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Gastrin and cholecystokinin peptide-based radiopharmaceuticals: an *in vivo* and *in vitro* comparison‡

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The development of suitable radioligands for targeting CCK-2 receptor expressing tumors, such as medullary thyroid carcinoma, is of great clinical interest. In the search for the best CCK-2R binding peptides, we have synthesized, evaluated and compared the CCK8 peptide (Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe–NH_2) and two gastrin analogs commonly referred to as MG0 (DGlu-Glu(5)-Ala-Tyr-Gly-Trp-Met-Asp-Phe–NH_2) and MG11 (DGlu(1)-Ala-Tyr-Gly-Trp-Met-Asp-Phe–NH_2). The N-terminal portion of the three peptide sequences was derivatized by introducing the DTPAGlu or DOTA chelators to allow radiolobeling with ^{111}In(III) and ^{68}Ga(III), respectively. Saturation binding and cellular internalization experiments were performed on A431 cells overexpressing CCK2R (A431-CCK2R). All compounds showed Kd values in the nM range and were internalized with similar rates in CCK2 receptor overexpressing cells. Biodistribution experiments showed higher specific uptake of both MG0-based compounds compared to conjugates containing the CCK8 and MG11 peptide sequences. The higher retention levels of MG0-based peptides were associated with markedly elevated and undesired kidney uptake compared to the other compounds. Current indications suggest that the 5 Glu N-terminal residues while improving peptide stability and receptor-mediated tumor uptake cause unacceptably high kidney retention. Although displaying lower absolute tumor uptake values, the DOTA-coupled CCK8 peptide provided the best tumor to kidney uptake ratio and appears more suitable as lead compound for improvement of radiopharmaceutical properties. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: minigastrin and CCK8 peptide conjugates; Ga and In complexes; nuclear medicine

Introduction

A number of receptor systems are being evaluated in order to develop peptide-based radiopharmaceuticals for diagnostic and therapeutic purposes [1]. This field is of particular interest for applications in oncology. Many of these receptor systems have been shown to be overexpressed in particular types of human cancers. The naturally occurring ligands for these receptors are, in many instances, small peptides. A number of potential receptor systems that can be utilized as molecular targets are under investigation [2]. An excellent example of the successful clinical application of a peptide/receptor system has been the use of the radiolabeled somatostatin analog, octreotide, which has been utilized in a number of clinical studies to identify tumors and other pathological conditions in which specific subtypes of the somatostatin receptor are overexpressed [3]. This approach is also being extensively applied to the treatment of somatostatin receptor expressing tumors with peptides labeled with β-emitters such as ^{90}Y or ^{177}Lu [4].

We are evaluating the CCK family of receptors. Two major CCK-R subtype have been identified (CCK1R and CCK2R also referred to as CCKAR and CCKBR, respectively). The CCK2R is the gastrin receptor which promotes acid secretion in the fundal mucosa of the stomach. The CCK-R is present in a number of different tissues with rather selective expression of one or the other subtype. The CCK1R subtype is expressed in the pancreas where it has been found to be involved in enzyme secretion in pancreatic acini and insulin secretion from islet cells. It is also found in the gall bladder and other areas of smooth muscle in the gastrointestinal tract, in the gastric mucosa and in select areas of the central nervous system such as vagal afferent neurons. CCK2R is expressed throughout the

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Abbreviations used: *CCK-R, cholecystokinin receptors; OTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate; DOTA(tBu)3, 1,4,7,10-tetraazacyclododecane-1,4,7,10- tetraacetate tert-butyl ester; DOTATOC, DOTA-[Tyr3]-octreotide; DTPAGlu, N,N-bis[2-[bis[2-acetate]-amino]ethyl]-L-glutamic acid; DTPAGlu(tBu)5, N,N-bis[2-[bis[2-(1,1-dimethyletoxy)-2- oxoethyl]-amino]ethyl]-L-glutamic acid1-(1,1-dimethylethyl)ester; GEP, gastroenteropancreatic; HATU, 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3- tetramethyluronium hexafluorophosphate; MIP, maximum intensity projection; MTC, medullar thyroid cancer; PET, positron emission tomography; PBS, phosphate buffer solution; TIS, tris-isopropylsilane.*

central nervous system as well as by various cell types of gastric mucosa: parietal cells responsible for acid production; chief cells responsible of pepsinogen production and enterochromaffin-like cells producing histamine [5].

Overexpression of both of these receptor subtypes has been demonstrated in certain human tumors [6]. The CCK1R has been found to be overexpressed in a number of pancreatic adenocarcinomas as well as in cell lines derived from such tumors and to a smaller extent in GEP tumors. The CCK2R has been found to be overexpressed in medullary thyroid cancers, as well as in other tumors of neuroendocrine origin such as small cell lung cancers and GEP tumors. The CCK2R has been found on cell lines derived from medullary thyroid carcinoma, such as the human cell line TT.

