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ORIGINAL ARTICLE

Comparison of biological stability and metabolism of CCK2 receptor targeting peptides, a collaborative project under COST BM0607

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Abstract
Purpose Stability of radiolabelled cholecystokinin 2 (CCK2) receptor targeting peptides has been a major limitation in the use of such radiopharmaceuticals especially for targeted radionuclide therapy applications, e.g. for treatment of medullary thyroid carcinoma (MTC). The purpose of this study was to compare the in vitro stability of a series of peptides binding to the CCK2 receptor [selected as part of the COST Action on Targeted Radionuclide Therapy (BM0607)] and to identify major cleavage sites.
Methods Twelve different 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA)-minigastrin/CCK conjugates were provided within an European COST Action (BM0607) by different laboratories and radiolabelled with ^{177}Lu. Their in vitro stabilities were tested in fresh human serum. Radiochemical yields (RCY) and intact radioligands for half-life calculations were determined by radio-HPLC. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis of metabolites was performed to identify cleavage products using conjugates labelled with excess stable ^{nat}Lu, incubated in serum at 37°C. Urine metabolite analysis after injection in normal mice was performed by radio-HPLC analysis.

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Results Variable stability in human serum was found for the different peptides with calculated half-lives between 4.5± 0.1 h and 198±0.1 h (n=2). In urine of normal mice only metabolised peptide fragments were detected even at short times after injection for all peptides. MALDI-TOF MS revealed a major cleavage site of all minigastrin derivatives between Asp and Phe-NH_2 at the C-terminal end.
Conclusion Development of CCK2 receptor ligands especially for therapeutic purposes in patients with MTC or small cell lung cancer (SCLC) is still ongoing in different laboratories. This comparative study provided valuable insight into the importance of biological stability especially in the context of other results of this comparative trial within the COST Action BM0607.

Keywords Cholecystokinin receptor · ^{177}Lu · Gastrin · CCK · In vitro stability · In vivo stability · Metabolism

Introduction

The development of peptide-based radiopharmaceuticals (radiopeptides) has a great potential especially for targeted therapy of cancer [1, 2]. The cholecystokinin 2 (CCK2)/gastrin receptor is of interest as a target for diagnostic and therapeutic purposes in patients with medullary thyroid carcinoma (MTC) and small cell lung cancer (SCLC) [3, 4] using radiolabelled receptor ligands. Other tumour types, such as gastrointestinal neuroendocrine tumours, stromal ovarian cancer, astrocytoma and gastrointestinal stromal tumours are also potential candidates for targeted therapy using radiolabelled CCK2 receptor ligands [5].

Various research groups have investigated gastrin and CCK analogues binding to CCK2 receptors to widen the potential of receptor targeting with radiotracers [4, 6–12]. Gastrin analogues were initially proposed for imaging and treatment of metastatic MTC [6], especially for patients that cannot be treated with radiolabelled somatostatin analogues as a result of somatostatin receptor expression decrease in advanced dedifferentiated stages of the disease [13].

Radiopeptides must reach their target site without being degraded and therefore should exhibit sufficient in vivo stability. Instability will not only affect targeting efficiency, but may also result in an additional radiation dose to normal organs as radioactive degradation products may accumulate in non-target tissue. This is of particular concern when it comes to therapeutic applications [14–16]. Instability may result from chemical instability of the radiolabelled construct [17, 18] and can be overcome by choosing the right chemistry for the application which is well established for most radionuclides [19, 20]. In contrast, the metabolic stability of the peptide itself is not as easy to control. During systemic circulation of peptides the most important compartments where enzymatic degradation takes place are blood, liver and kidney [21]. Due to rapid enzymatic degradation most unmodified, natural peptides do not circulate in the blood for more than a few minutes. Therefore most peptide-based drug candidates have to be stabilised and a number of stabilisation strategies are available, such as replacing L-amino acids by their corresponding D-amino acid, designing cyclic analogues, modifying the side chains of some of the amino acids involved or modifying N/C termini [21, 22].

Low in vivo stability has been a major concern in the development of radiolabelled CCK/gastrin analogues for diagnosis and therapy of CCK2/gastrin receptor-expressing tumours. In addition to enzymatic cleavage these peptides hold the risk of being oxidised at the methionine residue resulting in loss of receptor binding. In the present study we aimed to evaluate and compare the biological stability and metabolism of a number of different CCK2/gastrin analogues provided within the collaborative European project, COST Action BM0607. This included the determination of half-lives in vitro and comparison of metabolic profiles with the intent to characterise the metabolites using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). In this way, the most stable candidates and cleavage sites could be identified and exploited for further stabilisation strategies.

