention of ^{111}In -DOTA-MG0 was remarkably higher (~30% versus ~3% IA/g at 24 h p.i.) [38].

The high kidney uptake of radiolabeled MG analogs was related to the pentaglutamate (penta-Glu) sequence in the linear peptide sequence. Preclinical studies in TT-xenografted female nu/nu mice showed that the kidney uptake of ^{111}In -DTPA-MG0 was efficiently reduced by up to 90% through co-injection of penta-Glu [45]. Also the commercially available gelatin-based plasma expander Gelofusine showed to be useful in this respect and allowed to reduce renal accumulation of ^{111}In -DTPA-MG0 by 45% in male Wistar rats [46]. Truncation of the penta-Glu sequence in ^{111}In -DOTA-MG11, led to significantly lower kidney uptake in comparison with ^{111}In -DOTA-MG0 (<0.4% versus 10% IA/g at 24 h p.i.), as studied in AR42J tumor-bearing Lewis rats. However, the depletion of the penta-Glu sequence was connected with a lower serum stability, indicating that the lower tumor uptake of ^{111}In -DOTA-MG11 (0.3% versus 0.6% IA/g observed with ^{111}In -DOTA-MG0 at 24 h p.i.) was related to lower metabolic stability [47]. Similar results were obtained with $^{99\text{m}}\text{Tc}$ -labeled peptide analogs based on MG or CCK [36,48–52]. The drawbacks related to high kidney retention or low metabolic stability hindered the development of a radiolabeled peptide analog suitable for therapeutic use. In the attempt to improve the pharmacokinetics

and tumor targeting profile a variety of peptide analogs with different modifications, such as the introduction of linkers or unnatural amino acids, modification of peptide bonds, as well as multimerization or cyclization of the peptide sequence was developed by different research groups [31,32,53–58]. With the major goal of developing a radiolabeled CCK2R targeting ligand for clinical use in PRRT nine European research groups from Athens, Basel, Freiburg, Innsbruck, Ljubljana, London, Naples, Nijmegen and Rotterdam evaluated a series of different peptide analogs within a research network funded by the European Cooperation in Science and Technology (COST BM0607: Targeted Radionuclide Therapy). The study included twelve DOTA-conjugated CCK2R targeting peptide analogs, which were shared between the groups to perform different steps of preclinical testing in side by side comparative studies under standardized conditions [41,59,60]. Besides comprising DOTA-MG0 and DOTA-MG11 for comparison, different peptide derivatives with several modifications, such as addition, elimination or substitution of amino acids, cyclisation or dimerization of the peptide sequence, were included in the study. The different amino acid modifications were mainly applied in the N-terminal part of the peptide sequence. In the CCK analog G-CCK-8, a glycine residue was introduced as a spacer in the peptide sequence of CCK-8. The same peptide analog was previously evaluated using the linear chelator DTPAGlu coupled to N-terminal Gly spacer through the carboxyl function of the side chain of Glu [48]. For APH070 DGlu in MG11 was replaced by the L-form of the amino acid and two histidine residues were introduced in the aim to reduce the peptide reabsorption in the kidneys [61]. Another attempt to decrease kidney retention, focused on the replacement of the penta-Glu sequence by different combinations of D-glutamine or DGlu residues in DOTA-PP-F6, DOTA-PP-F10, DOTA-PP-F11 and DOTA-PP-F16. These peptide analogs were chosen from a previous study investigating the influence of different nonionic spacers, such as PEG or D-amino acids like D-Ser and D-Gln, on the metabolic stability and pharmacokinetic profile. Especially hydrophilic uncharged Gln spacers were found to be most promising to improve pharmacokinetics. The reduction of negative charges led to decreased kidney uptake, whereas the increased metabolic stability was related to the adoption of a more stable secondary structure [58]. To improve the pharmacokinetic profile in terms of stability against enzymatic degradation, cyclisation of the linear sequence of MG11 was achieved by incorporating DGlu in position 1 through the γ -carboxylic group and replacing glycine with D-lysine in position 4 allowing the introduction of an internal amide bond leading to DOTA-cyclo-MG1 [56,57]. A few C-terminal substitutions focused on the replacement of oxidation sensitive methionine (Met) by homopropargylglycine (HPG) in the CCK-8 analog SA106, a peptide analog which is additionally modified with a sulfonate isostere of tyrosine sulfate (Phe(p-CH₂SO₃H)). The influence of the introduction of Phe(p-CH₂SO₃H) in combination with the exchange of Met for Nle or HPG on the CCK2R affinity and tumor targeting properties was studied previously [62,63]. In sargastrin, derived from human gastrin-I, as well as in MGD5, a dimeric form of MG11, Met was replaced by norleucine (Nle). The possibility of replacing Met in the C-terminal receptor-specific region by Nle was reported also earlier [43,47,56]. HPG and methoxinine (O-methyl-L-homoserine) are other surrogates of Met allowing to maintain CCK2R affinity [64], whereas for replacement by isoleucine, methionine-sulfoxide and methionine sulfone loss of receptor affinity was reported [47,65]. Some of the N-terminal modifications introduced in the series of peptide analogs studied led to an increased enzymatic stability as

well as increased tumor-to-kidney ratios in *in vivo* biodistribution studies performed in female BALB/c nude mice tumor xenografted with A431-CCK2R and A431-mock cells, as compared to ^{111}In -DOTA-MG11 or ^{111}In -DOTA-MG0. Three peptide derivatives, the dimeric peptide conjugate MGD5, the cyclic peptide DOTA-cyclo-MG1 and the linear peptide DOTA-PP-F11 with the penta-Glu moiety exchanged for the D-isomeric form, showed a favorable biodistribution profile with tumor values of ~10% IA/g at 1 h p.i. combined with improved tumor-to-kidney ratio [41,59,60]. For these three derivatives, however, different enzymatic cleavage sites have been reported, mainly in the receptor-specific C-terminal sequence. No intact radiopeptide was detectable in the blood and urine of female BALB/c mice intravenously injected with the ^{177}Lu -labeled peptide derivatives already at 10 min after injection [59].

^{111}In -DOTA-PP-F11 was selected for clinical translation and further preclinical evaluations of its pharmacology, pharmacokinetics and toxicology were carried out [66,67]. A kit formulation with two different doses of DOTA-PP-F11 (10 μg and 50 μg) for the straightforward preparation of ^{111}In -DOTA-PP-F11 in the clinical setting was developed. By special attention on reducing metal traces, such as copper, zinc or ferric ions, as well as addition of ascorbic acid and Met to suppress Met oxidation a high shelf life of the kit as well as a high radiochemical purity and stability of the radiolabeled peptide analog was obtained [66]. Furthermore, biodistribution and dosimetry studies, as well as an extended single dose toxicity study in rats, based on the microdosing concept described in the ICH guideline M3(R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals (CPMP/ICH/286/95), was performed. In these studies, a very low toxicity of DOTA-PP-F11 with a median lethal dose (LD50) 178.5 $\mu\text{g}/\text{kg}$ body weight and a no-observed-adverse-effect-level (NOAEL) of 89 $\mu\text{g}/\text{kg}$ body weight was confirmed. For calculating the human equivalent dose (HED) a safety factor of 10 was considered recommending a starting dose (MRSD) for a first-in-human clinical trial of 1.4 $\mu\text{g}/\text{kg}$. In the biodistribution studies performed with ^{111}In -DOTA-PP-F11 in male Swiss albino mice up to 72 h p.i., a rapid blood clearance of the radiopeptide and predominant uptake in CCK2R-positive stomach and in the kidneys as main route of excretion was observed. Dosimetric calculations were based on extrapolating animal to human data and assuming a radioactivity dose of 220 MBq predicting a whole body radiation dose of 10 mSv and identified the kidneys as the organ with the highest absorbed dose (0.124 mGy/MBq) [67]. Kidney uptake was efficiently reduced by co-injection of the plasma expander Gelofusine (~2% versus ~6% IA/g at 4 h p.i.) [67].