There is particularly high incidence of CCK2R overexpression in certain neuroendocrine tumors such as medullary thyroid cancer (MTC, >90%) [6]. On this basis, development of CCK2R targeting radiopharmaceuticals has gained relevant interest. A number of CCK and gastrin derivatives, showing high affinity for the CCK2R, have been characterized over the past years for the purpose of *in vivo* receptor targeting. Among these, the C-terminal CCK octapeptide amide (CCK26–33 or CCK8) is capable of binding CCK2R and has been chosen by our group for the development of CCK2R targeting ligands. Our initial work has characterized the CCK8 peptide coupled to a DTPA derivative [7]. Behe and Behr have evaluated DTPA-coupled derivatives of minigastrin for CCK2R targeting [8–10]. Reubi described several DTPA- and DOTA-coupled CCK analogs [11] with high affinity for the CCK2R. One of these analogs DTPA [D-Asp26, Nle28,31]CCK(26–33) has been characterized in a rat tumor model [12] and in a clinical trial in patients with MTC [13]. Derivatives of minigastrin labeled with ^{99m}Tc have also recently been evaluated [14] and some clinical applications have also been described [15].

While specific targeting to CCK2R has been demonstrated with most compounds tested to date, there are a number of issues that may be improved for both imaging and therapeutic applications. The use of positron emitters for imaging applications would allow higher spatial resolution and sensitivity through the use of PET. Imaging of somatostatin receptor expressing tumors with DOTA-coupled octreotide derivatives such as DOTATOC labeled with the positron emitter ^{68}Ga appears more sensitive than ^{111}In-Octreoscan [16,17]. Furthermore, the quantitative nature of PET scanning may allow more accurate calculations of tumor and normal organ dosimetry in treatment planning. One major issue encountered in the development of these peptides is the elevated kidney accumulation that may cause toxicity problems, particularly in therapeutic applications. This appears to be a significant problem with DTPA-coupled minigastrin [18,19] although co-infusion of polyglutamic acid appears to greatly reduce kidney accumulation [20]. Kidney accumulation, however, appears much lower for DTPA-coupled CCK derivatives such as sulfated CCK8 [18] and DTPA[D-Asp26, Nle28,31]CCK (26–33) [12] and with DTPAGlu-G-CCK8 tested by our group [7]. Nevertheless, initial clinical trials utilizing ^{90}Y labeled minigastrin [8] have been carried out and have shown kidney and bone marrow toxicities.

When developing radiopharmaceuticals for tumor imaging and radionuclide therapy, one must find the best compromise between the highest possible uptake and retention in the target organ and the lowest possible uptake and retention in non-target tissues. In this work, we attempt to make a direct comparison using the same cellular binding assays and animal model between three known CCK2R binding peptide sequences, one derived from CCK (CCK8) and two derived from gastrin (MG0 and MG11). These peptide sequences were coupled to two different chelators: one is the DTPAGlu chelator which is used for ^{111}In labeling and for which we have previously reported *in vitro* and *in vivo* properties of DTPAGlu-G-CCK8 [7]. Peptides labeled in this manner can be utilized for gamma camera imaging applications. This chelating agent has one more negatively charged carboxylic function compared to DTPA commonly used in peptide labeling applications. The other is the DOTA chelator that is widely used for applications in PET using the short half-life positron emitter ^{68}Ga and for radionuclide therapy with β-emitting isotopes such as ^{90}Y or ^{177}Lu. The *in vitro* and *in vivo* properties of these six derivatives were compared in detail and the use of these compounds as radiopharmaceuticals is presented.

Materials and Methods

Peptide Conjugate Synthesis

Protected Nα-Fmoc-amino acid derivatives, coupling reagents and Rink amide MBHA resin were purchased from Inbios srl (Napoli, Italy) and Calbiochem-Novabiochem (Laüfelfingen, Switzerland). [DTPAGlu(tBu)$_5$] was prepared according to reported procedures [21]. [DOTA(tBu)$_3$] was purchased from Macrocyclics (Dallas, TX, USA). The synthesis of DTPAGlu-G-CCK8 has been previously reported [7]. All other chemicals were commercially available by Sigma–Aldrich (Bucks, Switzerland) and were used as received unless otherwise stated. Solid phase peptide synthesis was performed on a 433A Applied Biosystems automatic synthesizer (Foster City, CA, USA). Analytical RP-HPLC runs were carried out on a HP Agilent Series 1100 apparatus (Santa Clara, CA, USA) using a Phenomenex (Torrance, CA, USA) C18 column, 4.6 mm × 250 mm with a flow rate of 1.0 ml/min. For all the RP-HPLC procedures, the system solvent used was 0.1% TFA in water (A) and 0.1% TFA in CH_3CN (B). The column was eluted with two linear gradients at 1.0 ml/min flow rate from 5 to 70% B in 30 min followed by 70–95% B in 10 min. Preparative RP-HPLC was carried out on a Shimadzu 8A (Kyoto, Japan) apparatus equipped with an UV Shimadzu detector using a Phenomenex (Torrance, CA, USA) C4 column, 22 mm × 250 mm with a flow rate of 20 ml/min, eluted with a linear gradient of 0.1% TFA in water (A) and 0.1% TFA in CH_3CN (B) as described above at 20 ml/min flow rate. Mass spectral analyzes were carried out on MALDI-TOF Voyager-DE mass spectrometer Perseptive Biosystems (Framigham, MA, USA).

