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Original article

Novel ^{99m}Tc '4 + 1' peptide conjugates: Tuning the biodistribution by variation of coligands

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ABSTRACT

A sophisticated coligand strategy is presented for peptide-derived radioconjugates based on ^{99m}Tc '4 + 1' mixed-ligand complexes. The new pharmacologically active coligands are assessed for ^{99m}Tc-labeling of the RGD-peptide cyclo(Arg-Gly-Asp-D-Tyr-Lys) which is an established vehicle to target $\alpha_v\beta_3$ integrins playing a crucial part in tumor pathogenesis.

Complexes of the general formula [^{99m}Tc(NS$_3$R)X] were synthesized and evaluated, in which Tc(III) is coordinated by NS$_3$R, a derivative of the tetradentate chelator 2,2′,2″-nitrilotriethanethiol (NS$_3$), and by X, a monodentate binding isocyanide bearing the biomolecule. The novel tetradentate chelators (NS$_3$R = NS$_3$crown, NS$_3$en, NS$_3$(COOH)$_3$) constitute NS$_3$ with a crown ether, an amine or a tricarboxylic acid as pharmacological modifiers. The isocyanides (X = L2-RGD, L2-Lys) contained the linker isocyanobutanoic acid (L2) coupled to N^6-Lys of the RGD-peptide and additionally to a single Lys.

The lipophilicity (distribution coefficient log $D_{O/W}$, pH = 7.4) of the RGD-containing radiotracers decreased in the order of the coligands NS$_3$crown (−1.7 ± 0.1), NS$_3$en (−2.7 ± 0.1) and NS$_3$(COOH)$_3$ (−3.3 ± 0.1). In the same order of the coligands, the biodistribution of the series [^{99m}Tc(NS$_3$R)(L2-RGD)] in normal rats showed a decrease of hepatobiliary and an increase of urinary excretion.

The ratio of specifically to unspecifically uptaken activity (sum of surface bound and internalized activity) in $\alpha_v\beta_3$ integrin-expressing M21 cells was in the range of approximately 4–5 and comparable for all [^{99m}Tc(NS$_3$R)(L2-RGD)] tracers. The biodistribution of [^{99m}Tc(NS$_3$en)(L2-RGD)] in v/v mice bearing M21 and M21L (control) tumor xenografts exhibited a specific tumor uptake with a low target-background ratio.

The metabolic stability of the [^{99m}Tc(NS$_3$R)(L2-RGD)] tracers in normal rats was high, since 75–87% of the radioactivity in the plasma extract was assigned to the injected radiotracers 60 min after intravenous application in a rat. The hypothetical metabolites [^{99m}Tc(NS$_3$R)(L2-Lys)] were not found.

These results demonstrate a considerable improvement of *in vivo* properties of ^{99m}Tc '4 + 1' peptide conjugates and open up the possibility of applying the labeling approach for further radiodiagnostic peptides.

1. Introduction

In the search for new radioactive technetium and rhenium labeled biomolecules for radiopharmaceutical purposes, our group has focussed on the development of '4 + 1' mixed-ligand complexes. The '4 + 1' framework [M(NS$_3$)X] consists of Tc/Re(III) coordinated by a tetradentate tripodal chelator NS$_3$ (2,2′,2″-nitrilotriethanethiol) and a monodentate isocyanide or phosphine (X) [1–3]. Radioconjugates containing '4 + 1' complexes attached to biomolecules including serotonin receptor ligands [4], fatty acids [5–9], quinazolines [10] and peptides [11–13] have been investigated. These studies demonstrated a primarily lipophilic nature of the '4 + 1' framework. This is an inherent characteristic, since the metal is well shielded by the '4 + 1' donor atom arrangement. The

