

# <sup>99m</sup>Tc-labelled HYNIC-minigastrin with reduced kidney uptake for targeting of CCK-2 receptor-positive tumours

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## Abstract

**Purpose** Different attempts have been made to develop a suitable radioligand for targeting CCK-2 receptors *in vivo*, for staging of medullary thyroid carcinoma (MTC) and other receptor-expressing tumours. After initial successful clinical studies with [DTPA<sup>0</sup>,D<sup>1</sup>Glu<sup>1</sup>]minigastrin (DTPA-MG0) radiolabelled with <sup>111</sup>In and <sup>90</sup>Y, our group developed a <sup>99m</sup>Tc-labelled radioligand, based on HYNIC-MG0. A major drawback observed with these derivatives is their high uptake by the kidneys. In this study we describe the preclinical evaluation of the optimised shortened peptide analogue, [HYNIC<sup>0</sup>,D<sup>1</sup>Glu<sup>1</sup>,desGlu<sup>2-6</sup>]minigastrin (HYNIC-MG11).

**Methods** <sup>99m</sup>Tc labelling of HYNIC-MG11 was performed using tricine and EDDA as coligands. Stability experiments were carried out by reversed phase HPLC analysis in PBS,

PBS/cysteine and plasma as well as rat liver and kidney homogenates. Receptor binding and cell uptake experiments were performed using AR4-2J rat pancreatic tumour cells. Animal biodistribution was studied in AR4-2J tumour-bearing nude mice.

**Results** Radiolabelling was performed at high specific activities and radiochemical purity was >90%. <sup>99m</sup>Tc-EDDA-HYNIC-MG11 showed high affinity for the CCK-2 receptor and cell internalisation comparable to that of <sup>99m</sup>Tc-EDDA-HYNIC-MG0. Despite high stability in solution, a low metabolic stability in rat tissue homogenates was found. In a nude mouse tumour model, very low unspecific retention in most organs, rapid renal excretion with reduced renal retention and high tumour uptake were observed.

**Conclusion** <sup>99m</sup>Tc-EDDA-HYNIC-MG11 shows advantages over <sup>99m</sup>Tc-EDDA-HYNIC-MG0 in terms of lower kidney retention with unchanged uptake in tumours and CCK-2 receptor-positive tissue. However, the lower metabolic stability and impurities formed in the labelling process still leave room for further improvement.

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## Introduction

Targeting receptors on human tumours using radiolabelled somatostatin analogues has been very successful in neuroendocrine tumours [1]. Up to now, receptor-targeted imaging and radionuclide therapy are mainly limited to these rather uncommon tumours, which represent only 2% of all malignant gastrointestinal neoplasms [2]. Many researchers are currently investigating whether receptors of other regulatory peptides are overexpressed in more

common human cancers, and several other regulatory peptides are under study for their potential diagnostic and/or therapeutic clinical application in nuclear medicine [3]. Among these, gastrin and CCK analogues that bind to cholecystokinin-2/gastrin (CCK-2) receptors have been intensively studied [4–10]. Gastrin derivatives with superior selectivity and affinity for the CCK-2 receptor represent the preferred radiolabelled analogues for in vivo targeting of CCK-2 receptor-positive tumours [5].

$^{131}\text{I}$  gastrin I and  $[\text{DTPA}^0, \text{D}^1\text{Glu}^1]$ minigastrin (DTPA-MG0) radiolabelled with  $^{111}\text{In}$  and  $^{90}\text{Y}$  were initially proposed for imaging and treatment of metastatic medullary thyroid carcinoma (MTC) when treatment with radiolabelled somatostatin analogues is not possible because of a decreased level of somatostatin receptor expression with increased dedifferentiation [11]. Beside MTC, other tumour entities, such as small cell lung cancer [12] and gastrointestinal neuroendocrine tumours, especially if somatostatin receptor scintigraphy is negative [13], are potential targets for diagnosis and therapy using radiolabelled gastrin analogues. CCK-2 receptors are frequently also present in stromal ovarian cancers, astrocytomas [14] and gastrointestinal stromal tumours [15].

Especially regarding the diagnosis of CCK-2-expressing malignancies, a  $^{99\text{m}}\text{Tc}$ -labelled peptide analogue would be of additional value, considering the advantages of the  $^{99\text{m}}\text{Tc}$  label (high image quality, low radiation exposure, availability on demand and cost-effectiveness). In previous work on the development of a  $^{99\text{m}}\text{Tc}$ -labelled MG0 analogue performed by our group, of the two different labelling approaches tested the  $[\text{Tc}]\text{HYNIC}$  core resulted in higher specific activities and a most promising in vivo behaviour [16]. In this complex, hydrazinonicotinic acid (HYNIC) occupies a coordination site via a Tc-diazenido linkage and a coligand such as ethylenediaminediacetic acid (EDDA) completes the coordination sphere. In the preclinical evaluation,  $^{99\text{m}}\text{Tc}$ -EDDA-HYNIC-MG0 showed the best tumour targeting properties, but also very high kidney uptake, which was the limiting factor for further clinical evaluation. Similar findings were obtained with  $^{90}\text{Y}$ -DTPA-MG0, where for radiotherapy nephrotoxicity proved a major concern to be addressed [17]. In another study, MG0 analogues with open chain tetraamines at the N-terminus radiolabelled with  $^{99\text{m}}\text{Tc}$  also showed high renal uptake, which was significantly reduced by co-injection of an excess of parent peptide [10]. Renal protection strategies developed so far are co-injection of polyglutamic acid, as the accumulation in the kidneys seems to be related to the polyglutamic acids in the peptide chain [18], and optimisation of the peptide sequence by depletion of the five glutamic acid molecules in positions 2–6 [19].