Peptide synthesis was carried out in solid phase under standard conditions using Fmoc strategy on Rink amide MBHA resin. After the assembling of the peptide sequences on the solid support, and Fmoc removal from the N-terminal residue, two equivalents of the full protected chelating agents [DTPAGlu(tBut)$_5$] or [DOTA(tBu)$_3$], preactivated with two equivalents of HATU and four equivalents of DIPEA, dissolved in DMF, were added to the vessels. The coupling time was 1 h under a N_2 stream at room temperature.

The peptide derivatives were cleaved from the solid support by suspending the resin in 10 ml TFA/TIS/H_2O (95.5/2/2.5) for 120 min. Free peptide derivatives were precipitated by adding cold ethyl ether. The crude compounds were purified by preparative RP-HPLC. Mass spectral analysis was carried out on MALDI-TOF. The desired compounds were obtained at HPLC purity higher than 95% and in a final yield between 30 and 40%. The mass and HPLC retention time are reported in Table 1.

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Table 1. Mass spectrometry and HPLC data of the studied minigastrins and CCK8 conjugates

Peptide conjugate	Sequence	Mass (amu)	Rt (min)
DOTA-G-CCK8	DOTA-Gly-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe–NH_2	$(m/z) = 1520$	17.9
DTPAGlu-G-CCK8	DTPAGlu-Gly-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe–NH_2	$(m/z) = 1566$	24.6
DOTA-MG11	DOTA-**DGlu**-Ala-Tyr-Gly-Trp-Met-Asp-Phe–NH_2	$(m/z) = 1401$	19.0
DTPAGlu-MG11	DTPAGlu-**DGlu**-Ala-Tyr-Gly-Trp-Met-Asp-Phe–NH_2	$(m/z) = 1485$	20.5
DOTA-MG0	DOTA-**DGlu**-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe–NH_2	$(m/z) = 2046$	21.1
DTPAGlu-MG0	DTPAGlu-**DGlu**-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe–NH_2	$(m/z) = 2130$	21.1

Radiolabeling

Indium-111 was purchased from Covidien (Petten, NL, USA) in the chemical form of $[^{111}In]Cl_3$ (740 MBq/ml). Gallium-68 was obtained from an in-house $^{68}Ge/^{68}Ga$ generator, based on TiO_2 phase (30 mCi, f-Con Pharma, Berlin). This type of $^{68}Ge/^{68}Ga$ radionuclide generators yields "ionic" $^{68}Ga^{3+}$ in 0.1 M HCl. This initial eluate was adsorbed onto a cation exchange resin (Bio-Rad AG 50 W-X8 <400 mesh) [22] and washed with a 80% $(CH_3)_2CO$–0.15 M HCl solution in order to remove possible impurities. Four hundred microliters of a 98% $(CH_3)_2CO$–0.05 M HCl solution was slowly flowed through the resin in order to recover the $^{68}Ga^{3+}$. This fraction was used directly for labeling.

All peptide conjugates were dissolved in H_2O containing 20% CH_3CN at a concentration of 0.1–1 mM for radiolabeling. All solutions were prepared by weight, and their molar concentrations measured by UV–vis spectrometry.

For labeling of the DTPAGlu-coupled conjugates with ^{111}In, the solution of $[^{111}In]Cl_3$ was buffered by adding 10 volumes of 0.3 M sodium citrate immediately prior to labeling. The peptide–conjugate solution was then mixed with 10 volumes of [^{111}In]citrate to a final concentration of peptide between 1 and 10 μM and incubated for at least 1 h at room temperature.

Gallium-68 labeling of DOTA-coupled conjugates was carried out in pure water, the processed activity was added to preheated water (3.8 ml) in an open standard glass vial containing 15 μl (10 μg) of DOTA-coupled peptide. Radiolabeling was performed in a heating block set at 124 °C for 10 min. The reaction mixture was passed through a small C18 cartridge (Phenomenex Strata-X Tubes, 30 mg). After washing the cartridge with 5 ml of H_2O, the ^{68}Ga-labeled peptide was recovered with 400 μl of pure ethanol, dissolved in 0.9% saline solution and sterilized by filtration through a 0.22 μm membrane filter. For all peptide conjugates, labeling efficiency was determined by silica gel-TLC using 0.3 M Na-citrate as eluent, or RP-HPLC which was performed on a Gilson 305 system (Gilson Inc. Middleton, WI, USA) with an analytical Vydac C_{18} column connected to a Beckman 170 radiodetector (Beckman Coulter Inc. Brea, CA, USA), using an H_2O/0.1% TFA and CH_3CN/0.1% TFA gradient (5–70% CH_3CN in 18 min, flow rate 0.9 ml/min).