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application to the ^{99m}Tc-labeling of comparatively hydrophilic biomolecules like peptides is therefore limited. The concept of '4 + 1' radioconjugates opens the possibility of varying the biobehavior by using tetradentate coligands grafted with pharmacokinetic modifiers. In the present paper we address these variation possibilities and describe the synthesis and characterization of three novel NS_3-chelators modified with a crown ether, an amine or a tricarboxylic acid (NS_3crown, NS_3en, $NS_3(COOH)_3$). These units are intended to decrease the lipophilicity under physiological conditions by a neutral hydrophilic, a positively charged and a negatively charged group and may considerably improve the *in vivo* characteristics of a labeled peptide bearing a ^{99m}Tc '4 + 1' mixed-ligand complex. The '4 + 1' complexes containing the new coligands were assessed conjugated to the RGD-peptide cyclo(Arg-Gly-Asp-D-Tyr-Lys).

Radioconjugates containing the RGD-motive have exhibited a great potential for radiopharmaceutical applications as reviewed by Haubner [14], Meyer et al. [15], Dijkgraaf and Boerman [16], Schottelius et al. [17] and Liu [18] for example. In particular, the RGD interacts with the target $\alpha_v\beta_3$ integrin that is involved in pathophysiological processes such as tumor metastasis, neoangiogenesis, osteoporosis, restenosis and inflammation. RGD-based radiotracers have already been studied with a large variety in the RGD-containing unit, in a modifying unit such as carbohydrate or polyethylene glycol, and in the labeling system including the nuclides ^{64}Cu, ^{18}F, ^{68}Ga, ^{123}I, ^{125}I, ^{111}In, ^{177}Lu, ^{99m}Tc and ^{90}Y [14–18].

In this paper, the cyclo(Arg-Gly-Asp-D-Tyr-Lys) was selected as biomolecule since its radioconjugates, consisting of a radiometal complex bound via Lys to the peptide, have been shown to target integrins with high affinity over-expressed in carcinomas [12,19,20]. Further, the cyclo(Arg-Gly-Asp-D-Tyr-Lys) exhibited a high *in vivo* stability as deduced from previously described metabolism studies [21,22].

The RGD-peptide was modified with 4-isocyanobutanoic acid (L2) as linker conjugated to N^6-Lys and then the conjugates [^{99m}Tc(NS_3crown)(L2-RGD)], [^{99m}Tc(NS_3en)(L2-RGD)], [^{99m}Tc($NS_3(COOH)_3$)(L2-RGD)] were prepared. The compounds were characterized by means of chromatography, determination of distribution coefficients, biodistribution and metabolism studies. Previous *in vivo* metabolism studies of radiolabeled RGD-derivatives containing cyclo(Arg-Gly-Asp-D-Phe-Lys) in mice exhibited the formation of one main metabolite of the respective radiotracer but the metabolites were not identified [21,22]. For the metabolism studies of the [^{99m}Tc(NS_3R)(L2-RGD)] series the formation of [^{99m}Tc(NS_3R)(L2-Lys)] as metabolites was assumed. Therefore the conjugates [^{99m}Tc(NS_3crown)(L2-Lys)], [^{99m}Tc(NS_3en)(L2-Lys)] and [^{99m}Tc($NS_3(COOH)_3$)(L2-Lys)] containing Lys in place of the RGD-peptide were prepared and served as reference compounds.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of coligands

The new tripodal tetradentate ligands to form the '4 + 1' complexes of the isocyanide functionalized biomolecules were designed as $N(CH_2{-}CH_2{-}SH)_3$-chelator ('NS_3-chelator') bearing three different pharmacokinetic modifiers – a crown ether, an amine and a tricarboxylic acid. These NS_3-chelators were synthesized by coupling (DCC-activation) the modifier molecules to $N(Sbz)_3COOH$ followed by deprotection (Scheme 1).