We herein describe the radiolabelling, in vitro characterisation, including receptor binding, and biodistribution

of the new analogue HYNIC-MG11 missing the penta-glutamic sequence in the peptide chain, in an attempt to improve pharmacokinetics and receptor targeting properties.

## Materials and methods

### Materials

Unless otherwise stated, reagents were purchased from Aldrich-Sigma Chemical Co.

$[\text{HYNIC}^0, \text{D}^1\text{Glu}^1, \text{desGlu}^{2-6}]$ minigastrin (HYNIC-MG11) and  $[\text{HYNIC}^0, \text{D}^1\text{Glu}^1]$ minigastrin (HYNIC-MG0) were synthesised by piChem (Graz, Austria) and characterised by reversed phase high-performance liquid chromatography (RP-HPLC; purity >95%) and mass spectrometry.

$\text{Na}^{99\text{m}}\text{TcO}_4$  was obtained from a commercial  $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$  generator (Ultratechnekow, Mallinckrodt, The Netherlands).

### Analytical methods

**HPLC** A Dionex 680 pump with variable UV detector and Bioscan radiometric detection were used for RP-HPLC analysis. A Macherey & Nagl Nucleosil 120-5 C18 250×4.6 mm column, flow rates of 1 ml/min, and UV detection at 220 nm were employed with the following gradient: acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA)/ $\text{H}_2\text{O}$ : t: 0–3 min 0%ACN, 3–5 min 0–25%ACN, 5–20 min 25–40%ACN, 20–25 min 40–60%ACN, 25–28 min 60–0%ACN, 28–33 min 0%ACN.

### $^{99\text{m}}\text{Tc}$ labelling

#### *Tris(hydroxymethyl)methylglycine (tricine) as coligand*

In a rubber-sealed vial, 5  $\mu\text{g}$  of HYNIC-MG11 was incubated with 0.5 ml of tricine solution (70 mg/ml in water), 0.5 ml of  $^{99\text{m}}\text{TcO}_4^-$  solution (>200 MBq) and 20  $\mu\text{l}$  of tin(II) solution (10 mg of  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  in 10 ml 0.1 N HCl) at 80°C for 20 min.

#### *Ethylenediaminediacetic acid (EDDA) as coligand*

**Direct labelling** In a rubber-sealed vial, 10  $\mu\text{g}$  of HYNIC-MG11 was incubated with 0.5 ml of EDDA solution (20 mg/ml in water), 400 MBq  $^{99\text{m}}\text{TcO}_4^-$  solution and 20  $\mu\text{l}$  of tin(II) solution (10 mg of  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  in 10 ml 0.1 N HCl), pH adjusted to pH 6 with 0.2 N  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  in a total volume of 1 ml at 80°C for 30 min.

**Tricine/EDDA exchange labelling** In a rubber-sealed vial, 20 µg of HYNIC-MG11 was incubated with 1 ml of EDDA/tricine solution (20 mg/ml tricine, 10 mg/ml EDDA in water), 800 MBq of  $^{99m}\text{TcO}_4^-$  solution and 20 µl of tin (II) solution (10 mg of  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  in 10 ml 0.1 N HCl), pH adjusted to pH 6 with 0.2 N  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  in a total volume of 2 ml at 100°C for 10 min.

Radiolabelling yields were determined by HPLC, and for in vitro assays, purification of the radiolabelled peptide was performed by solid phase extraction (SPE). For this purpose the radiolabelling mixture was passed through a C18-SepPak-Light cartridge (Water, Milford, MA). The cartridge was washed with 5 ml saline, and the radiolabelled peptide eluted with 50% ethanol. This method efficiently removed all hydrophilic, non-peptide-bound impurities (mainly  $^{99m}\text{TcO}_4^-$ ,  $^{99m}\text{Tc}$  coligands).

#### In vitro evaluation of radioligands

##### Stability studies

The stability of the radiolabelled peptide in aqueous solution was tested by incubation of the SPE-purified reaction mixtures at a concentration of 200–1,000 pmol peptide/ml in phosphate buffer pH 7.4 (PBS), in PBS containing a 10,000-fold molar excess of cysteine over the peptide and in fresh human plasma at 37°C for up to 24 h. After incubation, plasma samples were precipitated with acetonitrile and centrifuged (1,750 g, 5 min). Degradation of the  $^{99m}\text{Tc}$  complexes was assessed by HPLC.

For incubation in liver and kidney homogenates, liver or kidneys freshly excised from rat were rapidly rinsed and homogenised in 20 mM HEPES buffer pH 7.3 with an Ultra-Turrax T25 homogenator for 1 min at room temperature.  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11 was incubated with fresh 30% homogenates at a concentration of 250–500 pmol peptide/ml at 37°C for up to 2 h. Samples were precipitated with acetonitrile, centrifuged (1,750 g, 5 min) and analysed by HPLC.

##### Receptor binding studies

The binding affinity of the cold peptide conjugate was tested in a competition assay against [ $^{125}\text{I}$ -Tyr $^{12}$ ]-gastrin I (Perkin Elmer Life Science, Boston, MA). Rat pancreatic tumour (AR4-2J) cell membranes were used as a source for gastrin receptors. Membrane preparation was performed as previously described [16]. In a Multiscreen well plate (Millipore Corporation, Bedford, MA), 50 µl competitor solution of increasing concentrations (0.0001–1000 nM in 1% BSA/10 mM  $\text{MgCl}_2$ /10 µM Bacitracin), 50 µl of radioligand solution (50,000 cpm in 1% BSA/10 mM  $\text{MgCl}_2$ /10 µM

Bacitracin) and 100 µl of membrane solution (50 µg protein/tube) were incubated in triplicate for 2 h at room temperature. Incubation was interrupted by filtration of the medium and rapid rinsing with ice-cold washing buffer (1×200 µl, followed by 1×50 µl 15 mM TRIS/139 mM saline pH 7.4), and filters were counted in a gamma counter.  $\text{IC}_{50}$  values were calculated following non-linear regression with Origin software (Microcal Origin 5.0, Northampton, MA).