Binding and Cellular Internalization Experiments

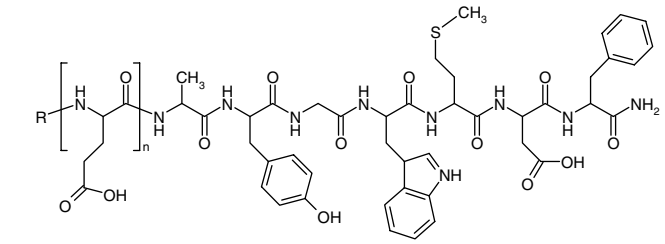
In vitro cellular assays were performed on A431 cells that had been stably transfected with the plasmid pCR3.1 containing the full coding sequence for the human CCK2R [7]. Cells were maintained in medium containing the neomycin analog G418 at a concentration of 500 μg/ml. Binding assays were performed on cells that had been plated at a density of 1–200 000 cells/well in 12-well multi-well plates 2 or 3 days prior to the experiments. These conditions allowed for the cells to be almost confluent at the time of the assay. To assess binding affinity of the different compounds, duplicate wells were incubated with serial dilutions of labeled conjugates in culture medium at 4 °C for 1 h, with concentrations ranging from 0.5 to 500 nM. To assess non-specific binding, 100–1000-fold excess unlabeled peptide was added to some of the wells. Cell-associated radioactivity was recovered from the wells by trypsinization after two rapid washes in ice-cold PBS. Radioactivity was then counted, normalized for the number of cells and non-specific binding was subtracted. Binding curves were analyzed using a Macintosh computer and Kaleidagraph software (Abelbeck Software, Version 3.0.5, distributed by Synergy Software, Reading, PA, USA). Dissociation constants (Kd) and apparent number of binding sites per cell (B_{max}) were derived by fitting the data to the following equation:

$$\text{Bound} = \frac{B_{max} \times [\text{peptide conjugate}]}{\text{Kd} + [\text{peptide conjugate}]}.$$

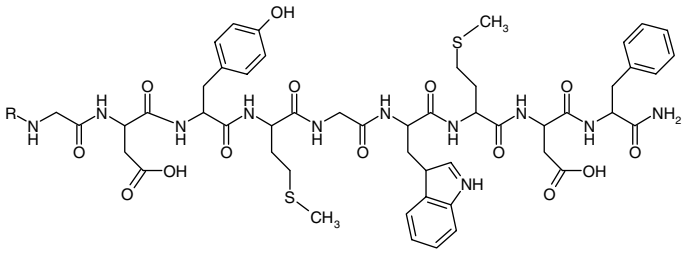
Cellular internalization was determined by comparing differences in accumulation of the compound incubated with A431-CCK2R cells at 4 °C, temperature at which most metabolic processes are blocked, and at 37 °C, where all metabolic processes including receptor internalization are active. Cells were incubated with a fixed concentration of the peptide conjugate (15 nM in culture medium) at the two temperatures, for 60 and 120 min. After 120 min of incubation, some wells were rinsed with PBS to remove unbound radioactivity and were subsequently incubated for an additional hour at 4 °C with 15 μM unlabeled peptide, in order to displace any surface-bound radioactivity. Radioactivity associated to cells was determined after washing away unbound radioactivity and subsequently normalized for protein content.

Biodistribution and Imaging Experiments in Mice

Mice bearing xenografts of A431-CCK2R and A431-control cells were generated by injecting 100 μl of the respective cell suspensions at a density of 2×10^7/ml in PBS in opposite flanks of 6-week-old CD-1 nude mice (weight 17–23 g). Tumors were allowed to grow for 10–14 days. Final tumor weight was between 0.5 and 1 g at the time of the biodistribution and imaging experiments. Tissue and tumor distribution of the radiolabeled compounds was determined 1 h following tail vein injection of labeled peptide in the lateral tail vein ($n \geq 5$ animals per compound, 0.5–2 nmol peptide). Blood, lungs, liver, spleen, kidneys, stomach, intestines, muscle and xenograft samples were weighed and radioactivity determined in the gamma counter. Dilutions of the injected compound were simultaneously counted for accurate determination of the injected dose. The relative amount of radioactivity in the organs was calculated and expressed as percentage of the injected dose/gram tissue (%ID/g) normalized

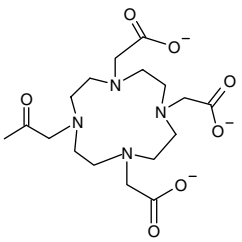


MG11n = 1
MG0n = 5

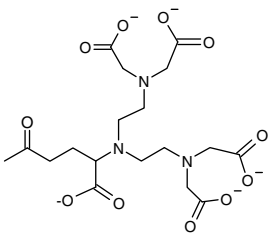


G-CCK8

R =



DOTA



DTPAGlu

Figure 1. Structural formula of the peptide conjugates being characterized.

to a 20-g mouse. Imaging of ^{68}Ga distribution was performed in some animals using a SIEMENS ECAT47 clinical PET scanner (Siemens, Knoxville, TN) in 2D mode with measured attenuation correction (Siemens, Erlangen, Germany) in order to visualize distribution in CCK2R positive tissues (one bed position, 5-min scanning time).