The synthesis of $N(Sbz)_3COOH$ has been previously described [11]. The linker $H_2N{-}C(-CH_2{-}O{-}CH_2{-}CH_2{-}COO{-}Me)_3$ was prepared as described by Newkome et al. [23]. Finally, NS_3crown, NS_3en and $NS_3(COOH)_3$ were obtained (Scheme 1). These compounds were obtained as aminium chlorides, since the crude products were purified by extraction into the aqueous phase using an acidified product solution. Therefore the obtained coligand may contain a certain amount of sodium chloride which was judged as marginal for the intended use of the coligands. The identity of the obtained ligands was confirmed by ^{1}H NMR and ^{13}C NMR.

2.1.2. Isocyanide modification

The RGD-peptide c(RGDyK) and Lys were selected as molecules for monodentate attachment to the '4 + 1' framework. The RGD-peptide was obtained by customized standard peptide synthesis and its identity and purity were confirmed by ESI–MS and RP-HPLC. The monodentate ligands were formed by functionalization via the N^6 of Lys with 2,3,5,6-tetrafluorophenyl 4-isocyanobutanoate (L2-BFCA) which was designed as a bifunctional coupling agent (BFCA) [13]. For the modification of Lys, N^2 was protected by Fmoc, which was removed by piperidine. The obtained conjugates L2-RGD and L2-Lys are shown in Schemes 2 and 3.

The prepared conjugates were purified by RP-chromatography using a basic mobile phase, since isocyanides are unstable under acidic conditions and therefore RP-chromatography using an acidic TFA containing mobile phase is not possible. The conjugates were then lyophilized. The obtained ESI–MS data of the products were consistent with the molecular weights calculated for each compound.

2.1.3. ^{99m}Tc-labeling

The ^{99m}Tc-labeling by forming the '4 + 1' complexes [^{99m}Tc(NS_3en)(L2-RGD)], [^{99m}Tc(crown)(L2-RGD)], [^{99m}Tc($NS_3(COOH)_3$)(L2-RGD)], [^{99m}Tc(NS_3en)(L2-Lys)], [^{99m}Tc(NS_3crown)(L2-Lys)], and [^{99m}Tc($NS_3(COOH)_3$)(L2-Lys)], displayed in Fig. 1, was performed in a single step procedure (Scheme 4) that was recently described [24].