Materials and methods

Twelve different 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA)-minigastrin/CCK derivatives (COST BM0607 peptides) were provided within an European COST Action (BM0607) by different laboratories (Table 1). $^{177}LuCl_3$ was purchased from ITG (Garching, Germany) and IDB (Petten, The Netherlands). Stable lutetium chloride (^{nat}Lu) was obtained from Sigma-Aldrich as $LuCl_3.6H_2O$. All other reagents were purchased from Merck or Fluka.

Analytical methods

HPLC A Dionex P580 pump (Dionex, Vienna, Austria) with Bioscan radiometric detection was used for RP-HPLC analysis. A Nucleosil 120-5 C18 Column 4.0×250 mm (SRD, Vienna, Austria), flow rate 1.5 ml×min^{-1}, was employed with the following gradients: acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA)/H_2O: *t*: 0–2 min 0% ACN, 2–7 min 20–35% ACN, 7–7.01 min 35–60% ACN, 7.01–10 min 60% ACN, 10–11 min 60–0% ACN, 11–15 min 0%

Table 1 Acronyms, sequence and molecular weight of the peptides under study

	Name	Sequence	MW (g/mol)
1	G-CCK8	DOTA-Gly-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2	1,507.0
2	SA106	DOTA-DAsp-Phe(*p*-CH_2SO_3H)-HPG-Gly-Trp-HPG-Asp-Phe-NH_2	1,482.6
3	MG0	DOTA-DGlu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	2,037.0
4	Sargastrin	DOTA-Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2	2,483.6
5	MG11	DOTA-DGlu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	1,531.0
6	APH070	DOTA-His-His-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	1,677.8
7	PP-F10	DOTA-DGln-DGln-DGln-DGln-DGln-DGln-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	2,042.9
8	PP-F6	DOTA-DGln-DGln-DGln-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	1,658.7
9	PP-F16	DOTA-DGln-DGlu-DGln-DGlu-DGln-DGlu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	2,045.9
10	PP-F11	DOTA-DGlu-DGlu-DGlu-DGlu-DGlu-DGlu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2	2,048.8
11	Cyclo-MG1	DOTA-cyclo[γ-D-Glu-Ala-Tyr-D-Lys]-Trp-Met-Asp-Phe-NH_2	1,455.7
12	MGD5	DOTA-Gly-Ser-Cys-(Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2)$_2$	2,782.9

HPG homopropargyl glycine

ACN and *t*: 0–2 min 0% ACN, 2.01–17 min 20–50% ACN, 17.01–20 min 70% ACN, 20–21 min 70–0% ACN, 21–25 min 0% ACN.

MALDI-TOF MS MALDI-TOF MS was performed on a 4800 Plus MALDI-TOF/TOF Analyzer mass spectrometer using the 4000 Series Explorer Software V3.6. for data acquisition and processing (Applied Biosystems, Foster City, CA, USA). MS spectra were acquired in positive reflector mode by accumulation of single measurements from 2,000 laser shots. Sample preparation was carried out by mixing 5.0 μl peptide sample (RP-HPLC fractions) with 5.0 μl matrix solution consisting of 10.0 mg/ml alpha-cyano-4-hydroxycinnamic acid (CHCA) dissolved in 50:50 ACN-H_2O with 0.1% TFA. A volume of 0.7 μl of the peptide-matrix solution was deposited onto a MALDI sample plate; the matrix-analyte droplet was then slowly dried in air.

Radiolabelling

Radiolabelling was performed with 25–30 μg COST BM0607 peptides and 100–200 MBq $^{177}LuCl_3$ in 0.4 M ammonium acetate/0.24 M 2,5-dihydroxybenzoic acid (gentisic acid) buffer (pH 4.5) at 80°C for 20 min. Quality control (QC) of radiolabelling was performed using the HPLC system described above.

^{nat}Lu labelling

About 10-mol equivalents of $^{nat}LuCl_3$ were incubated with the corresponding DOTA-peptide (10–12 nmol) in 0.4 M ammonium acetate/0.24 M gentisic acid buffer (pH 4.5) for 20 min at 80°C. QC of "cold" labelling was performed using MALDI-TOF MS.