Results and Discussion

Peptide Conjugate Synthesis

The peptide conjugates are based on native bioactive peptide sequences modified on the *N*-terminus to link the chelating agents. The amino acid sequences of the three different peptides and the chemical structures of the two chelating agents are reported in Figure 1. Previous studies have demonstrated that modifications on peptide *N*-terminal functions do not affect the receptor binding properties of the bioactive peptides [23]. The selected chelating agents, DOTA and DTPAGlu, are capable of coordinating Ga^{3+} and In^{3+} ions, respectively, with high affinity. DTPAGlu has one more carboxylic function compared to the more frequently utilized DTPA chelator. This feature improves stability of the indium complex and increases the negative charge of the conjugate. Therefore, peculiar biodistribution properties of DTPAGlu-coupled peptides are expected relative to DTPA conjugated compounds.

The peptide conjugate syntheses were performed by solid phase with Fmoc strategy following well established procedures. The conjugates, characterized by mass spectrometry for identity and HPLC for their purity, were obtained in high yields and high purity.

All compounds showed rapid and efficient labeling which was in all cases above 95% based on the quality control performed at the end of labeling. Figure 2 shows a typical radioactivity trace from the labeling of DOTA-G-CCK8 with ^{68}Ga (t_R = 12 min.).

Figure 3(A) shows results of the binding assays performed at 4 °C on A431 cells overexpressing the CCK2R with the DOTA-coupled peptides. The saturation curves show high affinity ligand–receptor interaction and saturable binding with dissociation constants (Kd) in the order of 10^{-8} M. Affinity measured for the DTPAGlu-coupled peptides was very similar with DTPAGlu-MG0 showing a Kd of 25 ± 5 nM (mean ± SD) and DTPAGlu-MG11 yielding 30 ± 9 nM. The Kd value for DTPAGlu-G-CCK8 in this same cell culture system is 23 ± 4 nM [7]. Non-specific binding, as assessed by incubation of the compound with non-receptor expressing cells or in receptor expressing cells in the presence of 100-fold excess unlabeled peptide, was very low for all compounds (not shown).

To evaluate if the peptides were being internalized by the cells, we performed parallel experiments in which A431-CCK2R cells were incubated with 15 nM labeled peptide at 4 °C (internalization blocked) or 37 °C (internalization active, Figure 3(B)). All compounds tested show progressive accumulation

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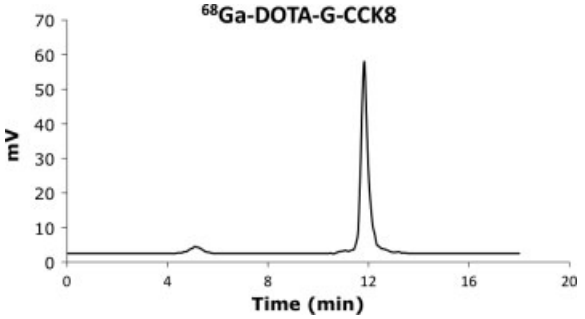

Figure 2. Radioactive trace of HPLC analysis of ^{68}Ga-DOTA-G-CCK8 (t_R = 12 min). Negligible amounts of free ^{68}Ga metal are detectable.

of radiolabel in cells incubated at 37 °C with labeled peptide alone, whereas, the levels of cell-associated radioactivity are constant in cells incubated at 4 °C for 60 and 120 min, suggesting that for all these compounds internalization is occurring to some extent at 37 °C. Co-incubation with excess cold peptide produced very low levels of cell-associated activity confirming specificity of interaction. After incubation for 120 min with labeled peptide alone, cells were incubated with excess cold peptide for 60 min at 4 °C. For all peptides, a large portion (80–95%) of the cell-associated radioactivity, was displaced by addition of the respective unlabeled peptide in cells previously incubated at 4 °C whereas a small amount (20–30%) was displaced in cells previously incubated at 37 °C, suggesting that the majority of the labeled peptide that is associated to cells has been internalized in cells incubated at 37 °C. Data for the DTPAGlu-coupled peptides is not shown.

Binding and internalization properties are very similar for the six compounds being compared and in agreement with the findings of other groups that have previously reported on similar compounds of this class [24]. These properties alone do not allow to single-out any particular compound which has more favorable characteristics.