Further studies were carried out to investigate the influence of the stereochemistry of the N-terminal penta-Glu sequence of DOTA-PP-F11 on the enzymatic stability and pharmacokinetics. In circular dichroism spectroscopy experiments a type II reverse turn conformation was reported for DOTA-PP-F11, whereas a preferential α -helical conformation was adopted when replacing the six DGlu residues by LGlu residues (DOTA-PP-F11-L). In the biodistribution studies performed in AR42J xenografted male Lewis rats, in line with the results obtained in binding affinity and internalization studies, an up to 2-fold lower tumor uptake was reported for ^{111}In -labeled DOTA-PP-F11 compared to DOTA-PP-F11-L. However also the kidney retention of ^{111}In -DOTA-PP-F11 was 20-fold lower

leading to the assumption that not only the charge but also the secondary structure of the peptide plays an important role for the kidney uptake and retention [68].

Considering a possible therapeutic use, the influence on the tumor uptake of the injected peptide amount was evaluated for DOTA-PP-F11. Biodistribution studies in A431-CCK2R xenografted female BALB/c nude mice with ^{111}In -DOTA-PP-F11 at an injected peptide amount in the range of 0.05 to 15 nmol revealed saturation effects leading to decreased uptake in tumor xenografts and stomach with a peptide amount >0.15 nmol, whereas kidney uptake was not affected. The dosimetry calculations, considering a similar biodistribution profile and the molar activities achievable for radiolabeling of DOTA-PP-F11 with different therapeutic radiometals, pointed out that for larger xenografts multiple injections of ^{90}Y -DOTA-PP-F11 are preferable, whereas for smaller xenografts alpha emitting bismuth-213 seems preferable over beta minus emitting lutetium-177 and yttrium-90 [69]. No therapeutic preclinical study was performed with DOTA-PP-F11 radiolabeled with a therapeutic radionuclide. Such a study was performed only for two ^{177}Lu -labeled cyclic MG analogs in A431-CCK2R/A431-mock xenografted BALB/c nude mice of both sexes. The study included three groups: a control group receiving no treatment (injection of physiological saline), a low activity group receiving 15 MBq of the ^{177}Lu -labeled peptide analogs corresponding to 0.6 nmol peptide and a high activity group receiving 30 MBq corresponding to 1.2 nmol peptide. For both groups treated with the radiolabeled peptide analogs, a significantly reduced tumor growth was reported for A431-CCK2R xenografts, whereas in CCK2R-negative A431-mock xenografts tumor growth was reduced to a lesser extent in comparison with the control group receiving no treatment. The treatment was connected with a transient medullary toxicity as well as increased serum creatinine and urea levels [70]. In another study, the risk of nephrotoxicity was preclinically evaluated for different ^{111}In -labeled peptide analogs, including ^{111}In -DOTA-MG0, after injection of a rather high radioactivity of 40 MBq. An absorbed kidney dose of more than 40 Gy was connected with long-term nephrotoxicity [71]. No further preclinical studies analyzing the long-term toxicity of radiolabeled CCK2R targeting peptides were carried out.

For potential imaging studies with the diagnostic radiometals gallium-68, indium-111 and copper-64, PP-F11 was conjugated to the alternative macrocyclic chelators, 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), and 1-(1,3-carboxypropyl)-4,7-carboxymethyl-1,4,7-triazacyclononane (NODAGA). The biodistribution and imaging profile of all chelator/radionuclide combinations was evaluated in female SCID mice bearing A431-CCK2R tumor xenografts. With the ^{64}Cu -labeled peptide analogs, highest tumor uptake was observed. However, instability of the ^{64}Cu -labeled chelator complexes also led to high uptake in non-target tissue. Interestingly, the different chelators did not show a high influence on the biodistribution profile of ^{111}In - and ^{68}Ga -labeled PP-F11. From the radiopeptides studied ^{68}Ga -DOTA-PP-F11 seemed most promising for PET/CT imaging [72]. LC-MS studies on the complexes of DOTA-PP-F11 with the natural isotopes of gallium, yttrium and lutetium revealed that for the complexation of yttrium and lutetium three carboxylic groups of DOTA are involved, whereas two carboxylic groups are involved in the coordination of gallium [73].

The peptide sequence of DOTA-PP-F11 contains a Met residue in the C-terminal receptor binding sequence which poses the risk of oxidation during the radiolabeling process connected with loss of receptor affinity [46,74]. Substitution of Met by Nle, a commonly known concept to overcome this disadvantage [43,47,56], was applied in DOTA-PP-F11N in the aim to develop a chemically stabilized therapeutic agent. A lower susceptibility against enzymatic degradation was found *in vitro* for ^{177}Lu -DOTA-PP-F11N, when compared to ^{177}Lu -DOTA-MG11 and ^{177}Lu -DOTA-PP-F11. However, the biodistribution studies in female nude mice revealed a very similar tumor uptake of ~7% IA/g at 4 h p.i. for ^{177}Lu -DOTA-PP-F11 and ^{177}Lu -DOTA-PP-F11N in A431-CCK2R xenografts [42].

3 Clinical use of Radiolabeled CCK2R Targeting Peptide Analogs

The very first imaging study with a radiolabeled CCK2R targeting peptide analog was reported in 1998. This study was performed with ^{131}I -gastrin-I. Scintigraphic whole body imaging in a 71-year-old male patient with metastatic MTC, beside confirming the physiological uptake in CCK2R expressing tissues, such as stomach, pancreas and gallbladder, visualized mediastinal lymph node and lung metastases [34,43].