Additionally, a saturation assay was performed for  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11. For this purpose, AR4-2J cells were seeded at a density of  $1 \times 10^6$  cells per well in six-well plates (Greiner Labortechnik, Germany) and grown to confluency for 48 h. On the day of the experiment, the cells were incubated in triplicate with radiolabelled peptide conjugate of increasing concentrations (0.2–40 nM) at room temperature for 2 h and treated as previously described [16]. Non-specific binding was determined by a parallel series containing 0.5 µM minigastrin I human (MGh).  $K_d$  values were calculated following non-linear regression and Scatchard plot (linear regression) with Origin software (Microcal Origin 5.0, Northampton, MA).

##### Cell uptake studies

For internalisation experiments, AR4-2J cells were seeded at a density of  $1 \times 10^6$  cells per well in six-well plates (Greiner Labortechnik, Germany) and grown to confluency for 48 h. On the day of the experiment, cells were incubated in triplicate with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11 (corresponding roughly to 200 fmol total peptide) alone (total series) or with 5 µM MGh (non-specific series) at 37°C for each time point of 5 min, 15 min, 30 min, 1 h and 2 h incubation time and treated as previously described [16]. The collected fractions were counted in a gamma counter and mean specific values were calculated. The specifically internalised fraction was expressed in relation to the total activity added (% of total activity) as well as in relation to the activity bound to the cells, i.e. internalised plus membrane-bound fraction (% of cell bound activity). Internalisation studies additionally were performed on reaction side products separated by HPLC.

For externalisation experiments (efflux studies), AR4-2J cells were treated as described for internalisation experiments and incubated with the radioligand for 2 h. Incubation was interrupted by removal of the medium and rapid rinsing with ice-cold internalisation medium two times, followed by an acid wash (with 50 mM glycine buffer pH 2.8, 0.1 M NaCl) for 5 min to remove membrane-bound radioligand. The cells were supplied with fresh medium alone or with 0.5 µM MGh, taking out small aliquots of supernatant and reconstituting the volume with fresh medium for each time point of 5 min, 15 min, 30 min, 45 min, 1 h and 1.5 h. Finally, cells were lysed by treatment with 1 N NaOH and collected

(internalised radioligand fraction). All fractions collected were counted in a gamma counter to determine the externalised fraction, expressed in relation to the specifically internalised radioligand fraction (% of internalised activity).

#### In vivo evaluation of radiolabelled peptides

All animal experiments were conducted in compliance with the Austrian animal protection laws and with the approval of the Austrian Ministry of Science. Biodistribution studies were performed in female nude mice (Charles River, Germany). For the induction of tumour xenografts, AR4-2J cells were subcutaneously injected at a concentration of  $10 \times 10^6$  cells/mouse and tumours were allowed to grow until they had reached a size  $>0.2$  ml (10–15 days). On the day of the experiment, the animal received  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11 (1 MBq/mouse, corresponding to  $\sim 0.05$   $\mu\text{g}$  peptide) intravenously into the tail vein, with or without co-injection of 50  $\mu\text{g}$  MGh. They were sacrificed in groups of three animals by cervical dislocation 1 or 4 h post injection. Tumours and other tissues (blood, lung, heart, stomach, spleen, liver, pancreas, kidneys, muscle, intestine) were removed. The amount of radioactivity was determined with a gamma counter. Results were expressed as percentage of injected dose per gram of tissue (% ID/g). Paired *t* test (significance level 0.05) was used for statistical analysis and tumour to organ ratios were calculated.

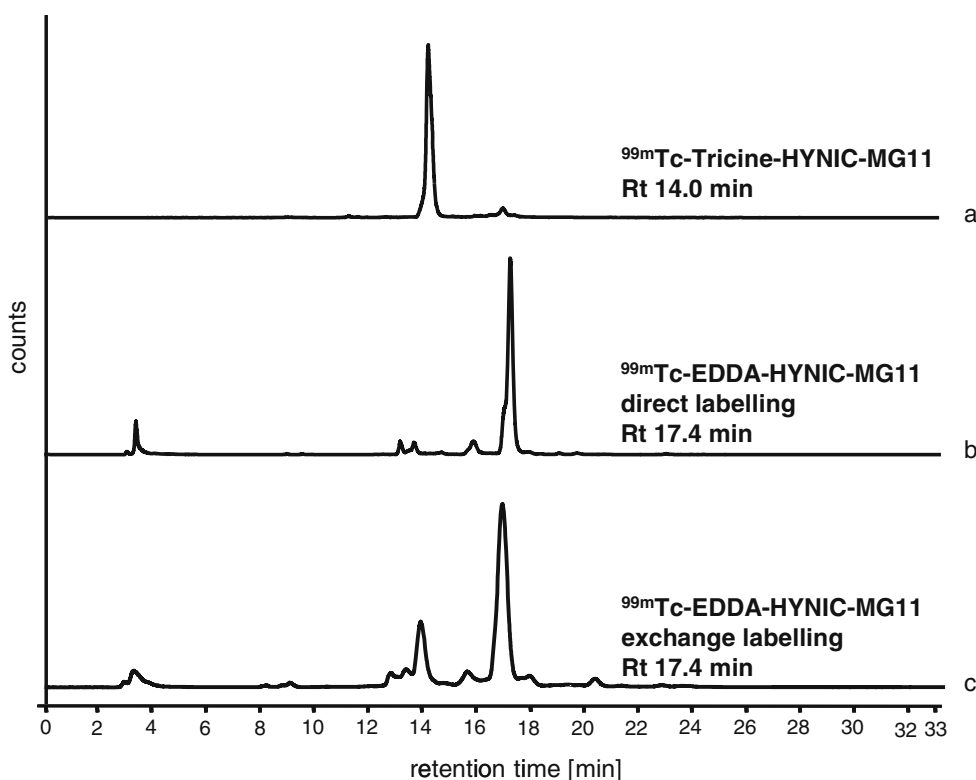
Tumour uptake studies were additionally performed on reaction side products separated by RP-HPLC.