In vitro metabolic stability studies

Influence of non-N_2-purged and N_2-purged serum on methionine oxidation in serum stability assay To investigate whether methionine oxidation for methionine-containing minigastrin/CCK derivatives could be minimised in stability assays by purging the serum with nitrogen, fresh serum was prepared from human blood [23], dispensed in fractions of 1 ml in closed vials and purged or non-purged for 5 min with nitrogen. One of the ^{177}Lu-labelled methionine-containing DOTA-peptides (^{177}Lu-PP-F11) was incubated in N_2-purged and non-N_2-purged serum. At different time points (0, 0.5, 1, 2, 4 and 24 h) samples were taken, treated and analysed as described below. Results were expressed as percentage intact peptide of the total extracted activity.

Radiopeptide stability in serum The half-life of the different ^{177}Lu-DOTA-peptides was determined in vitro in human serum. Blood was collected in a non-heparinised syringe and centrifuged (Heraeus Labofuge 400R) at 4°C for 10 min to separate the serum [23]. Then the ^{177}Lu-DOTA-peptide was added to 1 ml of freshly prepared serum and incubated at 37°C. At different time points (0.5, 1, 2, 4 and 24 h) samples were taken and the proteins precipitated with ACN (1:1), then vortexed. The activity in the precipitate was determined and was <20% for all peptides except for MGD5 where >50% were precipitated. The precipitate was separated by centrifugation at 2,000 *g* for 5 min (Eppendorf centrifuge 5424). For the HPLC analysis, the supernatant was diluted with double distilled water (1:1).

Radiopeptide degradation in tissue homogenates For incubation in liver/kidney homogenates, the organs were excised from rats and immediately frozen at −80°C. On

the day of the experiment liver/kidneys were defrosted and homogenised in 20 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer pH 7.3 with an Ultra-Turrax T 25 homogenator for 5 min and placed on ice. The ^{177}Lu-DOTA-peptides were incubated with 10% tissue homogenates at a constant concentration of peptide (4 μM) at 37°C in a water bath for 30–100 min. Samples were precipitated with ACN (1:1), vortexed and then centrifuged (2,000 *g*, 5 min). The supernatant was transferred into an Eppendorf tube and recentrifuged (2,000 *g*, 5 min) to ensure good separation from tissue residues. For HPLC analysis, the supernatant was diluted with double distilled water (1:1).

In vivo metabolic stability studies

All animal experiments were conducted in compliance with the Austrian animal protection laws and with approval of the Austrian Ministry of Science. In vivo metabolic stability studies were performed in female BALB/c mice. The ^{177}Lu-DOTA-peptides (~3 μg, 15 MBq) were administered into the tail vein. Urine was collected 10 min post-injection (p.i.), diluted with double distilled water and immediately analysed by HPLC. After collection of a urine sample, mice were sacrificed by cervical dislocation. Blood was collected, proteins precipitated with ACN (1:1) and then vortexed. The precipitate was separated by centrifugation (2,000 *g*, 10 min) and diluted with double distilled water (1:1) for HPLC analysis.

Evaluation of enzymatic cleavage sites of radiolabelled peptides

To identify serum radiometabolites the different ^{177}Lu-DOTA-peptides were incubated in serum for 24 h at 37°C. After 24 h a sample was withdrawn and analysed by HPLC as described above. HPLC fractions were collected and measured in a well counter. The fractions that contained radiometabolites were identified and their retention times noted. The same study was then repeated incubating ^{nat}Lu-DOTA-peptides in serum. Fractions containing ^{nat}Lu-labelled metabolites were collected at the retention times noted previously for the eluted radiometabolites and these were analysed by MALDI-TOF MS. By comparison of detected masses with calculated masses cleavage products were identified.

Results

The per cent of radiochemical yields (RCY %) of ^{177}Lu-DOTA-peptides are shown in Table 2. For methionine-containing DOTA-peptides oxidation after radiolabelling was always <10% and therefore they were used without further purification for stability studies.