Soon thereafter, a chelator-based labeling approach with radiometals was developed allowing the use of indium-111 with superior physical imaging characteristics when compared to iodine-131. In analogy to ^{111}In -labeled DTPA-OCT (^{111}In -DTPA-OCT), finding increasing clinical use for the diagnosis of neuroendocrine tumors at the time, the linear chelator DTPA was introduced into the amino acid sequence of MG as well as a peptide analog derived from CCK-8. Scintigraphic imaging with ^{111}In -DTPA-MG visualized known metastatic lesions in two patients (lymph node, lung, liver and bone metastases) as well as an unknown liver lesion in a patient with occult disease, which could be confirmed by conventional CT imaging only four weeks later [43,75]. ^{111}In -DTPA-CCK was studied in seven MTC patients, from which only two had radiological evidence of metastases [37,76]. Only for one of these two patients some of the known liver lesions could be visualized, whereas the known lesions in liver and bone were missed in the second patient. Scintigraphy of the five patients with occult disease suggested a possible pathology in one additional patient. With both radiotracers physiological uptake in the CCK2R expressing stomach together with predominant renal excretion and minor intestinal activity was observed. The uptake of ^{111}In -DTPA-CCK in liver and kidneys was lower when compared to ^{111}In -DTPA-OCT [37,76]. However, a relatively high soft tissue accumulation was observed at 4 h p.i., leading to visualization of the breast and nipples. Thus, a better visualization of the lesions was possible at 24 and 48 h p.i. [76]. ^{111}In -DTPA-MG showed a lower liver uptake in comparison with ^{111}In -DTPA-OCT favoring the possible detection of liver metastases [43].

^{111}In -DTPA-MG0 with Leu in position 1 replaced by DGLu was studied in healthy volunteers in comparison with ^{111}In -DTPA-MG. Scintigraphic imaging with both radiotracers revealed main physiological uptake in stomach, together with minor uptake in gallbladder and breast tissue. Additional uptake occurred in the kidneys as main route of excretion together with transient bowel activity indicating additional biliary excretion [35,75,77]. In line with improved complex stability, scintigraphic imaging with ^{111}In -DTPA-MG0 confirmed a significantly lower liver and bone marrow uptake when compared to the

Leu or DLeu containing derivative [77]. The development of a chelator-based radiolabeling approach, allowing stable radiolabeling with different trivalent radiometals was an important step also towards the further development of CCK2R targeted PRRT.

In subsequent studies with ^{111}In -DTPA-MG0 a total of 45 MTC patients was examined [77,78]. In 23 patients with known metastatic MTC, already at 1 h p.i. all lesions known from conventional imaging modalities could be visualized. The tumor-to-background activity ratios increased over time. Thus, at 24 h p.i. the best contrast was obtained. In 20 of 22 patients with occult metastatic MTC at least one lesion was visualized, therefore reaching a very promising sensitivity of 91%. Furthermore, almost no physiological uptake occurred in liver and spleen, suggesting that the detection of metastatic lesions can be highly improved when compared to ^{111}In -DTPA-CCK, as well as somatostatin receptor scintigraphy with ^{111}In -DTPA-OCT.

Similar results were reported also in a consecutive study including 27 MTC patients [79]. In a subgroup of 19 patients undergoing both gastrin receptor scintigraphy with ^{111}In -DTPA-MG0 and somatostatin receptor scintigraphy with ^{111}In -DTPA-OCT, 94.2% of the lesions known from other imaging methods were detected with ^{111}In -DTPA-MG0 and 40.7% of the lesions were detected with ^{111}In -DTPA-OCT. Another subgroup of 26 patients was examined with ^{111}In -DTPA-MG0 and ^{18}F -FDG. In this subgroup, 87.3% of the lesions confirmed by two imaging methods were detected with ^{111}In -DTPA-MG0, 76.1% by CT and 67.2% by ^{18}F -FDG PET. Using the combination of ^{111}In -DTPA-MG0 and CT 96.7% of the lesions could be localized. In the subgroup of 6 patients with occult MTC only one unknown lesion could be detected with ^{111}In -DTPA-MG0. Using ^{111}In -DTPA-MG0 also patients with other tumor entities were examined, reporting CCK2R-specific uptake in the liver lesions of a patient with metastatic carcinoid as well as in a patient with grade III astrocytoma [77]. A broader study was carried out comparing gastrin and somatostatin receptor scintigraphy in patients with neuroendocrine tumors [80]. Sixty-one patients with histologically verified neuroendocrine tumors were included in the study, comprising 51 carcinoid tumors, 3 gastrinomas, 2 glucagonomas, 1 insulinoma, 3 paragangliomas, as well as 1 presumed pulmonary carcinoid which proved to be small cell lung cancer and was therefore excluded from the study. From the 60 patients evaluated, 54 had known metastatic disease, 5 recurrent disease and one no known metastases. In this study, an overall tumor-detection rate of 73.7% and 82.1% was reported for ^{111}In -DTPA-MG0 and ^{111}In -DTPA-OCT, respectively. Interestingly, in 6 of 11 patients showing no uptake of ^{111}In -DTPA-OCT, positive uptake was seen with ^{111}In -DTPA-MG0. In additional 6 of 49 patients with positive ^{111}In -DTPA-OCT uptake, a total of 18 additional lesions was visualized with ^{111}In -DTPA-MG0. Conventional imaging methods were used to confirm lesions that were detected only by ^{111}In -DTPA-MG0. When considering the number of tumor sites and degree of uptake, ^{111}In -DTPA-MG0 was superior to ^{111}In -DTPA-OCT in 21.7% of the patients. The authors, therefore, concluded that gastrin receptor scintigraphy should be performed in selected patients with neuroendocrine tumors showing equivocal or absent ^{111}In -DTPA-OCT uptake.

Based on the promising imaging results with ^{111}In -DTPA-MG0, an initial therapeutic study was carried out with ^{90}Y -DTPA-MG0 in eight patients with advanced metastatic MTC. The study was designed as dose escalation study with increasing activity dose levels. The first

group of patients received up to four injections of 1110 MBq/m² in intervals of 4–6 weeks. Subsequently, the activity injected was increased to 1480 MBq/m² and 1850 MBq/m² in the second and third group, respectively [77]. Treatment of the first three patients with 1110 MBq/m² was well tolerated with no signs of toxicity. Dose escalation in the subsequent three patients to 1480 MBq/m² was connected with transient hematologic toxicity (grade 3) in all three patients. One patient without signs of renal toxicity during treatment developed chronic renal failure requiring hemodialysis one year after treatment. In the group treated with 1850 MBq/m² only two patients were included. In one of these two patients transient hematologic toxicity (grade 3) was observed, whereas the second patient developed a combined hematologic (grade 4) and reversible renal toxicity (grade 1) followed by myelodysplastic syndrome and chronic myelomonocytic leukemia two years after treatment. In terms of therapeutic effect, in one patient a partial remission and in another patient a minor response was observed, four additional patients showed a stabilization of the disease lasting for 6 to 36 months [81]. Due to the observed severe nephrotoxic side effects, no further treatments were carried out with ⁹⁰Y-DTPA-MG0.