The data obtained in the biodistribution experiments is displayed in Figure 4. Specific targeting of the A431-CCK2R xenografts is evident for all the compounds being compared as higher values of accumulation are observed in these tumors compared to the control xenografts. The two Glu(5)-minigastrin derivatives show the highest accumulation in the CCK2R positive xenografts (2.9%ID/g for the ^{111}In labeled DTPAGlu-coupled derivative and 3.1%ID/g for the ^{68}Ga labeled DOTA-coupled compound). The higher target tumor accumulation of these two

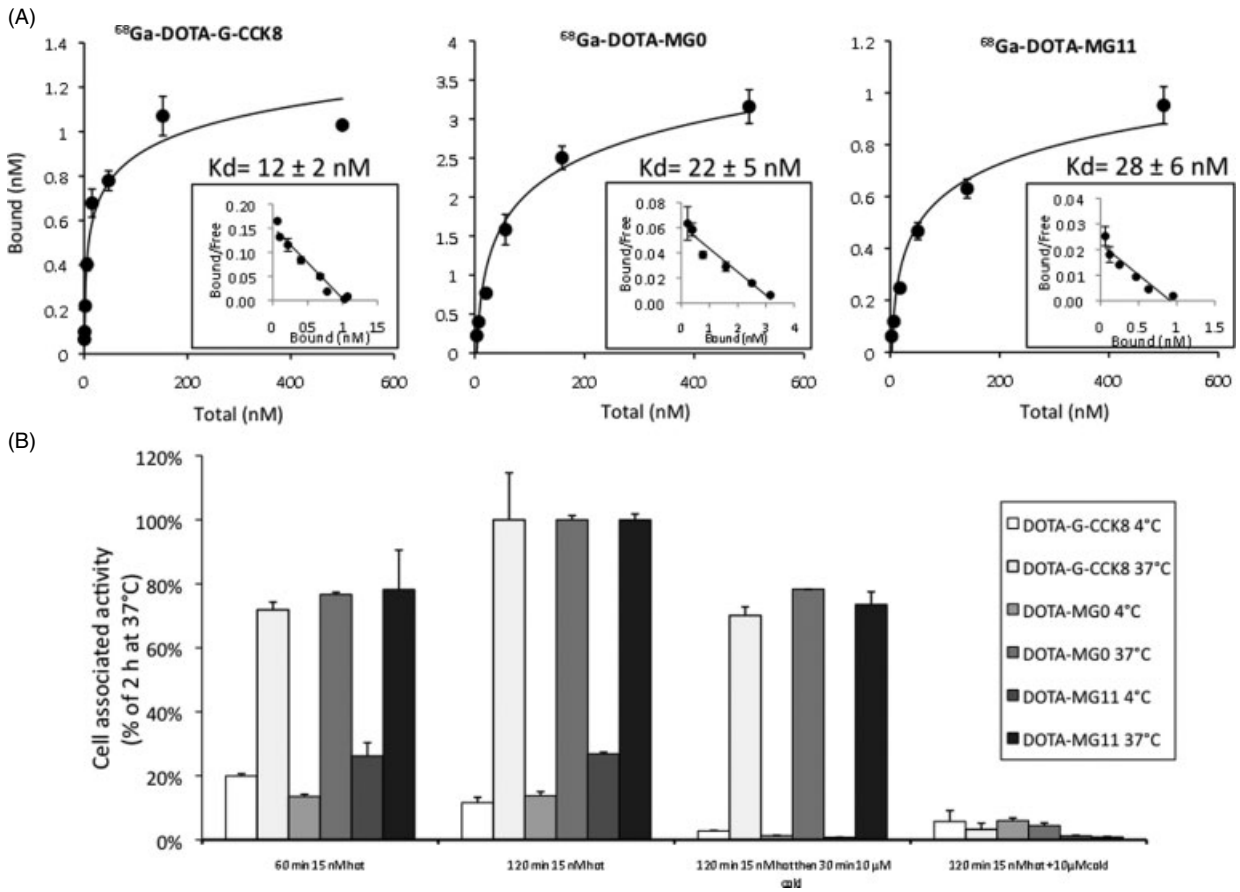

Figure 3. (A) Saturation binding curves of ^{68}Ga-DOTA-G-CCK8, ^{68}Ga-DOTA-MG0 and ^{68}Ga-DOTA MG11 on A431-CCK2R cells at 4 °C. There is similar high affinity binding for all peptides tested. (B) Internalization of ^{68}Ga-DOTA-G-CCK8, ^{68}Ga-DOTA-MG0 and ^{68}Ga-DOTA-MG11 on A431-CCK2R cells (see Materials and Methods). There is progressive accumulation of activity in cells incubated at 37 °C compared to cells incubated at 4 °C with the same compound. After accumulating radiolabeled peptide for 120 min (third set of bars from the left) addition of cold peptide at high concentrations (10 µM) displaces virtually all radioactivity from cells previously incubated at 4 °C, whereas most activity in cells incubated at 37 °C is not displaced indicating that it is internalized. Co-incubation of hot and excess cold peptide produces minimal accumulation of radioactivity indicating specific receptor-mediated cell uptake. There are virtually no differences in the biological behavior of the three peptides.