Table 2 RCY % of the ^{177}Lu-labelled DOTA-peptides

	Name	RCY % (n=3)
1	^{177}Lu-SA106	99.00±1.03
2	^{177}Lu-MG0	92.80±3.29
3	^{177}Lu-Sargastrin	99.83±0.12
4	^{177}Lu-MG11	90.93±3.44
5	^{177}Lu-APH070	95.49±1.80
6	^{177}Lu-PP-F10	93.49±1.68
7	^{177}Lu-PP-F6	94.39±0.54
8	^{177}Lu-PP-F16	91.07±2.09
9	^{177}Lu-PP-F11	93.02±2.61
10	^{177}Lu-Cyclo-MG1	99.02 ±1.51
11	^{177}Lu-MGD5	98.37±1.65

In vitro metabolic stability studies

Influence of N_2-purged and non-N_2-purged serum on the methionine oxidation in serum stability assays No significant difference in the degree of methionine oxidation of ^{177}Lu-PP-F11 was seen when using N_2-purged serum as opposed to non-N_2-purged serum at any of the time points (0, 0.5, 1, 2, 4 and 24 h incubation). Methionine oxidation was 10.9 and 10.2% after 30 min incubation and increased to 15.3 vs 17.3% 24 h after incubation in N_2-purged and non-N_2-purged serum, respectively. Therefore all further studies were carried out without nitrogen purging.

Radiopeptide stability in serum Variable stability in human serum was found for the different ^{177}Lu-DOTA-peptides tested. ^{177}Lu-PP-F10 ($T_{1/2}$=198±0.3 h, n=2) and ^{177}Lu-MG11 ($T_{1/2}$=4.5±0.1 h, n=2) resulted in being the radiopeptides that had the highest and lowest stability in serum, respectively. A summary of the results of the in vitro stability of all the ^{177}Lu-DOTA-peptides in serum is shown in Fig. 1.

Radiopeptide degradation in tissue homogenates In 10% kidney homogenate ^{177}Lu-PP-F11 ($T_{1/2}$=61.2 min, n=1) and ^{177}Lu-MG0 ($T_{1/2}$=0.5 min, n=1) showed the highest and lowest stability, respectively. In 10% liver homogenate these radiopeptides were found to be ^{177}Lu-PP-F11 ($T_{1/2}$=144 h, n=1) and ^{177}Lu-PP-F10 ($T_{1/2}$=12.1 min, n=1). Only a limited correlation was found between serum stability and stability in kidney (R=0.58) and liver (R=0.47) homogenate (Fig. 2a, b). However, if the two outliers, ^{177}Lu-PP-F10 and ^{177}Lu-PP-F11, are not considered, the

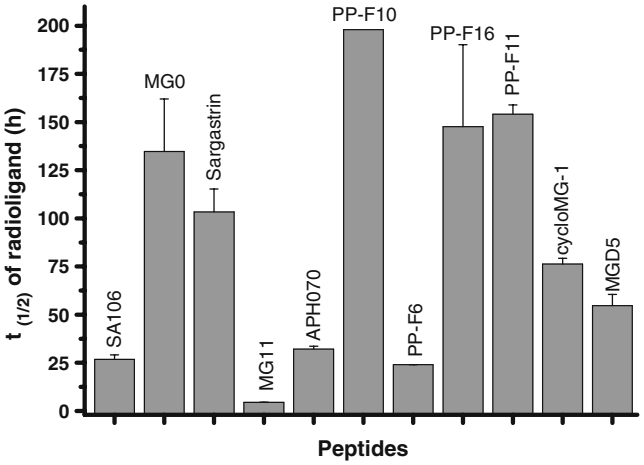

Fig. 1 In vitro serum stability of ^{177}Lu-DOTA-peptides, mean value of n=2, *error bars* indicate the range

correlations increases to R=0.94 in kidney and R=0.71 in liver, showing good correlation for all other peptides.

In vivo metabolic stability studies

At 10 min p.i. of the ^{177}Lu-DOTA-peptides in BALB/c mice no intact peptide could be found in urine. Radio-HPLC degradation profiles of the radiopeptides in urine are shown in Fig. 3. For the most (^{177}Lu-PP-F10) and least (^{177}Lu-MG11) stable radiopeptide in serum (in vitro) no intact radiopeptide could be detected in the blood in vivo (Fig. 4). To verify these results two other radiopeptides (^{177}Lu-PP-F6 and ^{177}Lu-SA106) were chosen randomly and again 10 min p.i. no intact radioligand was found in the blood (Fig. 4).