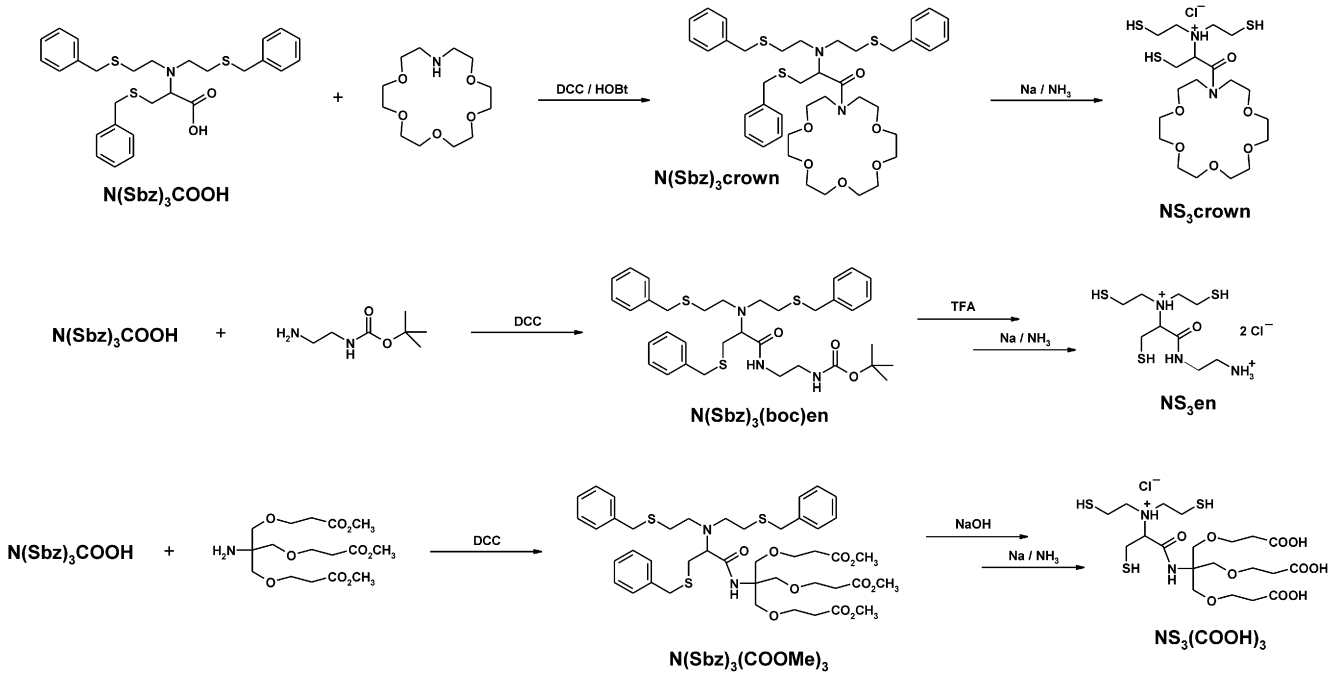
Accordingly, a labeling mixture was prepared containing eluate of a ^{99}Mo/^{99m}Tc generator, the NS_3-chelator, the isocyanide modified biomolecule, $SnCl_2$, Na_2EDTA, mannitol, ascorbic acid and ethanol, and allowed to react for about 60 min at room temperature. As previously described, this procedure enables a ^{99m}Tc-labeling with a radiochemical yield higher than 95% without the need for a purification step, if at least 50 nmol isocyanide and 500 nmol NS_3-chelator are used [24]. Lower amounts of the ligand were used in the present work, primarily because the synthesized NS_3-chelators contained a certain amount of NaCl that could not completely separated by the described preparation method. Therefore, the radioconjugates were purified by HPLC to give a radiochemical purity of higher than 95%. The ^{99m}Tc-labeled compounds used in animal studies were purified by RP-HPLC using a mobile phase containing water, ethanol and TFA. Ethanol was used in place of acetonitrile to prevent the contamination of the injection solution with the toxic acetonitrile.

The radioconjugates were stable in the HPLC eluate and in the injection solution within the test period of 24 h.

A comparison of the HPLC profiles of the ^{99m}Tc-containing radiotracer with those of analogous Re compounds which eluted at the same retention times gave a strong indication of the identity of the formed radioconjugates.

2.1.4. Rhenium reference compounds

The '4 + 1' Re compounds [Re(NS_3R)(BM)], NS_3R = NS_3crown, NS_3en, BM = L2-RGD, L2-Lys, (Fig. 1) were synthesized by exchanging the thiourea ligands of $[Re(tu)_6]Cl_3$ against NS_3R and PMe_2Ph resulting in the formation of [Re(NS_3R)(PMe_2Ph)]. Then, the phosphine was replaced by the appropriate isocyanide and purified by RP-HPLC. In order to synthesize [Re($NS_3(COOH)_3$)(L2-RGD)] and [Re($NS_3(COOH)_3$)(L2-Lys)], the same procedure was applied, resulting in