## Results

#### Radiolabelling

HYNIC-MG11 could be radiolabelled with  $^{99m}\text{Tc}$  at high specific activities ( $>70$  GBq/ $\mu\text{mol}$ ) in high yields; however, some coligand-related differences could be observed. Radiolabelling with tricine as coligand resulted in high radiochemical purity (RCP)  $>90\%$  with a single peak, whereas radiolabelling with EDDA as coligand showed a labelling yield strongly influenced by the labelling approach. With the previously reported exchange labelling from tricine to EDDA at  $100^\circ\text{C}$  for 15 min, labelling yields were  $<90\%$ . Only by direct labelling, with incubation at  $80^\circ\text{C}$  for 30 min, a RCP  $>90\%$  could be achieved. Figure 1 shows typical HPLC radiochromatograms of different reaction mixtures. Beside the main peak with a retention time (Rt) of 17.4 min, corresponding to the radiolabelled peptide, some additional minor peaks are observed, which are related to hydrophilic impurities, such as free pertechnetate and  $^{99m}\text{Tc}$ -EDDA, eluting at an early Rt of 2.8–4.0 min, and peptide-related peaks with an Rt of 13.6 and 16.0 min.

**Fig. 1** HPLC radiochromatograms of  $^{99m}\text{Tc}$ -labelled HYNIC-MG11: **a** direct labelling with tricine as coligand; **b** direct labelling with EDDA as coligand; **c** exchange labelling from tricine to EDDA showing incomplete conversion of the tricine complex into the EDDA complex



Stability studies

Results from stability studies are summarised in Table 1 in comparison with previous results obtained with <sup>99m</sup>Tc-EDDA-HYNIC-MG0 [16]. Incubation of SPE-purified peptide in PBS, PBS/cysteine and plasma for up to 24 h revealed high stability of both radiolabelled peptides. At 24 h after incubation, <sup>99m</sup>Tc-EDDA-HYNIC-MG11 showed higher stability (95%) in solution compared with <sup>99m</sup>Tc-tricine-HYNIC-MG11 (77.5%), while its stability in plasma and towards cysteine challenge was lower (89.4% and 85.8%, respectively) than that of <sup>99m</sup>Tc-EDDA-HYNIC-MG0 (90.9% and 96.4%, respectively). Based on the low stability of <sup>99m</sup>Tc-tricine-HYNIC-MG11 and other experiences with tricine as coligand [16, 20], further studies were performed with <sup>99m</sup>Tc-EDDA-HYNIC-MG11 only.

Investigation of the metabolic stability of <sup>99m</sup>Tc-EDDA-HYNIC-MG11 in rat kidney and liver homogenates showed a very rapid decrease in radioactivity related to the original peptide peak, which was much more pronounced compared with <sup>99m</sup>Tc-EDDA-HYNIC-MG0. For incubation in liver homogenate, less than 5% intact peptide could be detected after 30-min incubation (Fig. 2a), whereas with kidney homogenate this value was already reached 10 min after incubation (Fig. 2b).

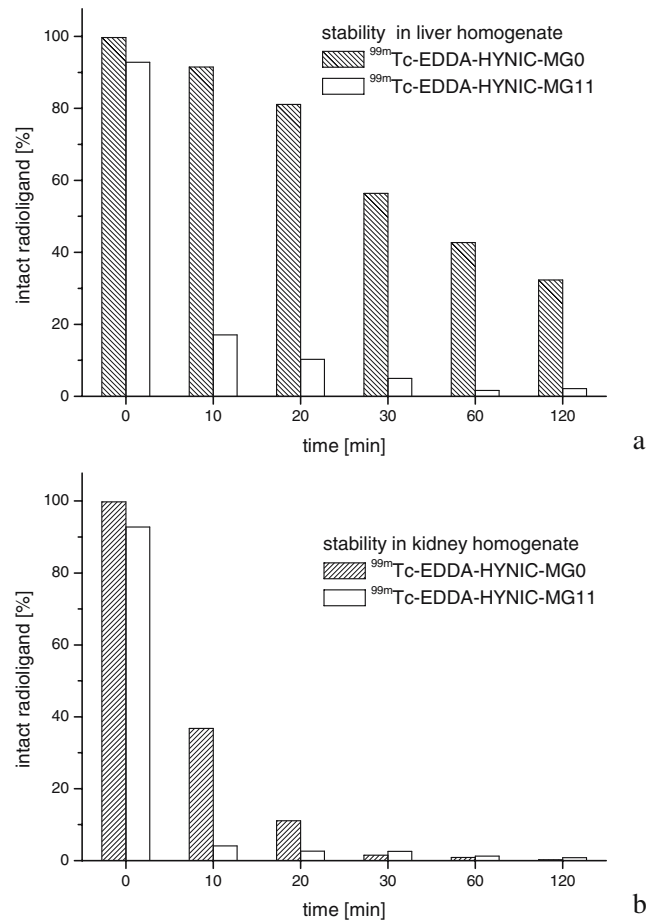
Receptor binding and internalisation

Displacement of [<sup>125</sup>I-Tyr<sup>12</sup>]-gastrin I showed high affinity of HYNIC-MG11 to the gastrin/CCK-2 receptor, with an IC<sub>50</sub> value in the nanomolar range (<2 nM). This finding