In seek of alternative radioligands, truncated DOTA-MG11 missing the penta-Glu sequence and displaying improved tumor-to-kidney ratios in preclinical animal studies [47], was therefore selected for further clinical studies. Furthermore, also a ^{99m}Tc-labeled MG0 derivative conjugated to an open chain tetraamine chelator (^{99m}Tc-Demogastrin-2) with most favorable tumor-to-nontarget ratios seemed promising for clinical translation [52]. In a comparative study investigating the diagnostic performance of ¹¹¹In-DOTA-MG11, ¹¹¹In-DOTA-CCK and ^{99m}Tc-Demogastrin-2 in the same six patients, ^{99m}Tc-Demogastrin-2 was clearly superior to the ¹¹¹In-labeled peptide analogs. Only with ^{99m}Tc-Demogastrin-2 all known tumor lesions as well as additional unknown lesions in neck (2 patients), brain (1 patient), bone (1 patient), and liver (1 patient) were visualized. From the lesions detected with ^{99m}Tc-Demogastrin-2, some of the neck and liver lesions were not visualized with ¹¹¹In-DOTA-MG11 (2 patients) and ¹¹¹In-DOTA-CCK (4 patients). The lower diagnostic performance of the ¹¹¹In-labeled peptides was attributed to suboptimal imaging characteristics of indium-111, lower injected radioactivity as well as lower stability against metabolic degradation [74,82]. The uptake of the DOTA-conjugated peptides in the tumor lesions was, however, too low to consider a potential application in PRRT. Similar disappointing results were observed with a ^{99m}Tc-labeled MG11 derivative conjugated to the monodentate ligand hydrazinonicotinic acid (^{99m}Tc-HYNIC-MG11). In a comparative study, the diagnostic performance of gastrin receptor scintigraphy with ^{99m}Tc-HYNIC-MG11 and somatostatin receptor scintigraphy with ^{99m}Tc-labeled HYNIC-TOC (^{99m}Tc-HYNIC-TOC) was evaluated in 30 MTC patients. Somatostatin receptor scintigraphy was positive in 20 patients (66.7%), whereas gastrin receptor scintigraphy was positive only in 11 patients (36.7%). The lower diagnostic performance of ^{99m}Tc-HYNIC-MG11 was attributed to possible oxidation of Met in the C-terminal receptor specific region during the heating step of the radiolabeling process leading to loss of receptor affinity. Physiological CCK2R related uptake in stomach was observed only in 19 of the 30 patients studied. However, a high intestinal tracer accumulation occurred in all patients potentially influencing the interpretation of the scans. Uptake in spleen, liver and kidneys was lower when compared to ^{99m}Tc-HYNIC-TOC [84].

The use of ^{68}Ga -labeled somatostatin analogs for PET/CT staging of neuroendocrine tumors greatly improved the staging and follow-up of neuroendocrine tumors. In a prospective study, including 84 patients with known or suspected neuroendocrine tumors, the diagnostic performance of ^{68}Ga -labeled DOTA-TOC (^{68}Ga -DOTA-TOC) PET was compared to conventional somatostatin receptor scintigraphy and CT. In this study ^{68}Ga -DOTA-TOC PET clearly showed a higher diagnostic efficacy compared to SPECT and CT [14]. In the attempt to use the improved detection rate of PET imaging also for CCK2R based receptor targeting, a first PET/CT imaging study with ^{68}Ga -DOTA-PP-F10 was performed in a 59-year-old female patient with metastatic MTC. The revealed receptor positive liver metastasis was in accordance with a previous ^{68}Ga -DOTA-TOC PET/CT. Standardized uptake values (SUVs) in the range of 0.5-1.0 were found for liver, lung and gluteus muscle, whereas the SUV for stomach mucosa was 3.1 [58]. Furthermore, a PET/CT imaging study with ^{68}Ga -DOTA-PP-F11 was performed in a 75-year-old male patient diagnosed with MTC by fine-needle aspiration biopsy [85]. Preoperative staging was additionally performed with the ^{68}Ga -labeled DOTA-TATE (^{68}Ga -DOTA-TATE) for comparison. The PET/CT images obtained with both PET tracers confirmed increased uptake in the right thyroid mass without signs of lymph node metastases. ^{68}Ga -DOTA-PP-F11 showed physiological uptake in the stomach, together with highly reduced uptake in liver, spleen and kidneys compared to ^{68}Ga -DOTA-TATE [85].

The intense preclinical research undertaken over the last years focused on the further optimization of the peptide sequence and led to new developments. Based on the promising preclinical results obtained with DOTA-PP-F11 and DOTA-PP-F11N, recently new clinical trials have been initiated. In a phase I multi-center study ^{111}In -DOTA-PP-F11 is currently investigated in patients with advanced MTC (ClinicalTrials.gov Identifier: NCT03246659) at two different peptide dose levels, 10 μg (as a safety step in first administrations) and 50 μg (suitable also for PRRT). The study goals include the evaluation of the safety of the intravenous administration and of the diagnostic potential to detect tumor lesions, as well as of the dosimetry to identify critical organs and of the co-administration of Gelofusine to reduce kidney retention [86,88]. Overall 16 patients were enrolled in the study, confirming the safe administration of 200 MBq ^{111}In -DOTA-PP-F11 at both peptide dose levels as well as a high sensitivity and specificity for the detection of unknown MTC lesions. Co-administration of the gelatin-based plasma expander Gelofusine resulted in a reduction of kidney retention and could therefore also be important for the future therapeutic assessment of ^{177}Lu -DOTA-PP-F11. In a phase 0 single-center proof of principle study (ClinicalTrials.gov Identifier: NCT02088645) 6 patients with advanced MTC received two administrations of 1 GBq ^{177}Lu -DOTA-PP-F11N with and without co-administration of Gelofusine, in a random cross-over order within 4 weeks [87]. The short infusion over 5 min of a peptide dose of ~ 100 μg was well tolerated with minor signs of toxicity, such as hypotension, flushing, hypokalemia. In four patients ^{177}Lu -DOTA-PP-F11N SPECT/CT could be compared to ^{18}F -DOPA PET/CT, a PET tracer with higher sensitivity and specificity in the characterization of MTC lesions when compared to ^{18}F -FDG PET [89]. With ^{177}Lu -DOTA-PP-F11N 41 of the 49 lesions identified with ^{18}F -DOPA could be visualized, confirming a good correlation of the two imaging methods. The dosimetry calculation revealed that with ^{177}Lu -DOTA-PP-F11N a lower tumor radiation dose (0.88 Gy/

GBq) is reached in comparison with ^{177}Lu -labeled DOTA-TATE (^{177}Lu -DOTA-TATE) in patients with neuroendocrine tumors [90]. The tumor accumulation seems, however, sufficiently high for a potential therapeutic application, as the median absorbed doses to kidneys (0.11 Gy/GBq) and bone marrow (0.028 Gy/GBq) were at least three times lower in comparison with ^{177}Lu -DOTA-TATE. The stomach was identified as the main dose-limiting organ (0.42 Gy/GBq) whereas co-administration of Gelofusine did not affect the tumor-to-kidney ratio. When combined with a renal-protective agent, treatment with ^{177}Lu -DOTA-TATE resulted to be associated with transient hematologic events, whereas no evidence of renal toxic effects occurred within the median duration of follow-up of 14 months [15]. A similarly low toxicity profile can be expected also for the treatment with ^{177}Lu -labeled CCK2R targeting peptides.