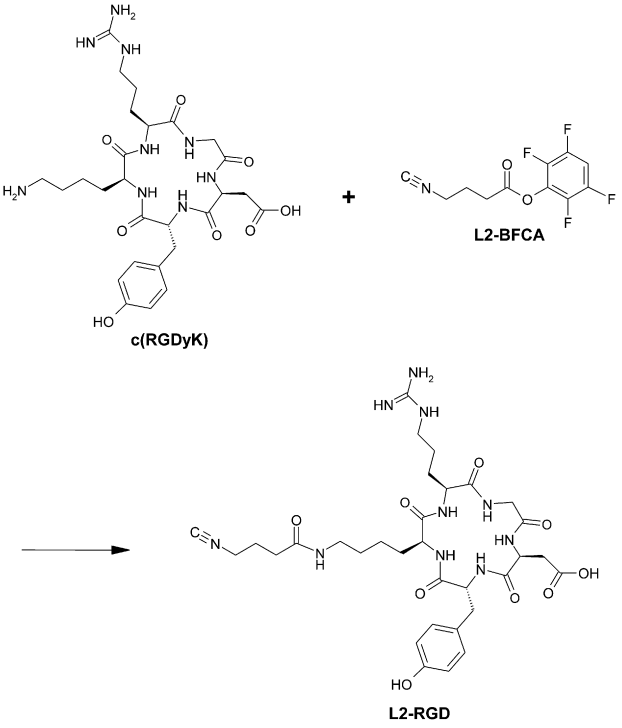


Scheme 1. Reaction routes to modified NS_3-ligands.

esterification of the three carboxylic acid groups that afforded a sapofinication of the isocyanide containing complex (Scheme 5). The obtained ESI–MS data of the products were consistent with the molecular weights calculated for each compound.

2.1.5. Lipophilicity

The lipophilicity of the labeled RDG-peptides was assessed by measuring their distribution in an octanol/water system buffered at pH 7.4. The results are shown in Table 1. The lipophilicity decreased in the order of the modifiers crown ether, ethylenediamine and tricarboxylic acid. In addition, the analogous complex containing NS_3COOH as coligand exhibited a log $D_{O/W}$ of −2.3 at pH 7.4, which shows that [^{99m}Tc($NS_3(COOH)_3$)(L2-RGD)] is considerably less lipophilic [12]. The determined distribution coefficients for [^{99m}Tc(NS_3en)(L2-RGD)] and [^{99m}Tc($NS_3(COOH)_3$)(L2-RGD)] are in the range of RGD-peptides labeled with ^{99m}Tc using the HYNIC approach [12,25] and are lower compared to the cyclo(Arg-Gly-Asp-D-Tyr-Lys) derivatives labeled via a ^{99m}Tc-tricarbonyl or a ^{99m}Tc-nitrido complex as previously reported [12].

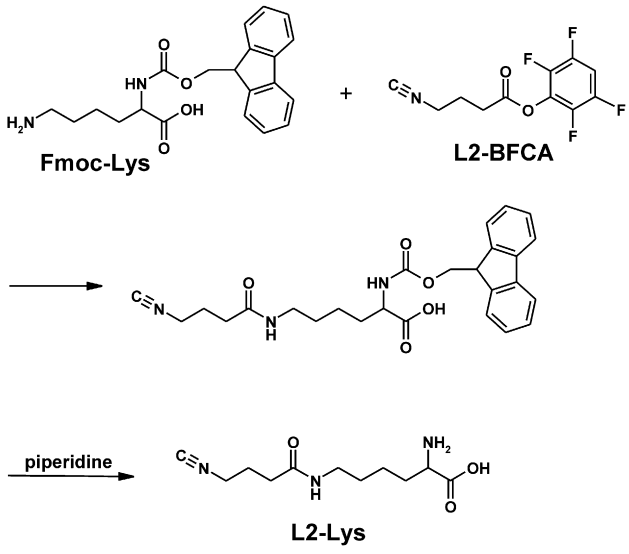


Scheme 2. Introduction of isocyanide onto RGD-peptide.

2.2. Pharmacology

2.2.1. Overall biodistribution

Selected biodistribution and elimination data after i.v. injection into rats of the radioconjugates [^{99m}Tc(NS_3R)BM],



Scheme 3. Introduction of isocyanide onto Lys.