Evaluation of enzymatic cleavage sites

A number of radiometabolites in serum could be identified by analysis of MALDI-TOF MS of corresponding HPLC fractions after serum incubation of ^{nat}Lu-DOTA-peptides and are summarised in Table 3. For methionine-containing peptides variable oxidation was found; for the sake of simplicity no differentiation between oxidised and non-oxidised metabolites was made. A common cleavage site of all radiopeptides was found between Asp and Phe-NH_2 at the C-terminal end. For some peptides other cleavage sites closer to the amino terminus resulting in smaller peptide fragments could be identified. Radiometabolites identified in serum by means of MALDI-TOF MS were compared with the corresponding retention times of metabolites identified in urine. From this comparison the main urinary metabolites were identified and are summarised in Table 4. Except for cyclo-MG1 and MGD5 (a dimeric peptide) the metabolites were found to be cleavage products resulting in Tyr or Gly as the C-terminal fragment.

Discussion

To obtain reliable data in biological stability assays, good standardisation is required. We recently showed that a number of parameters including type and age of serum used for incubation may influence the outcome parameters such as serum half-life [23]. In this study we additionally investigated the influence of oxygen in incubation media by purging the serum with nitrogen, with no significant effect on the outcome of the stability assay.

In the present study we tested the in vitro stability of a series of ^{177}Lu-labelled minigastrin/CCK analogues in human serum as well as rat tissue homogenates (liver and kidneys). When comparing this series of peptides with potential use in targeted radionuclide therapy, variable in vitro stability results were found. There was a good correlation between serum stability and stability in tissue homogenates except for two peptides, ^{177}Lu-PP-F10

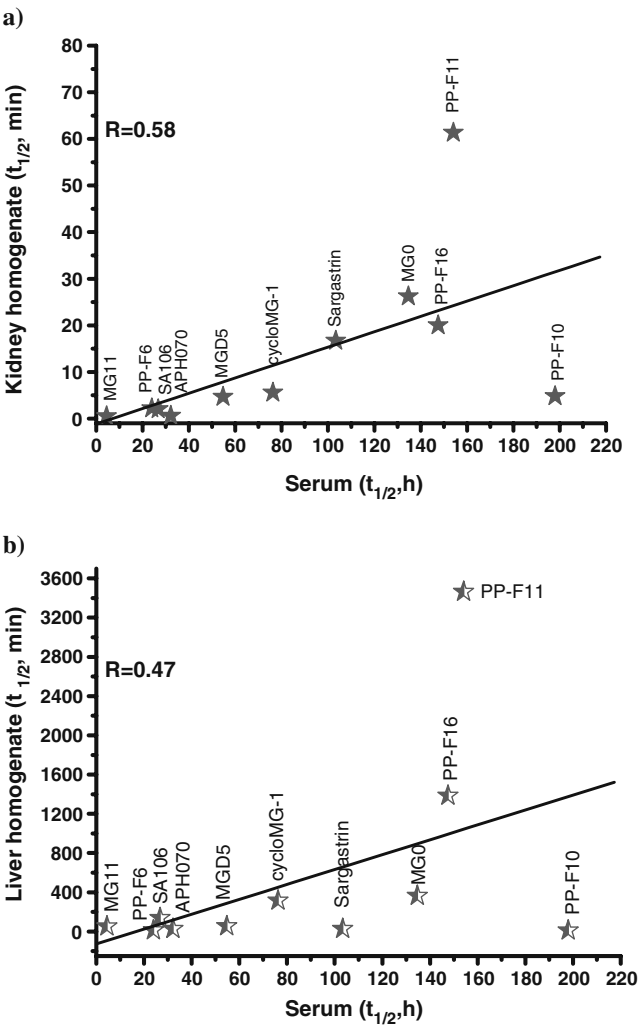

Fig. 2 Correlation of the half-lives of the radiopeptides calculated from incubation in serum and kidney (**a**) and liver (**b**) homogenate