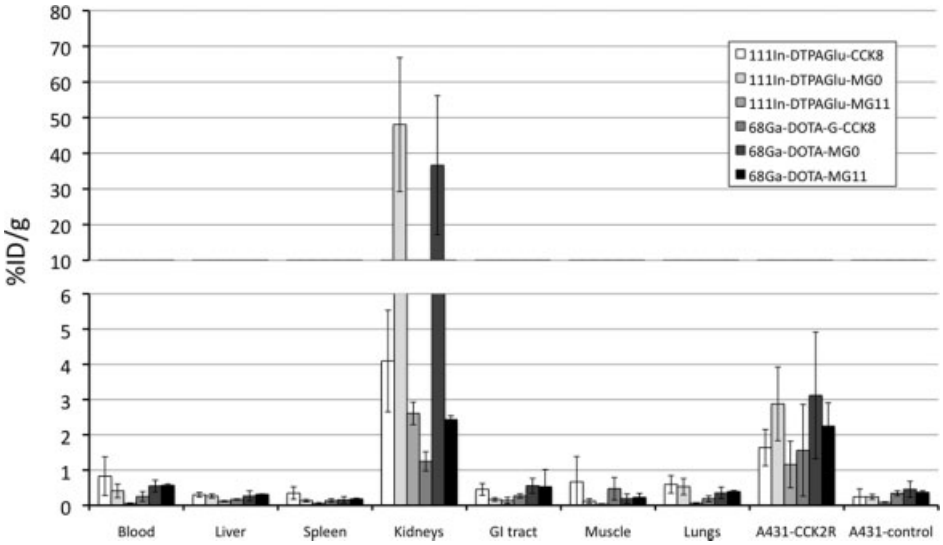

Figure 4. Biodistribution of the six peptide conjugates 1 h after injection ($n = 5$ animals per time point) in nude mice bearing A431-CCK2R and control xenografts.

Table 2. Summary of biodistribution properties at 1-h post-injection of the six peptides and binding affinities

Biodistribution at 1 h post-injection	^{111}In-DTPAGlu-G-CCK8	^{111}In-DTPAGlu-MG0	^{111}In-DTPAGlu-MG11	^{68}Ga-DOTA-G-CCK8	^{68}Ga-DOTA-MG0	^{68}Ga-DOTA-MG11
Kidneys (%ID/g)	4.1	48.1	2.6	1.2	36.6	2.4
Receptor positive xenograft (%ID/g)	1.6	2.9	1.2	1.5	3.1	2.2
Control xenograft (%ID/g)	0.4	1.3	0.7	0.3	0.5	0.4
Positive xenograft/kidney ratio	0.4	0.1	0.4	1.3	0.1	0.9
Positive to control xenograft ratio	3.7	2.2	1.7	4.6	6.7	5.2
Binding affinity *in vitro* (Kd, n**M**)	23	25	30	12	22	28

compounds is likely related to longer circulation times perhaps due to their higher *in vivo* stability as previously indicated [10]. This is also supported by the higher blood concentrations of radioactivity observed for these two compounds at the time point. One drawback, however, is that these two derivatives show the highest levels of uptake in the kidneys (48.1%ID/g and 36.6%ID/g respectively) which is undesired as high kidney accumulation greatly increases the possibility of kidney toxicity in clinical applications of radionuclide therapy. Furthermore, having areas of very high activity concentration also impairs imaging studies by creating artefacts that reduce picture quality. The high kidney accumulation of peptides containing elevated number of Glu residues is well known and appears related to the high concentration of negative charges on the peptide [18,19]. There are several reports on how this undesired renal accumulation can be reduced by co-injection of large amounts of negatively charged amino-acids [20] or other compounds [25].

The MG11 and CCK8 derivatives show very similar biodistribution features. For these compounds kidney accumulation is much lower than for the MG0 derivatives. One marked improvement of [^{68}Ga]DOTA-G-CCK8 compared to the previously published [^{111}In]-DTPAGlu-G-CCK8 [7] was found to be its extremely low kidney retention (1.2% vs 4.1% ID/g, respectively). Target tumor accumulation also occurs at lower levels for these derivatives compared to the two MG0-based peptides.

Accumulation of the compounds in the remaining organs, including the liver that is a major route of excretion of lipophilic compounds and metabolites, was negligible. For all compounds, the bulk of the excess radioactivity appears to be cleared through the kidneys, i.e. in general, a desired feature as it is more rapid than hepatobiliary clearance, although there is extremely high retention of the MG0 derivatives in the kidneys as pointed out earlier. Compounds that have prevailing hepatobiliary clearance are retained in the gut for prolonged periods of time making it difficult to identify areas of specific uptake in the abdomen using imaging techniques, and are thus less desirable.