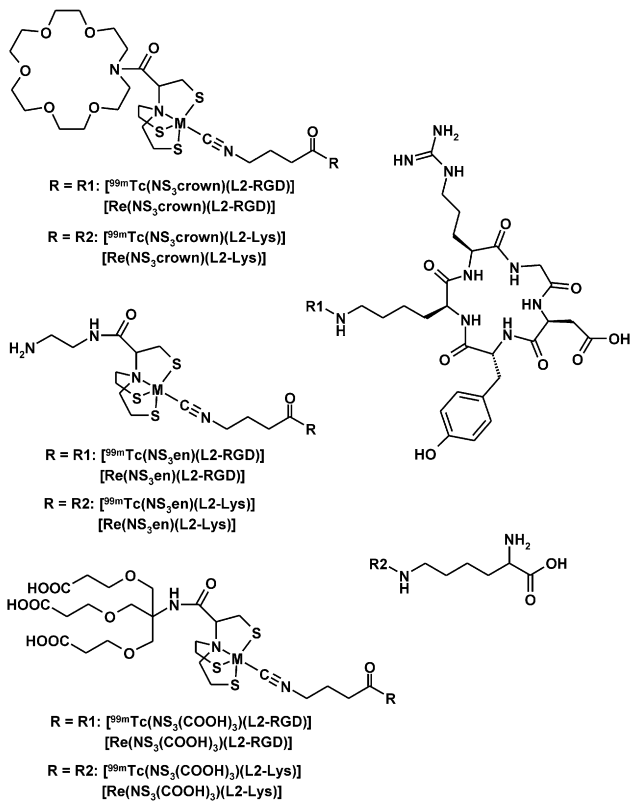
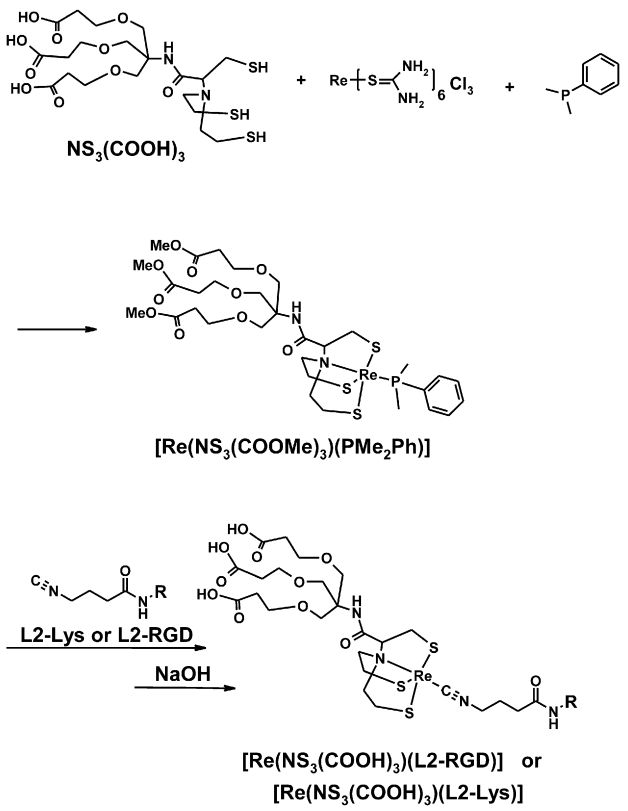


Fig. 1. ^{99m}Tc and Re '4 + 1' complex-bearing RGD-peptide or Lys (M = ^{99m}Tc or Re).



Scheme 5. Exemplary reaction route to rhenium-reference compounds.

NS_3R = NS_3crown, NS_3en, $NS_3(COOH)_3$, BM = L2-RGD, L2-Lys, are shown in Figs. 2–5. The values of %ID, %ID/g, and SUV (ID = injected dose; SUV = standardized uptake value) are reported in detail in the Supporting Information. The SUV as a measure for the activity concentration was used to normalize the biodistribution data and calculated as described in the experimental section.

The biodistribution data show distinctive differences between the activity uptake into the kidney and liver, respectively the hepatobiliary and urinary excretion. For the RGD conjugates, in the order of the modifiers crown ether, ethylenediamine and tricarboxylic acid, a decrease of the hepatobiliary and an increase of the urinary excretion were observed. This is consistent with the order of the determined distribution coefficients. The conjugates had a rapid clearance demonstrated by a decrease of the activity contents in the organs between 5 and 60 min p.i. and an increase in the intestinal uptake.