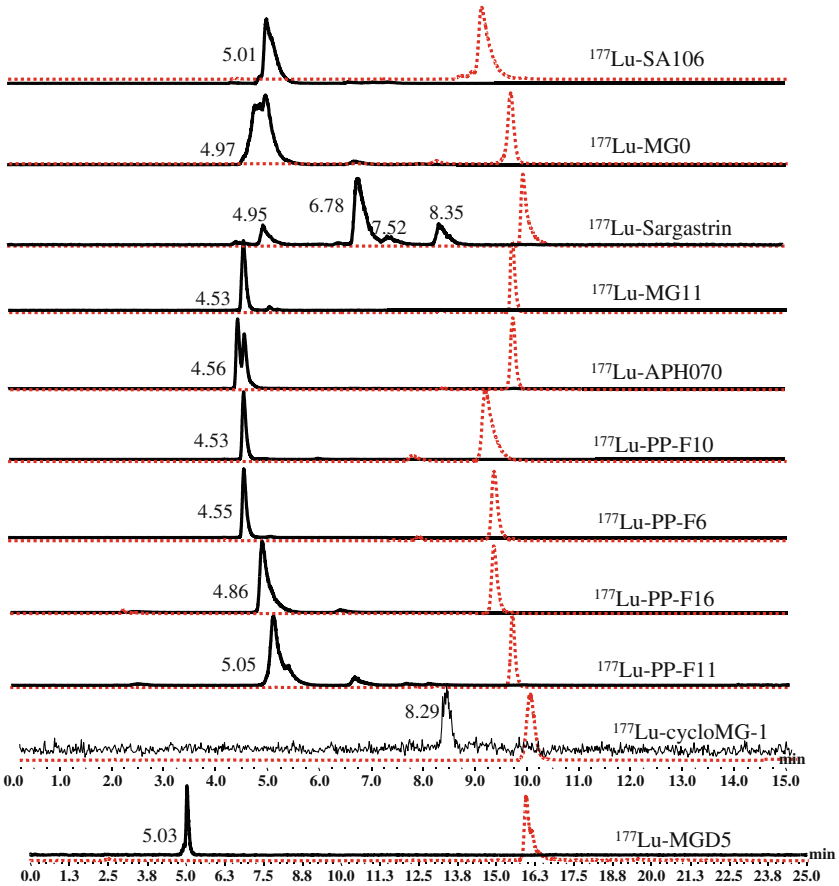

Fig. 3 Radio-HPLC profiles of urine collected 10 min after injection of ^{177}Lu-DOTA-peptides (*black trace*) compared with the radiochromatograms prior to injection (*red dashed trace*)

and ^{177}Lu-PP-F11. In general, in tissue homogenates, the degradation of the radiopeptides was considerably faster compared to incubation in serum. ^{177}Lu-PP-F11 resulted in being the most stable radiopeptide in kidney ($T_{1/2}$=61.32 min) and liver ($T_{1/2}$=57.75 h) homogenate, whereas ^{177}Lu-MG11 (kidney: $T_{1/2}$=0.5 min; liver: $T_{1/2}$=53 min) and ^{177}Lu-PP-F10 (kidney: $T_{1/2}$=4.85 min; liver: $T_{1/2}$ =12.12 min) were found to be the least stable radiopeptides. In addition, our investigations showed a major difference between in vitro serum and in vivo urinary metabolites. None of the ^{177}Lu-DOTA-peptides resulted in being intact in urine collected 10 min after injection in BALB/c mice. The mechanism of this phenom-

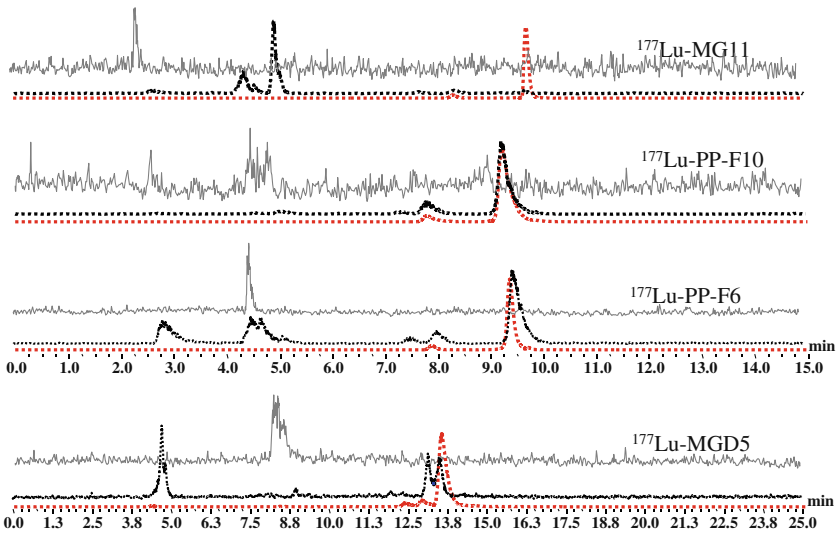

Fig. 4 Radio-HPLC profiles of ^{177}Lu-PP-F10, ^{177}Lu-MG11, ^{177}Lu-PP-F6 and ^{177}Lu-SA106 in blood ex vivo 10 min after injection in BALB/c mice (*grey trace*) compared to serum incubation in vitro (*black dashed trace*) and ^{177}Lu-DOTA-peptide radiolabelling solution (*red dashed trace*)