4 Ongoing Preclinical Investigations and Future Directions

When developing new radiopharmaceuticals based on regulatory peptides, different requirements have to be taken under consideration. The most important step at the beginning of any new development is to prove that the chosen target (e.g. a receptor or an enzyme) for the new radioligand is predominantly and highly overexpressed on the tumor tissue. A high incidence of CCK2R was reported for neuroendocrine tumors, in particular MTC (>90%), whereas only about 20% of gastroenteropancreatic (GEP) tumors express CCK2R. When investigating stromal tumors, all tissue samples of stromal ovarian cancers and ~50% of GIST expressed CCK2R. An incidence of about 50% was also found for SCLC and astrocytoma. A receptor density of >5000 dpm/mg tissue was found for MTC and GIST and also SCLC shows a high receptor density [16,18]. Therefore, these tumors seem most promising for CCK2R targeting. High receptor incidence and density, together with a rapid clearance of the radioligand from the blood and non-targeted tissues lays the basis for achieving sufficient target-to-non-target ratios [30]. These requirements are often fulfilled by regulatory peptide based radiopharmaceuticals acting as GPCR-agonists, which are internalized into the cell. Binding of a receptor agonist is followed by internalization of the peptide-receptor complex. After internalization, the receptor either undergoes degradation in the lysosome or is recycled to the cell surface. Radiometal-chelator conjugated amino acids formed by intralysosomal degradation of radiolabeled peptide analogs are not recognized by transport proteins and therefore cause accumulation and long-term retention of radioactivity in the target cells [75,77]. Internalization of the radiopeptide was assumed to be a basic requirement for successful diagnostic and therapeutic application [91]. Most of the radiolabeled CCK2R targeting peptide analogs developed are based on the natural ligands CCK and MG and are therefore agonists.

In the last years, several studies, especially focusing on somatostatin analogs, revealed that also non-internalizing receptor antagonists, due to their higher number of binding sites show a high potential to be used for nuclear medicine applications [92–94]. On the basis of benzodiazepines non-peptidic radioiodinated CCK2R antagonists haven been developed [95]. *Via* introduction of different spacers, the CCK2R antagonist Z-360 was conjugated to a chelating agent and radiolabeled with technetium-99m. Biodistribution studies in nu/nu mice xenografted with HEK293 cells stably transfected with CCK2R and CCK2i4svR revealed a high tumor uptake of 12% IA/g 4 h p.i., however, also the uptake in liver and kidneys was

comparably high [96]. The use of an antagonist would have the clear advantage of less side effects in comparison to the possible biological effects of an agonist. The administration of radiolabeled CCK2R targeting peptides acting as agonists can be associated with various side effects, including nausea, flushes, cough, sensations of pressure in the chest and abdomen, sensations of paresthesia in the extremities, dizziness, as well as temporary change in blood pressure and heart rate with no change of the ECG pattern. The effects reported were generally mild, lasted only a few minutes and resolved spontaneously without intervention. Slow injection of the radiopeptide is recommended to avoid such biological effects.

Not only the chelator, but the radionuclide too plays a crucial role in the pharmacological behavior of the radiolabeled peptide analog. Furthermore, the same chelator labeled with different radiometals can form metal chelates with varying charge and hydrophilicity influencing the biodistribution profile of the radioligand. Chelator and radiometal were shown to greatly affect the targeting properties of radiolabeled somatostatin antagonists [6]. For CCK2R targeting peptide analogs only a limited influence on the tumor uptake and tumor-to-kidney ratio was described, as exemplified by PP-F11 conjugated to different chelators and labeled with different radiometals [72].

The main limitation of the CCK2R targeting peptide analogs currently investigated in clinical trials remains their high sensitivity toward enzymatic degradation *in vivo* explaining the rather lower tumor radiation doses achievable with ^{177}Lu -DOTA-PP-F11 and ^{177}Lu -DOTA-PP-F11N. It seems therefore of particular importance to avoid fast degradation during blood circulation to allow that the radiopeptide can reach its target in an intact state. In preclinical investigations it was demonstrated for different peptide analogs, such as somatostatin, bombesin and gastrin analogs [32,97], that by increasing the *in vivo* stability and bioavailability of the radiopeptide through co-administration of enzyme inhibitors a highly improved targeting profile can be achieved. These findings give high promise that stabilization against enzymatic degradation may lead to higher diagnostic sensitivity as well as improved therapeutic efficacy. It has been shown in previous studies that angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP or neprilysin) and aminopeptidase A (APA) are major enzymes involved in the degradation of CCK and gastrin analogs [98,99]. Both, CCK and gastrin analogs, are potential substrates of ACE and NEP. However, MG analogs containing two or more Glu residues seem to be ACE resistant [32]. Radiolabeled peptide analogs with N-terminal conjugation of a chelator are protected against degradation by APA. Therefore, only the effect of ACE inhibition using lisiniprol (Lis) as well as NEP inhibition with phosphoramidon (PA) or thiorphan (TO) was evaluated for radiolabeled gastrin analogs [42,51,100,101]. With both NEP inhibitors a highly improved tumor targeting was achieved for ^{111}In -DOTA-MG11, which was higher for co-injection of PA versus TO, whereas no effect was observed for ACE inhibition with Lis [101]. Comparative studies comparing the effect of PA for three ^{111}In -labeled peptide analogs revealed that NEP inhibition was effective only for ^{111}In -DOTA-MG11 and resulted in 70% intact radiopeptide in the blood of male Swiss albino mice 5 min p.i. (versus <4% without PA). This stabilization also led to a significantly increased tumor uptake in xenografted male SCID mice with values of ~15% IA/g 4 h p.i. in AR42J xenografts and 10-16% IA/g in A431-CCK2R xenografts versus 2-3% IA/g without PA [101]. For ^{111}In -DOTA-MG0 and ^{111}In -

DOTA-PP-F11 containing the penta-Glu sequence only a minor effect on the *in vivo* stability (>85% intact radiopeptide versus 70% without PA) was observed. Still, the tumor uptake in A431-CCK2R xenografts increased from 8% to 16% IA/g at 4 h p.i. for ¹¹¹In-DOTA-PP-F11 and from 12% to 17% IA/g at 4 h p.i. for ¹¹¹In-DOTA-MG0, underlining that minor improvements in stability lead to highly improved targeting properties [100]. Similar results have been reported also for ^{99m}Tc-labeled gastrin analogs of different length [51]. When analyzing the susceptibility of ¹⁷⁷Lu-DOTA-PP-F11N, ¹⁷⁷Lu-DOTA-PP-F11 and ¹⁷⁷Lu-DOTA-MG11 against different extracellular and intracellular proteases *in vitro*, cleavage by NEP was confirmed for ¹⁷⁷Lu-DOTA-MG11 and ¹⁷⁷Lu-DOTA-PP-F11, whereas ¹⁷⁷Lu-DOTA-PP-F11N with replacement of Met by Nle was not susceptible to NEP. NEP inhibition with PA and TO *in vivo* was, however, effective only for ¹⁷⁷Lu-DOTA-MG11 leading to a highly improved targeting profile with more than 3-fold increased uptake in A431-CCK2R xenografts in female nude mice. For ¹⁷⁷Lu-DOTA-PP-F11 and ¹⁷⁷Lu-DOTA-PP-F11N only a slightly enhanced uptake was observed in A431-CCK2R xenografts, confirming the limited utility of enzyme inhibitors for the radiolabeled MG analogs currently investigated in clinical studies [42]. For PA no extensive toxicity studies are available and the clinically certified NEP inhibitors seem to show a lower effect on the targeting properties. Different issues such as solubility, safety, efficacy and potential route of administration still need to be addressed, hindering the clinical translation of this concept [100,101].