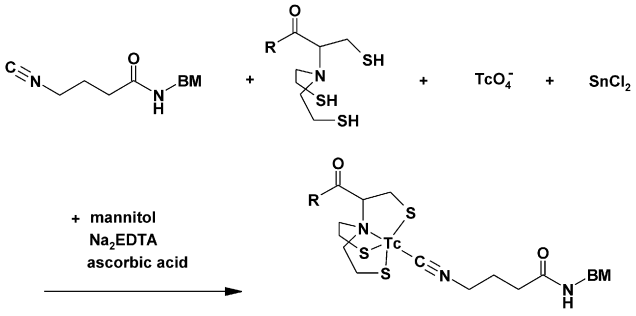
The Lys-containing radioconjugates exhibited a faster clearance compared to the labeled RGD derivatives. The grading between the conjugates containing NS_3en and $NS_3(COOH)_3$ were comparable for Lys and RGD. The biodistribution characteristic for [^{99m}Tc(NS_3crown)(L2-Lys)] was different, showing a quick excretion by the kidney and liver at about the same rate.

The activity concentration (SUV) of the investigated radioconjugates in the kidneys was the highest of all investigated organs and higher for the Lys than for the RGD conjugates. The SUV were lowest for the brain and testis and were roughly at blood level for the other organs (data shown in the Supporting Information).

The investigated conjugates exhibited a minimal ^{99m}Tc uptake into the stomach and the thyroid (data shown in the Supporting Information) indicating that there is no or marginal dissociation of ^{99m}Tc from the complex to produce $^{99m}TcO_4^-$.

Previous investigations using ^{99m}Tc '4 + 1' complexes containing NS_3 or NS_3COOH as coligands attached to cyclo(Arg-Gly-Asp-D-Tyr-Lys) [12] or bombesin derivatives [13] showed an extensive hepatobiliary excretion. Thereby, no pharmacological effect was observed to be caused by the one carboxylic group proposed as pharmacokinetic modifier in the radioconjugates containing NS_3COOH compared to the conjugates containing NS_3 [13].

In the present study, the more comprehensive pharmacokinetic modifiers display a considerable ability to shift the excretion pathway to a roughly similar rate of renal and hepatobiliary excretion for the tracers containing the coligands NS_3crown and NS_3en and further to a predominant renal excretion for compounds containing $NS_3(COOH)_3$.



Scheme 4. ^{99m}Tc-labeling reaction (BM = biomolecule).

Table 1
Distribution coefficients (log $D_{O/W}$) of the RGD-containing radioconjugates at pH 7.4 (mean ± SD, n = 4).

Radioconjugate	log $D_{O/W}$
[^{99m}Tc(NS_3crown)(L2-RGD)]	−1.7 ± 0.1
[^{99m}Tc(NS_3en)(L2-RGD)]	−2.7 ± 0.1
[^{99m}Tc($NS_3(COOH)_3$)(L2-RGD)]	−3.3 ± 0.1