enon of rapid in vivo degradation could be a result of degrading enzymes located on the cell surface, called ectoenzymes, known to be shed in blood and highly expressed in liver and kidneys [24], even though correlation between stability in liver and kidney homogenates was poor (R=0.56) in this series of peptides. It is not exactly known which enzymes are mainly responsible; an important enzyme in this respect could be the neutral endopeptidase 24.11, which has been shown to be able to cleave gastrin and CCK at various sites [25] including the Asp-Phe and Gly-Trp bond. Further studies, e.g. changes on stability using specific enzyme inhibitors, are required to elucidate this mechanism and may also address the question as to why in vivo degradation occurs much more rapidly than was expected from in vitro studies. This rapid degradation is indicated by the fact that even though the investigated peptides are small enough to be renally cleared, no intact peptide was detected in urine. Renal reabsorption and intracellular metabolism seem unlikely considering the residualising properties of a radiometal such as ^{177}Lu. This was also confirmed by analysing blood samples

Table 3 Cleavage sites of the DOTA-peptides in serum in vitro as determined by MALDI-TOF MS (black arrows indicate cleavage sites)

COST BM0607 peptides	Peptide sequence
SA106	DOTA-D-Asp-Phe(ρ-CH_2SO_3H)-HPG-Gly-Trp-HPG-Asp↓-Phe-NH_2
MG0	DOTA-DGlu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr↓-Gly↓-Trp-Met-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
MG11	DOTA-DGlu-Ala-Tyr↓-Gly↓-Trp-Met-Asp↓-Phe-NH_2
APH070	DOTA-His-His↓-Glu↓-Ala-Tyr-Gly-Trp-Met -Asp↓-Phe-NH_2
PP-F10	DOTA-$(DGln)_6$-Ala-Tyr↓-Gly↓-Trp-Met -Asp↓-Phe-NH_2
PP-F6	DOTA-$(DGln)_3$-Ala↓-Tyr↓-Gly↓-Trp-Met -Asp↓-Phe-NH_2
PP-F16	DOTA-DGln-DGlu-DGln-DGlu-DGln-DGlu-Ala-Tyr↓-Gly↓-Trp-Met -Asp↓-Phe-NH_2
PP-F11	DOTA-$(DGlu)_6$-Ala-Tyr-Gly-Trp-Met -Asp↓-Phe-NH_2
cycloMG1	DOTA-cyclo[γ-dGlu-Ala-Tyr-dLys]-Trp-Met-Asp↓-Phe-NH_2
MGD5	DOTA-GSC-Glu-Ala↓-Tyr↓-Gly-Trp-Nle-Asp↓-Phe-NH_2 succinimidopropionyl- Glu-Ala↓-Tyr↓-Gly-Trp-Nle-Asp↓-Phe-NH_2

of four peptide candidates including ^{177}Lu-PP-F10, the most stable peptide in serum in vitro, showing no intact peptide in blood already 10 min after injection. For all radiopeptides degradation in serum in vitro was considerably slower compared to incubation in tissue homogenates. This is a clear indication of the poor predictive value of serum stability studies alone to judge the metabolic stability in vivo of radiolabelled peptides and suggests that incubation in tissue homogenates in vitro may be a better reflection of the degrading peptidases in vivo. From this perspective ^{177}Lu-PP-F11, being very stable in tissue homogenates, might be the most suitable candidate in this series for further trials.

MALDI-TOF MS studies on the metabolites obtained from incubation in serum of the carrier-added ^{nat}Lu-DOTA-peptides revealed a common cleavage site of all DOTA-peptides between Asp and Phe-NH_2 (Table 3). Other prominent cleavage sites were found between Met-Asp and Tyr-Gly. The metabolites identified by means of MALDI-TOF MS were correlated with the corresponding retention time of radiometabolites in urine (Table 4). From this comparison, the major urinary metabolites were identified as being cleavage products resulting in Tyr and Gly as carboxy-terminal amino acid, except for cyclo-MG1 and MGD5 (a dimeric peptide), two peptides structurally different from the other peptides under investigation. These results may help to develop stabilising strategies for minigastrin/CCK derivatives for targeted radionuclide therapy applications which is a central issue as the carboxy-terminal aromatic amino acids are essential for binding to the CCK2 receptor [26].