The side by side comparison of the *in vitro* and *in vivo* properties of the six compounds being evaluated is summarized in Table 2. It appears that the DOTA-coupled derivatives show in general lower retention in the control xenografts compared to the DTPAGlu-coupled counterparts. This can be appreciated in the line regarding the positive xenograft to control xenograft ratio where it is clear that higher values are observed for the DOTA-coupled compounds. The lower background radioactivity seen with DOTA-coupled compounds may be related to the radioisotope utilized. The ^{111}In labeled metabolites derived from metabolic breakdown of the peptides may have different biological behaviors than ^{68}Ga breakdown products. It is well known that small quantities of ^{111}In may be chelated by iron-binding proteins such as transferrin [26]. The long plasma half-life of this protein may be causing the slightly higher background radioactivity levels compared to the ^{68}Ga labeled compounds. Other possibilities include more rapid clearance of [^{68}Ga]DOTA-coupled metabolites relative to [^{111}In] DTPAGlu breakdown products.

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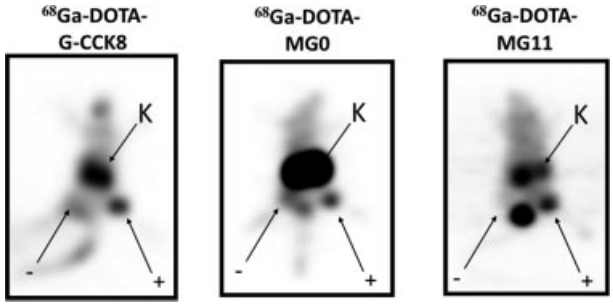

Figure 5. MIP-PET images obtained after injection of the three ^{68}Ga-labeled compounds. The A431-CCK2R expressing xenografts (+) and control xenografts (−) are located in opposite thighs of the animals. Concentration in the kidneys (K) is much higher for the ^{68}Ga-DOTA-MG0 compound compared to the other two peptides.

The ^{68}Ga labeled DOTA-coupled compounds appear in general to have better characteristics compared to the ^{111}In labeled compounds. Figure 5 shows MIP images from PET scans performed on the animals at 1-h post-injection of the three ^{68}Ga labeled compounds. The animals bear tumors on both flanks; however, in all cases only the CCK2R positive tumor (right flank) is visible compared to the background activity. The only other areas of significant activity visible in these images are the kidneys and the bladder in some cases. The [^{68}Ga]DOTA-MG0 compound shows remarkable concentration in the kidneys. This markedly high uptake greatly reduces the contrast between the CCK2R positive tumor and the kidneys. Furthermore, it seems like the entire abdominal cavity is occupied by this non-specific uptake due to the spill-over effect caused by the very high concentration of radioactivity relative to the other organs. The MG0 compounds therefore appear less useful than the MG11- and CCK8-based compounds at least for the purpose of imaging.

Another advantage of the use of DOTA-coupled compounds is the ability to image their distribution with PET by using isotopes such as ^{68}Ga as in this manuscript. This allows more accurate localization of areas of concentration and accurate quantitative measurements of radioactivity in tissues of interest. Furthermore, the same compound labeled with a different isotope may be utilized for radionuclide therapy giving the advantage of utilizing a single agent that can be radiolabeled with different isotopes based on the intended application.

Selection of an ideal peptide-based radiopharmaceutical for nuclear imaging or for radionuclide therapy relies on a combination of different characteristics where the best possible trade-off must be accepted. Imaging and therapy have different requirements in the biological characteristics of the radiopharmaceuticals utilized. In imaging applications, the absolute amount of radioactivity that is concentrated in a lesion or target tissue of interest is less important compared to radionuclide therapy, where tumor absorbed doses are directly related to how much radioactivity is concentrated in the given tissue. Successful imaging compounds rely more on the contrast between target and non-target tissues rather than absolute radioactivity concentration values. Furthermore, in radionuclide therapy, the time course of radioactivity concentration in the target tissue plays a key role in determining overall absorbed dose. Absorbed dose is dependent on the integrated activity in the tissue over time. Therefore, high uptake in a lesion at an early time point, while suitable for lesion detection in imaging applications, is not sufficient to guarantee elevated absorbed doses. Prolonged elevated retention of the radiolabeled compound is more important for therapeutic applications whereas high contrast between target and non-target tissues is necessary for imaging.

It has become apparent over the past few years that the major issue in the development of CCK2R targeted radiopharmaceuticals is how to best compromise between tumor and kidney concentrations. Mather *et al*. have recently described a comparison of several radiolabeled peptides that they have analyzed and selected based on the tumor to kidney uptake ratio [27]. Of the six compounds we compared in the current manuscript, only [^{68}Ga]DOTA-G-CCK8 shows higher uptake in the CCK2R positive xenograft compared to kidney (Table 2). This compound therefore appears to be most promising for imaging applications although improvement of the tumor uptake properties may be necessary to improve the possibility of radionuclide therapy applications.

Conclusions

We have directly compared the biological behavior of six radiolabeled peptide derivatives targeting the CCK2R. Our findings suggest that among the compounds we have tested [^{68}Ga]DOTA-G-CCK8 is the most promising for further development.

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