The goal to optimize CCK2R targeting seems more likely achievable through chemical stabilization of the radiolabeled peptide itself. The stabilization strategies of CCK2R targeting peptide analogs reported so far mainly focused on the introduction of stabilizing substitutions close to the N-terminus of the peptide, although enzymatic cleavage sites are also known within the C-terminal receptor binding sequence Trp-Met-Asp-Phe-NH₂ [42,59]. The different peptide derivatives developed still present enzymatic cleavage sites, thus a sufficient stabilization against enzymatic degradation seems to be achievable only by adequate stabilization of the receptor specific C-terminus. The development of a stabilization strategy for this particular region of the peptide sequence is challenging due to the risk of losing CCK2R affinity. An interesting approach directed to the stabilization of the peptide backbone is the amide-to-triazole substitution [102].

However, when comparing NEP-inhibition versus triazole substitution of different radiolabeled bombesin analogs, a higher *in vivo* stability of the radiopeptides was found for NEP-inhibition of the unsubstituted bombesin analog when compared to mono and bis-triazole-substituted analogs (88% intact radiopeptide versus 30-45% at 5 min p.i. in male Swiss albino mice). Biodistribution studies in SCID mice bearing human prostate adenocarcinoma PC-3 xenografts expressing the gastrin-releasing peptide receptor (GRPR) revealed a threefold higher tumor uptake for co-injection of PA in comparison to triazole-substitution (~25 versus ~8 %IA/g for h p.i.) [102]. Triazole substitution is currently investigated also for MG analogues with varying effects on receptor affinity and tumor uptake [104].

Based on evidence in the literature, that CCK analogs substituted within the C-terminal peptide sequence with unnatural amino acids containing bulky aromatic side chains or N-

methylated peptide bonds are highly resistant to enzymatic degradation [105], a new series of radiolabeled MG analogs has been developed recently. By applying specific amino acid substitutions, such as aromatic and N-methylated amino acids, within the receptor specific C-terminus of MG11 new radiopeptides with promising preclinical characteristics were developed [106–108] which are summarized in Table 4. Single substitution of phenylalanine (Phe) with 1-naphthylalanine (1-Nal) or N-methyl-phenylalanine ((N-Me)Phe) as well as additional substitution of Met with phenylglycine or N-methyl-norleucine ((N-Me)Nle) was investigated. Based on the preclinical investigations performed, including receptor affinity and cell uptake assays, as well as stability and biodistribution studies, specific amino acid substitutions were identified, that allow to retain receptor affinity. Furthermore it was found that two substitutions in the C-terminal region seemed to be required for efficient stabilization and improved tumor targeting [106]. For ^{111}In -DOTA-MGS4 showing replacement of Met by (N-Me)Nle and of Phe by (N-Me)Phe a promising *in vivo* stability (~80% intact radiopeptide 10 min p.i. in the blood of female BALB/c mice) as well as a high tumor uptake (~10% IA/g 4 h p.i. in A431-CCK2R xenografted female BALB/c nude mice) was observed. ^{111}In -DOTA-MGS4 therefore combines the high tumor uptake of ^{111}In -DOTA-MG0 with the reduced kidney uptake of ^{111}In -DOTA-MG11 leading to an improved tumor-to-kidney ratio of ~3. With ^{111}In -DOTA-MGS5 showing replacement of Met by (N-Me)Nle and of Phe by 1-Nal these promising results could be further improved. For ^{111}In -DOTA-MGS5 an unexpectedly high cell uptake was observed *in vitro* (~50% in A431-CCK2R cells after 2 h incubation). In addition, as illustrated in fig. 1, an improved *in vivo* stability was confirmed in the blood of female BALB/C mice injected with ^{111}In -DOTA-MGS5 (83% intact radiopeptide at 10 min p.i.; n=2). Similar results were obtained also for ^{177}Lu -DOTA-MGS5 (86% and 70% intact radiopeptide at 10 min and 1 h p.i., respectively) [107]. The combination of increased cell uptake and improved resistance against enzymatic degradation led to a further increase in tumor uptake independently of using indium-111, gallium-68 or lutetium-177 for labeling (19-25% IA/g in A431-CCK2R tumor xenografts, 1 h or 4 h p.i.).

A somewhat increased uptake was additionally observed in CCK2R expressing stomach and pancreas, underlining a receptor-specific effect [107]. Based on these promising results also two HYNIC-conjugated peptide conjugates labeled with technetium-99m were developed. Besides investigating HYNIC-conjugated MGS5, a second N-methylated peptide bond between aspartic acid and 1-Nal was introduced in HYNIC-MGS11 in the attempt to further stabilize the C-terminal region. $^{99\text{m}}\text{Tc}$ -HYNIC-MGS5 showed an *in vivo* stability (~70% intact radiopeptide in the blood of female BALB/c mice 10 min p.i.) and tumor uptake (~25% IA/g in A431-CCK2R/mock xenografted female BALB/c nude mice 1 h and 4 h p.i.) comparable to ^{111}In -DOTA-MGS5. The introduction of an additional N-methylated peptide bond in $^{99\text{m}}\text{Tc}$ -HYNIC-MGS11 led to a further increased resistance against enzymatic degradation (~95% intact radiopeptide) together with an almost doubled tumor uptake (~40% IA/g, 1 h and 4 h p.i.) when compared to $^{99\text{m}}\text{Tc}$ -HYNIC-MGS5. This increased tumor uptake was, however, also connected with increased kidney retention in comparison with $^{99\text{m}}\text{Tc}$ -HYNIC-MGS5 (~17% IA/g versus 8% IA/g at 4 h p.i.) resulting in comparable tumor-to-kidney ratios of 2-3 [108]. The new MG analogs clearly outperform the radiopeptides currently used in clinical trials. Of particular importance are the results

achieved with DOTA-MGS5 and HYNIC-MGS5 (see fig. 2). The two peptide analogs radiolabeled with different radiometals, indium-111 or technetium-99m for SPECT, gallium-68 for PET and lutetium-177 for PRRT, show a three-fold higher tumor uptake in comparison with ^{177}Lu -DOTA-PP-F11 and ^{177}Lu -DOTA-PP-F11N (20-24% IA/g versus ~7 in A431-CCK2R xenografted nude mice at 1-4 h p.i.) [42,107]. Furthermore, the tumor-to-kidney ratio with values of 6.5 for ^{177}Lu -DOTA-MGS5 at 4 h p.i. versus 1.2-1.6 reported for ^{177}Lu -DOTA-PP-F11 and ^{177}Lu -DOTA-PP-F11N is highly improved. DOTA-MGS5 therefore fulfils all important criteria for diagnostic and therapeutic use in nuclear medicine and might therefore be a powerful new peptide probe for the diagnosis and treatment of patients with CCK2R expressing tumors [109].