**Table 1** Stability of <sup>99m</sup>Tc-labelled HYNIC-MG in aqueous solutions

	Duration of incubation			
	1 h	2 h	4 h	24 h
<sup>99m</sup> Tc-tricine-HYNIC-MG0 (%)				
Phosphate	98.9	96.3	91.1	80.8
Cysteine	98.4	92.5	88.4	81.4
Plasma	98.9	98.4	96.7	94.6
<sup>99m</sup> Tc-EDDA-HYNIC-MG0 (%)				
Phosphate	99.4	99.1	99.4	94.8
Cysteine	99.2	98.7	99.1	96.4
Plasma	99.3	96.6	97.4	90.9
<sup>99m</sup> Tc-tricine-HYNIC-MG11 (%)				
Phosphate	98.6	98.6	nd	77.5
Cysteine	99.0	97.4	nd	88.2
Plasma	98.8	97.2	nd	87.7
<sup>99m</sup> Tc-EDDA-HYNIC-MG11 (%)				
Phosphate	99.6	98.9	98.6	95.0
Cysteine	99.7	96.0	91.2	85.8
Plasma	99.2	99.2	98.1	89.4

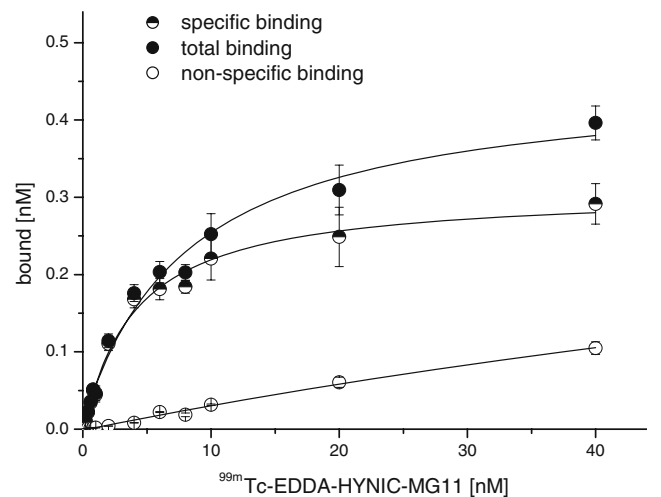
nd not determined



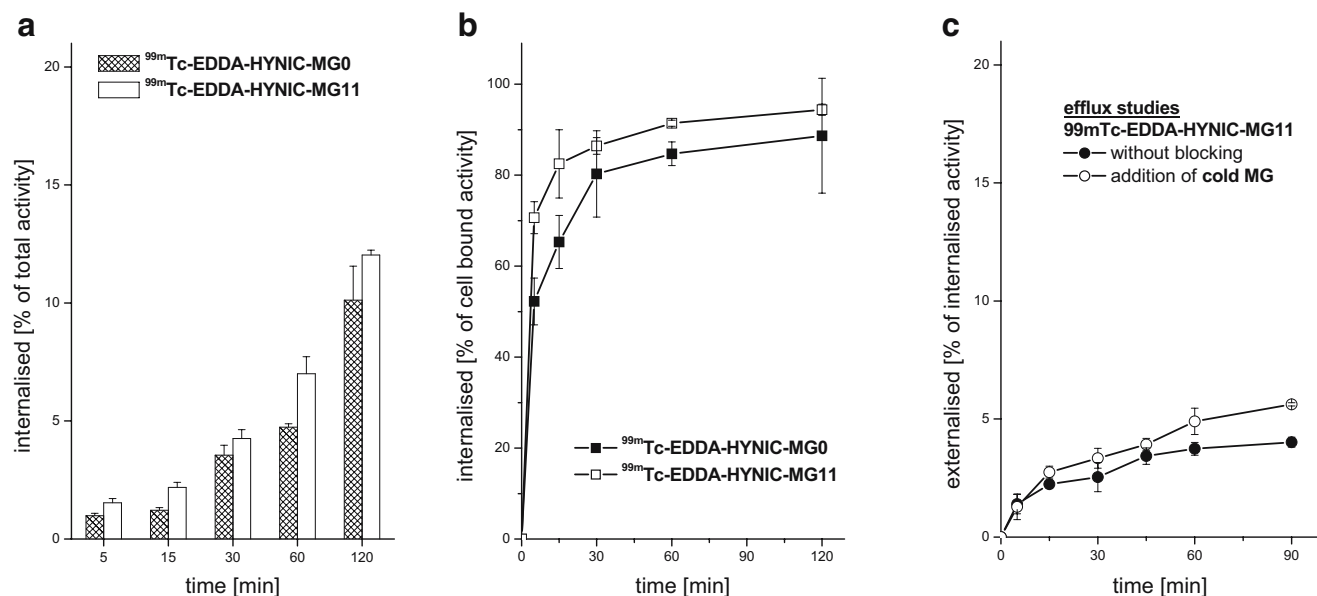
**Fig. 2** Stability of <sup>99m</sup>Tc-labelled HYNIC-MG in rat tissue homogenates: **a** liver, **b** kidney

was confirmed by a saturation assay for <sup>99m</sup>Tc-EDDA-HYNIC-MG11 revealing a K<sub>d</sub> of 3.97 nM (Fig. 3).