In summary, our results show that in vitro stability assays of the ^{177}Lu-DOTA-peptides were of limited value to estimate the stability in vivo. Overall a much more rapid degradation was found in vivo than expected from serum incubation studies, which is usually the only stability study performed in the development of radiolabelled peptides for nuclear medicine applications. Even though incubation in kidney and liver tissue homogenates revealed a more rapid degradation, no reliable prediction of in vivo metabolism could be given. However, in the direct in vitro comparison of a series of radiopeptides distinctions in terms of stability could be made, revealing compounds with considerably improved metabolic stability. Together with data from receptor binding, biodistribution and tumour uptake the results obtained added important information to the selection of the most suitable compounds for further evaluation. The major cleavage site for all of the peptides compromising their stability was found in the C-terminal region. Modification strategies, therefore, should focus on this region to further enhance metabolic stability both in vitro and in vivo.

Table 4 Radiometabolites detected by MALDI-TOF MS for ^{nat}Lu-COST BM0607 peptides in serum having corresponding retention times (rt) as the main metabolite found in urine of BALB/c mice injected with the radiopeptides

COST BM0607 peptides	Detected fragments
SA106	n.a.
MG0 (rt: 4.9–5.0)	-^{177}Lu-DOTA-$(DGlu)_6$-Ala-Tyr-Gly
	-^{177}Lu-DOTA-$(DGlu)_6$-Ala-Tyr
Sargastrin	n.a.
MG11 (rt: 4.5–4.6)	-^{177}Lu-DOTA-DGlu-Ala-Tyr-Gly
	-^{177}Lu-DOTA-DGlu-Ala-Tyr
APH070 (rt: 4.5–4.6)	-^{177}Lu-DOTA-His-His-Glu-Ala-Tyr-Gly
	- ^{177}Lu-DOTA-His-His-Glu-Ala
	-^{177}Lu-DOTA-His-His-Glu
	-^{177}Lu-DOTA-His-His
PP-F10 (rt: 4.5–4.6)	- ^{177}Lu-DOTA-$(DGln)_6$-Ala-Tyr-Gly
	-^{177}Lu-DOTA-$(DGln)_6$-Ala-Tyr
PP- F6 (rt: 4.5–4.6)	- ^{177}Lu-DOTA-$(DGln)_3$-Ala-Tyr-Gly
	- ^{177}Lu-DOTA-$(DGln)_3$-Ala-Tyr
PP-F16 (rt: 4.8–4.9)	- ^{177}Lu-DOTA-DGln-DGlu-DGln-DGlu-DGln-DGlu-Ala-Tyr-Gly
	- ^{177}Lu-DOTA-DGln-DGlu-DGln-DGlu-DGln-DGlu-Ala-Tyr
PP-F11	n.a.
Cyclo-MG1 (rt: 8.2–8.3)	- ^{177}Lu-DOTA-cyclo[γ-dGlu-Ala-Tyr-dLys]-Trp-Met-Asp
MGD5 (rt: 5.0–5.1)	^{177}Lu- DOTA-GSC-Glu-Ala | succinimidopropionyl- Glu-Ala

Conclusion

In the present study in vitro and in vivo stability of a series of ^{177}Lu-labelled DOTA-peptides was evaluated. When comparing the different radiopeptides studied variable in vitro stability results were found. ^{177}Lu-PP-F10 and ^{177}Lu-MG11 were found to be the most and least stable radiopeptides in serum, respectively, whereas ^{177}Lu-PP-F11 was the most stable compound in tissue homogenates, also showing high stability in human serum. These results will help to select the best candidate for clinical evaluation in CCK2 receptor-targeted radionuclide therapy of human tumours, corroborated by data from binding and internalisation studies, biodistribution experiments in tumour models and small animal imaging. Thus far, ^{177}Lu-PP-F11, the most stable radiopeptide in tissue homogenates, seems to be the most promising candidate for this purpose. However, using the knowledge of metabolites and cleavage sites identified in this study, it should be possible to design alternative stabilisation strategies to develop analogues that are more metabolically stable.

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Conflicts of interest None.

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