Conclusion

Over the past decades, a variety of CCK2R targeting peptide analogs has been developed for both diagnostic and therapeutic use in nuclear medicine applications. Based on first preclinical studies comparing radiopeptides derived from the natural ligands, MG and CCK, MG analogs were selected as most promising for further development. Kidney uptake was efficiently reduced by truncation of the penta-Glu sequence. In order to counteract the thereby caused high susceptibility towards enzymatic degradation *in vivo*, various stabilization strategies, such as the introduction of different linkers or unnatural amino acids, modification of peptide bonds, as well as multimerization or cyclization of the peptide sequence, were investigated. *In situ* stabilization with enzyme inhibitors as well as modification of the peptide backbone has shown to increase the bioavailability of MG analogs *in vivo*. Recent developments achieving radiolabeled MG analogs with highly improved stability against enzymatic degradation *in vivo* through site-specific modifications of the C-terminal receptor specific sequence Trp-Met-Asp-Phe-NH₂ promise a further improvement in tumor uptake while limiting the radiation dose delivered to the kidneys. Clinical studies investigating the diagnostic and therapeutic potential of DOTA-MGS5 radiolabeled with different radiometals, such as the use of ^{68}Ga -DOTA-MGS5 for high sensitivity PET/CT as well as PRRT with ^{177}Lu -DOTA-MGS5 in patients with progressive or metastatic MTC are warranted, to elucidate the potential of this new peptide analog in the clinical management of patients with CCK2R expressing tumors.

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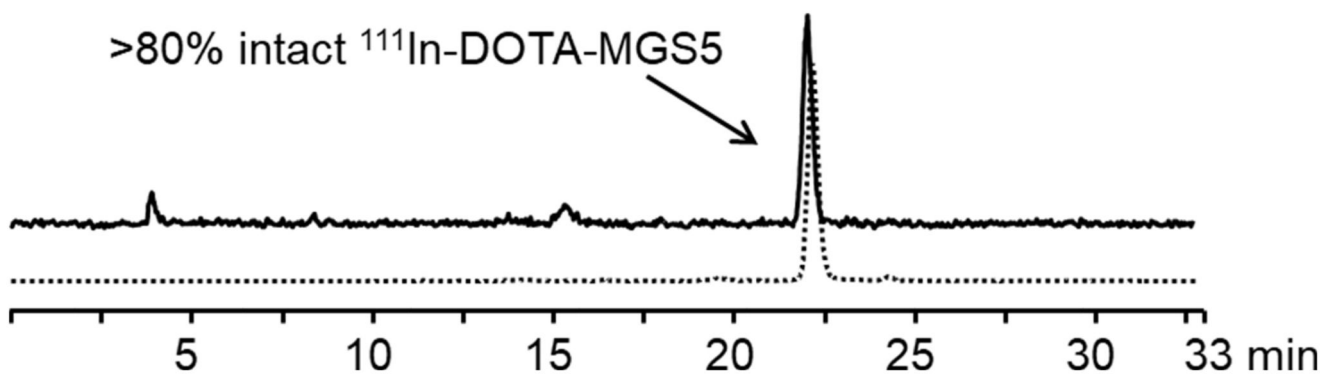


Fig. 1. Radio-HPLC analysis of the blood obtained from a BALB/c mouse 10 min after intravenous injection of ¹¹¹In-DOTA-MGS5 (~10 MBq, ~2 nmol); dashed line showing the radiochromatogram of the radioligand after preparation.

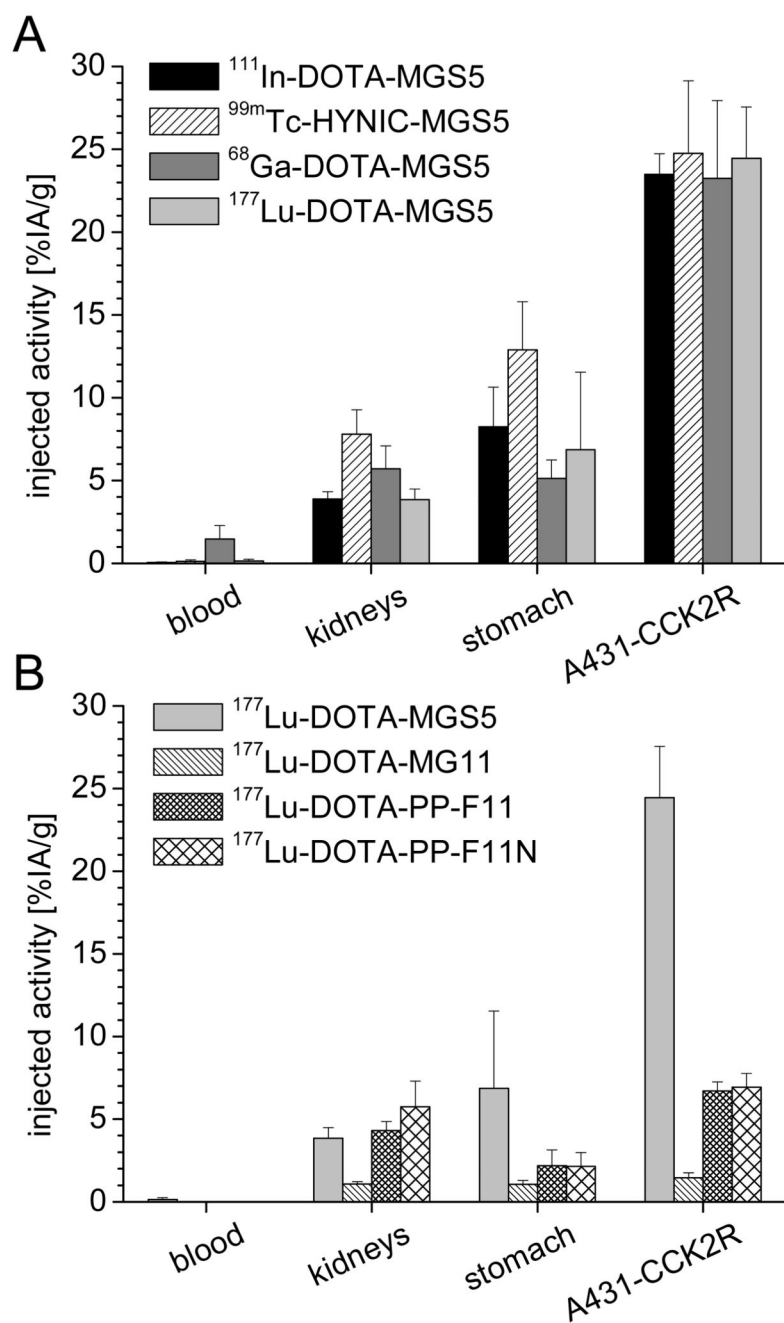


Fig. 2. Biodistribution profile in A431-CCK2R xenografted BALB/c nude mice of (A) DOTA-MGS5 radiolabeled with different radiometals ($^{111}\text{In}/^{177}\text{Lu}/^{99\text{m}}\text{Tc}$: 4 h p.i.; ^{68}Ga : 1 h p.i.) and (B) ^{177}Lu -DOTA-MGS5 in comparison with other radioligands at 4 h p.i. [42,107]; data are expressed as mean \pm SD.