Internalisation behaviour of <sup>99m</sup>Tc-EDDA-HYNIC-MG11 on AR4-2J cells, shown in Fig. 4, is comparable to



**Fig. 3** Receptor binding of <sup>99m</sup>Tc-EDDA-HYNIC-MG11 on AR4-2J cells: saturation curve of <sup>99m</sup>Tc-EDDA-HYNIC-MG11 showing total binding, non-specific binding and specific binding, with a calculated K<sub>d</sub> of 3.97 nM



**Fig. 4** Time-dependent cell uptake of  $^{99m}\text{Tc}$ -labelled HYNIC-MG in AR4-2J cells: **a** internalisation expressed as % of total activity, **b** internalisation expressed as % of cell-bound activity and **c** efflux expressed as % of internalised activity (mean $\pm$ SD,  $n=3$ )

previous findings with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0 [16]. More than 10% ( $12.0\pm 0.21\%$ ) of the total activity added is internalised after 2-h incubation time (Fig. 4a). A slightly increased internalisation rate of the specifically cell-bound activity corresponding to  $94.4\pm 1.22\%$  after 2-h incubation was observed, showing a plateau of more than 80% internalised fraction 15 min after incubation (Fig. 4b). Studies additionally performed on the reaction side products with Rt 13.6 min and 16.0 min showed an almost quantitative internalisation of the specifically membrane-bound fraction ( $>80\%$ ); however, less than 1% of the total activity was accumulated in the cells after 2 h (data not shown).

Efflux studies revealed a very low percentage of released radioactivity (Fig. 4c). Within 1.5 h only  $4.02\pm 0.21\%$  of the internalised activity was released, and this value was only slightly increased in the presence of MGh ( $5.62\pm 0.07\%$ ).

#### Biodistribution and tumour uptake

Results of biodistribution in nude mice bearing AR4-2J tumour xenografts 1 and 4 h p.i. are summarised in Table 2 and Fig. 5, again in comparison with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0 [16]. Generally, rapid elimination from most organs and mainly renal excretion could be observed. Despite slightly increased unspecific tissue uptake in comparison with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0, especially in spleen, intestine and liver, clearly less renal retention ( $2.44\pm 0.97\%$  and  $1.96\pm 0.14\%$  ID/g 1 and 4 h p.i.) was observed. This implies a reduction of kidney uptake of 98% compared with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0 ( $101\pm 3.47\%$  ID/g 4 h p.i.),

which was not further reduced significantly by co-injection of MGh.

Tumour uptake did not vary considerably, with  $4.77\pm 0.72\%$  and  $7.11\pm 0.22\%$  ID/g 1 and 4 h p.i., respectively, compared with  $8.09\pm 1.87\%$  ID/g 4 h p.i. of  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0. Tumour uptake was significantly blocked by co-injection of  $50\ \mu\text{g}$  MGh, reducing this value to  $1.37\pm 0.54\%$  and  $1.10\pm 0.26\%$  ID/g 1 and 4 h p.i., corresponding to a reduction of 71% and 85%, respectively. A significant reduction of tissue uptake by co-injection of MGh was also observed in receptor-expressing organs, stomach and pancreas, 1 h p.i.

Tumour uptake studies additionally performed on the reaction side products with Rt 13.6 and 16.0 min showed higher levels in almost all tissues 1 h p.i., in particular spleen ( $0.30\pm 0.08\%$  ID/g), intestine ( $4.60\pm 1.79\%$  ID/g) and liver ( $0.81\pm 0.16\%$  ID/g), whereas no specific tumour uptake could be found (Table 2 and Fig. 6).

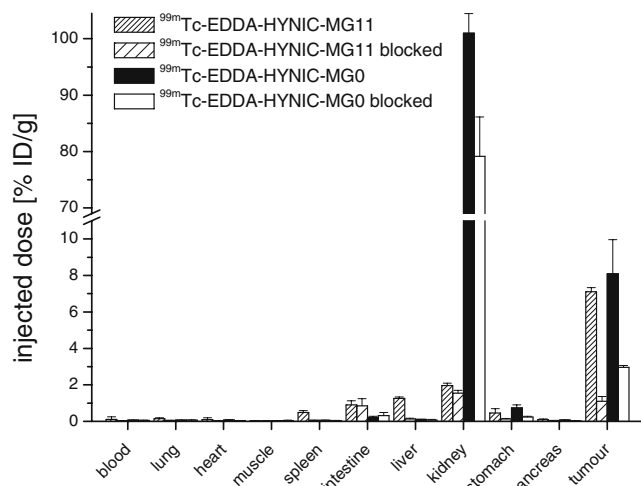
#### Discussion

The [Tc]HYNIC core is of particular interest for the radiolabelling of small peptide analogues with  $^{99m}\text{Tc}$ . HYNIC-derivatised peptides can be easily labelled with very high radiolabelling efficiency at high specific activity. The HYNIC moiety occupies only one coordination site via a Tc-diazenido linkage, and the coligands, tricine and EDDA, complete the coordination sphere. We could show that radiolabelling yields strongly depend on the optimisation of the labelling approach. For HYNIC-MG11 using

**Table 2** Biodistribution of <sup>99m</sup>Tc-EDDA-HYNIC-MG11 (1 and 4 h p.i., including peptide-related side products 1 h p.i.) in comparison with <sup>99m</sup>Tc-EDDA-HYNIC-MG0 (4 h p.i.) in AR4-2J tumour-bearing nude mice

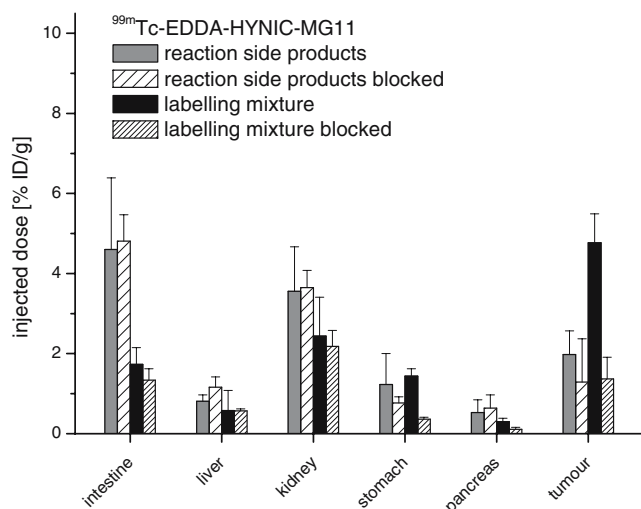
Organ	<sup>99m</sup> Tc-EDDA-HYNIC-MG11	<sup>99m</sup> Tc-EDDA-HYNIC-MG11 blocked	Side products of <sup>99m</sup> Tc-EDDA-HYNIC-MG11	Side products of <sup>99m</sup> Tc-EDDA-HYNIC-MG11 blocked
1 h p.i.				
Blood	0.40±0.12	0.47±0.47	0.82±0.50	0.99±0.003
Lung	0.51±0.18	0.26±0.03	0.73±0.34	0.96±0.20
Heart	0.19±0.07	0.13±0.01	0.46±0.15	0.49±0.06
Muscle	0.40±0.33	0.08±0.05	0.46±0.35	0.31±0.01
Spleen	0.13±0.02	0.12±0.02	0.30±0.08	0.39±0.12
Intestine	1.73±0.42	1.34±0.28	4.60±1.79	4.81±0.66
Liver	0.28±0.03	0.57±0.05	0.81±0.16	1.16±0.26
Kidney	2.44±0.97	2.18±0.40	3.56±1.11	3.65±0.43
Stomach	1.44±0.18*	0.36±0.05	1.23±0.77	0.77±0.15
Pancreas	0.30±0.09*	0.11±0.05	0.53±0.32	0.64±0.33
Tumour	4.77±0.72*	1.37±0.54	1.98±0.59	1.29±1.08
4 h p.i.				
Organ	<sup>99m</sup> Tc-EDDA-HYNIC-MG11	<sup>99m</sup> Tc-EDDA-HYNIC-MG11 blocked	<sup>99m</sup> Tc-EDDA-HYNIC-MG0	<sup>99m</sup> Tc-EDDA-HYNIC-MG0 blocked
Blood	0.11±0.14	0.02±0.02	0.06±0.002	0.05±0.01
Lung	0.15±0.05	0.05±0.001	0.06±0.01	0.07±0.01
Heart	0.09±0.10	0.02±0.01	0.06±0.03	0.04±0.002
Muscle	0.02±0.02	0.01±0.03	0.03±0.004	0.04±0.02
Spleen	0.49±0.10	0.05±0.02	0.05±0.01	0.04±0.01
Intestine	0.90±0.23	0.85±0.39	0.21±0.07	0.32±0.16
Liver	1.26±0.07	0.13±0.04	0.10±0.01	0.07±0.03
Kidney	1.96±0.14*	1.54±0.16	101.00±3.47*	79.14±6.98
Stomach	0.46±0.23	0.13±0.03	0.73±0.17*	0.23±0.04
Pancreas	0.09±0.04	0.02±0.03	0.07±0.01*	0.03±0.003
Tumour	7.11±0.22*	1.10±0.26	8.09±1.87*	2.96±0.10

\*Significant  $p=0.05$   
 Values are expressed as % ID/g = percentage injected dose/g tissue (means±SD,  $n=3$ )



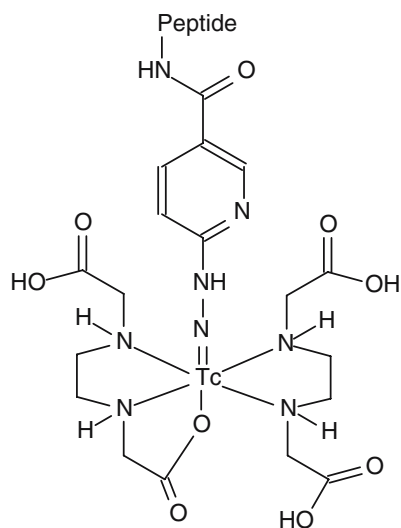
**Fig. 5** Biodistribution of <sup>99m</sup>Tc-labelled HYNIC-MG in AR4-2J tumour-bearing nude mice 4h after injection. Values are expressed as % ID/g (means±SD,  $n=3$ )

EDDA as coligand, only by direct labelling yields >90% could be achieved, while for the exchange labelling approach from tricine to EDDA only incomplete conversion of the Tc-tricine complex into the Tc-EDDA complex was found. This finding is in contrast to previous results with HYNIC-MG0 [16] and a HYNIC-derivatised somatostatin analogue [21], where high labelling yields with EDDA as coligand were achieved only when applying the exchange labelling approach. Still, the HYNIC technology can be regarded as a straightforward approach to achieve radiolabelling of peptides with <sup>99m</sup>Tc; however, some questions regarding the exact structure of the resulting Tc complex remain. We have recently shown that the <sup>99m</sup>Tc-EDDA-HYNIC complex is composed of two coligand molecules per peptide molecule: the suggested molecular structure is shown in Fig. 7. Additional peaks found in the radiochromatograms of <sup>99m</sup>Tc-EDDA-HYNIC-MG11 can be explained by isomerism, or an involvement of the carboxyl group of the dGlu residue at the amino terminus,



**Fig. 6** Biodistribution in AR4-2J tumour-bearing nude mice of peptide-related reaction side products of  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11 in comparison with the labelling mixture 1 h after injection. Values are expressed as % ID/g (means $\pm$ SD,  $n=3$ )

as reported for DTPA-MG0 [8]. This additional species may have only minor effects on the in vivo behaviour, as long as the interaction of the receptor binding sequence -Trp-Met-Asp-Phe-NH<sub>2</sub> with the CCK-2 receptor is not impaired. The presence of methionine in the peptide chain additionally poses the risk of formation of oxidative side products, especially when a heating step is included in the radiolabelling process. We could show that by-products have considerably reduced cell internalisation values and much lower tumour uptake in vivo, indicating a lower affinity of the side products for the CCK-2 receptor. We therefore believe that the side products observed are a result