Table 1
Amino acid sequence of the most important members of the gastrin/CCK family

Natural ligand	Amino acid sequence*
Gastrin-17 (gastrin-I)	Glp-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly- Trp-Met-Asp-Phe-NH₂
Gastrin-13 (minigastrin)	Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly- Trp-Met-Asp-Phe-NH₂
Cholecystokinin-8 (CCK-8)	Asp-Tyr-Met-Gly- Trp-Met-Asp-Phe-NH₂
Cholecystokinin-4 (CCK-4)	Trp-Met-Asp-Phe-NH₂

* bold letters = amino acids known to be involved in receptor binding

Table 2
Amino acid sequence of different CCK2R targeting peptide analogs suitable for labelling with radiometals

Peptide analog	Amino acid sequence
DTPA-minigastrin	DTPA-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-sCCK8	DOTA-Asp-Tyr(SO ₃ H)-Met-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-CCK	DOTA-DAsp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH ₂
DTPA-CCK	DTPA-DAsp-Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH ₂
DTPA-MG0	DTPA-DGlu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-MG0	DOTA-DGlu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-MG11	DOTA-DGlu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-G-CCK8	DOTA-Gly-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH ₂
SA106	DOTA-DAsp-Phe(<i>p</i> -CH ₂ SO ₃ H)-HPG-Gly-Trp-HPG-Asp-Phe-NH ₂ *
Sargastrin	DOTA-Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH ₂
APH070	DOTA-His-His-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-cyclo-MG1	DOTA-cyclo[γ -D-Glu-Ala-Tyr-D-Lys]-Trp-Met-Asp-Phe-NH ₂
DOTA-cyclo-MG2	DOTA-cyclo[γ -D-Glu-Ala-Tyr-D-Lys]-Trp-Nle-Asp-Phe-NH ₂
MGD5	DOTA-Gly-Ser-Cys-(Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH ₂) ₂
DOTA-PP-F6	DOTA-DGln-DGln-DGln-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-PP-F10	DOTA-DGln-DGln-DGln-DGln-DGln-DGln-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-PP-F16	DOTA-DGln-DGlu-DGln-DGlu-DGln-DGlu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-PP-F11/CP04	DOTA-DGlu-DGlu-DGlu-DGlu-DGlu-DGlu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-PP-F11-L	DOTA-Glu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH ₂
DOTA-PP-F11N	DOTA-DGlu-DGlu-DGlu-DGlu-DGlu-DGlu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH ₂

* HPG = homopropargylglycine

Table 3
Clinical studies with radiolabeled CCK2R targeting peptide derivatives in patients with MTC or other CCK2R expressing tumors

Peptide derivative	Radiionuclide	Radiopetide	Injected activity (MBq)	Injected peptide amount (µg)	Tumor type	Number of patients	Reference
gastrin-I	iodine-131	¹³¹ I-gastrin-I	370	0.5/kg BW	metastatic MTC	1	[34,43]
[DTPA ⁰]minigastrin	indium-111	¹¹¹ In-DTPA-MG	130-170	0.5/kg BW	metastatic MTC occult MTC	2 1	[43,75]
[DTPA ⁰ ,DAsp ¹ ,Nle ^{3,6}]CCK8	indium-111	¹¹¹ In-DTPA-CCK	158-211	7.2-8.5 1 x 18.7	metastatic MTC occult MTC	2 5	[37,76]
[DTPA ⁰ DGlu ¹]minigastrin	indium-111	¹¹¹ In-DTPA-MG0	185-259	5-10	metastatic MTC occult MTC metastatic carcinoid astrocytoma (grade III)	23 22 1 1	[77,78]
			150-200	5.5-7.3	metastatic MTC occult MTC	22 5	[79]
			150-200	5.5-7.3	neuroendocrine tumors	61	[80]
	yttrium-90	⁹⁰ Y-DTPA-MG0	1110-1850*	Not stated	advanced metastatic MTC	8	[77,78,81]
[DTPA ⁰ ,DAsp ¹ ,Nle ^{3,6}]CCK8 [DOTA ⁰ ,DGlu ¹ ,DesGlu ²⁻⁶]minigastrin [N ⁴ -Gly ⁰ ,DGlu ¹]minigastrin	indium-111 indium-111 technetium-99m	¹¹¹ In-DOTA-CCK ¹¹¹ In-DOTA-MG11 ^{99m} Tc-Demogastrin-2	216±19 186±34 773±97	11.1±0.6 10.8±1.7 9.5±1.5	metastatic MTC	6	[82]
			500-700	7-9	presurgical MTC postsurgical MTC	7 23	[83,84]
			347 MBq	~20	metastatic MTC	1	[58]
[HYNIC ⁰ ,DGlu ¹ ,DesGlu ²⁻⁶]minigastrin	technetium-99m	^{99m} Tc-HYNIC-MG11	Not stated	Not stated	presurgical MTC	1	[85]
[DOTA ⁰ ,DGlu ¹⁻⁶]minigastrin	gallium-68	⁶⁸ Ga-DOTA-PP-F10	200±20	10 or 50	advanced MTC	6	[86]
[DOTA ⁰ ,DGlu ¹⁻⁶]minigastrin	gallium-68	⁶⁸ Ga-DOTA-PP-F11	1000 [§]	<100	histologically proven MTC	16	[87]

* Up to 4 injections in intervals of 4-6 weeks (MBq/m²);

[§] two injections with/without Gefolofusine within 4 weeks

Table 4
Amino acid sequence of C-terminally stabilized CCK2R targeting peptide analogs
suitable for labeling with radiometals

Peptide derivative	Radionuclide	Radiopeptide
[DOTA ⁰ ,DGlu ¹ ,DesGlu ²⁻⁶ ,I-Nal ¹³]minigastrin	indium-111	¹¹¹ In-DOTA-MGS1
[DOTA ⁰ ,DGlu ¹ ,DesGlu ²⁻⁶ ,(N-Me)Nle ¹¹ , (N-Me)Phe ¹³]minigastrin	indium-111	¹¹¹ In-DOTA-MGS4
[DOTA ⁰ ,DGlu ¹ ,DesGlu ²⁻⁶ ,(N-Me)Nle ¹¹ ,I-Nal ¹³]minigastrin	gallium-68 indium-111 lutetium-177	⁶⁹ Ga-DOTA-MGS5 ¹¹¹ In-DOTA-MGS5 ¹⁷⁷ Lu-DOTA-MGS5
[HYNIC ⁰ ,DGlu ¹ ,DesGlu ²⁻⁶ ,(N-Me)Nle ¹¹ ,I-Nal ¹³]minigastrin	technetium-99m	^{99m} Tc-HYNIC-MGS5
[HYNIC ⁰ ,DGlu ¹ ,DesGlu ²⁻⁶ ,(N-Me)Nle ¹¹ ,(N-Me)I-Nal ¹³]minigastrin	technetium-99m	^{99m} Tc-HYNIC-MGS11