Peptide:

-D-Glu-Glu<sub>5</sub>-Ala-Tyr-Gly-Trp-Met-Asp-PheNH<sub>2</sub> (HYNIC-MG0)

-D-Glu-Ala-Tyr-Gly-Trp-Met-Asp-PheNH<sub>2</sub> (HYNIC-MG11)

**Fig. 7** Molecular structure proposed for  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG

of changes in the receptor binding sequence, specifically oxidation of methionine during radiolabelling, rather than structural variations of the Tc complex. Breeman et al. have recently shown that for DOTA-MG11 radiolabelled with  $^{111}\text{In}$ , oxidation can be reduced by optimising the reaction conditions [22].

In vitro stability studies revealed a generally high stability of  $^{99m}\text{Tc}$ -HYNIC-MG11 complexes, which was somewhat lower for tricine as coligand. Similar findings were obtained with HYNIC-MG0 in our previous study [16], where this lower stability for tricine as coligand led to an unfavourable biodistribution. We therefore decided to exclude  $^{99m}\text{Tc}$ -tricine-HYNIC-MG11 from further characterisation. Whereas the stability in PBS reflects the stability of the formed complex, the stability towards cysteine challenge and in plasma predict possible ligand exchange and stability in vivo. Both of these parameters seemed to be favourable for  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11: only a slightly increased degradation could be observed in comparison with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0. However, degradation in rat liver and kidney homogenates was markedly more rapid in comparison with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0, and these results might better reflect the situation in vivo regarding enzymatic degradation by peptidases.

The affinity for the CCK-2 receptor of HYNIC-MG11 was found to be in the nanomolar range, and saturation assays performed with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11 ( $K_d=3.97$  nM) revealed higher affinity than was found for  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0 ( $K_d=10.3$  nM) [16]. Internalisation studies showed rapid specific internalisation (>10% of the total activity added and >90% of the cell-bound activity 2 h after incubation), comparable to that observed with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0, and persisting retention of the internalised fraction in the tumour cells. Only very low efflux of radioactivity of about 5% at 90 min after incubation could be detected. In contrast to this finding, a higher externalisation rate up to 40% after 4 h has been reported for MG11 radiolabelled with  $^{111}\text{In}$ , using DTPA or DOTA as a bifunctional coupling agent [23]. In accordance with the finding of a more than tenfold decrease in CCK-2 receptor affinity for the oxidised form of  $^{111}\text{In}$ -DOTA-MG11 [22], the reaction side products of  $^{99m}\text{Tc}$ -HYNIC-MG11 with  $R_t$  13.6 min and 16.0 min showed very low cell uptake, underlining the importance of avoiding formation of oxidative side products.

Biodistribution studies in tumour-bearing nude mice revealed rapid renal excretion and low unspecific retention in most organs. Despite the low stability against enzymatic degradation found in vitro, very high tumour uptake of  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11 was found, with values of 4.77% and 7.11% ID/g 1 and 4 h p.i.; these values are similar to previous findings obtained with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0 (8.09% ID/g 4 h p.i.) [16]. In comparison,



Nock et al. characterised  $^{99m}\text{Tc}$ -labelled demogastrin 2 in the same animal model, and observed decreasing tumour uptake over time (from 5.50% ID/g 1 h p.i. to 2.88% ID/g 4 h p.i.) [10].  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11 showed clearly less renal uptake and retention (1.96% ID/g) than  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0 (101% ID/g), resulting in a reduction in renal uptake by 98%. Similar findings have been reported for  $^{99m}\text{Tc}$ -labelled MG0 based on the carbonyl labelling approach [16] and for DTPA- and DOTA-MG11 radiolabelled with  $^{111}\text{In}$  [23]. Behe et al. have shown that kidney uptake is driven by negatively charged amino acids and can be blocked significantly by co-infusion of penta-L-glutamic acids or longer chains [18]. This explains the reduction in kidney uptake of our MG11 derivative in comparison with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0, where the five glutamic acids cause this specific uptake. However, unspecific tissue uptake was somewhat increased, leading to lower tumour to organ ratios. This finding might in part be explained by the lower hydrophilicity of HYNIC-MG11 compared with HYNIC-MG0. One could also argue that the pentaglutamic acid sequence in MG0 not only is responsible for the specific uptake by the kidneys, but also protects enzymatic cleavage by peptidases. In addition to the findings of potential oxidative side products showing almost no specific tumour uptake, the higher metabolic instability of MG11 impairs the overall biological behaviour.  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG11 still shows advantages in comparison with  $^{99m}\text{Tc}$ -EDDA-HYNIC-MG0, especially regarding the reduction in kidney uptake. Tumour to organ ratios as calculated from the animal tumour model are somewhat lower, except for the kidneys, but are still comparable to other radioligands of widespread use in nuclear medicine [20]. Further improvements in terms of increasing stability using, for example, cyclic peptide derivatives and replacement of methionine by other amino acids such as norleucine and leucine [17] should be considered to avoid the formation of oxidative side products in the radiolabelling process and to improve the metabolic stability.

## Conclusion

$^{99m}\text{Tc}$ -EDDA-HYNIC-MG11, with high tumour uptake and reduced kidney uptake, is a promising new radioligand for the diagnosis of MTC and other CCK-2-expressing malignancies. However, the presence of oxidative side products in the radiolabelling process and the comparatively low metabolic stability still leave room for further improvement.

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