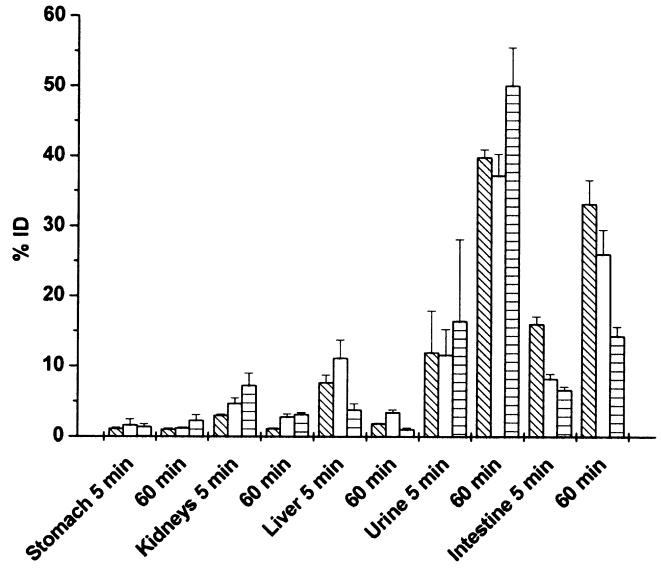


Fig. 2. Biodistribution of [^{99m}Tc(NS$_3$crown)(L2-RGD)], diagonal stripes; [^{99m}Tc(NS$_3$en)(L2-RGD)], no stripes; [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-RGD)], horizontal stripes, in rats after single intravenous application in a tail vain. Data are % ID (mean ± SD of 4 animals per group).

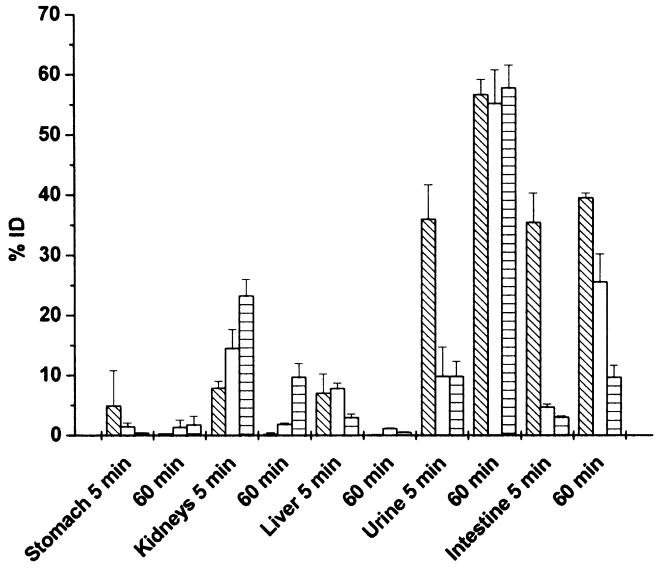


Fig. 4. Biodistribution of [^{99m}Tc(NS$_3$crown)(L2-Lys)], diagonal stripes; [^{99m}Tc(NS$_3$en)(L2-Lys)], no stripes; [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-Lys)], horizontal stripes, in rats after single intravenous application in a tail vain. Data are % ID (mean ± SD of 4 animals per group).

2.2.2. Cell uptake study

Results of the cell binding study are summarized in Fig. 6. The uptake into M21 cells expressing the $\alpha_v\beta_3$ integrin was compared to the unspecific uptake in M21 cells blocked with an excess of the non-labeled RGD-peptide, M21L tumor cells without $\alpha_v\beta_3$ integrin expression and blocked M21L cells. The highest specific uptake including surface bound and internalized radioactivity was found for [^{99m}Tc(NS$_3$en)(L2-RGD)] compared to [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-RGD)] and [^{99m}Tc(NS$_3$crown)(L2-RGD)]. However, the non-specifically internalized activity was also highest for [^{99m}Tc(NS$_3$en)(L2-RGD)]. The ratio of specifically to unspecifically internalized activity was in the range of about 6–8 and comparable for all tracers. The ratio of specific to unspecific uptake (sum of surface bound and internalized) activity was in the range of approximately 4–5 and also comparable for the investigated tracer series. These findings indicate similar receptor binding and internalization behavior of the investigated compounds.

2.2.3. Studies in tumor mice

The biodistribution of [^{99m}Tc(NS$_3$en)(L2-RGD)] was investigated in ν/ν mice bearing M21 and M21L (control) tumor xenografts. The radioactivity content in the M21 xenograft was 3-fold the activity in the control xenograft, proving a specific uptake of the tracer (Table 2). However, the target-background ratios were low and the radioactivity excretion into the intestine was very high. A considerable washout of the tumor-enriched activity was also observed 4 h after application.

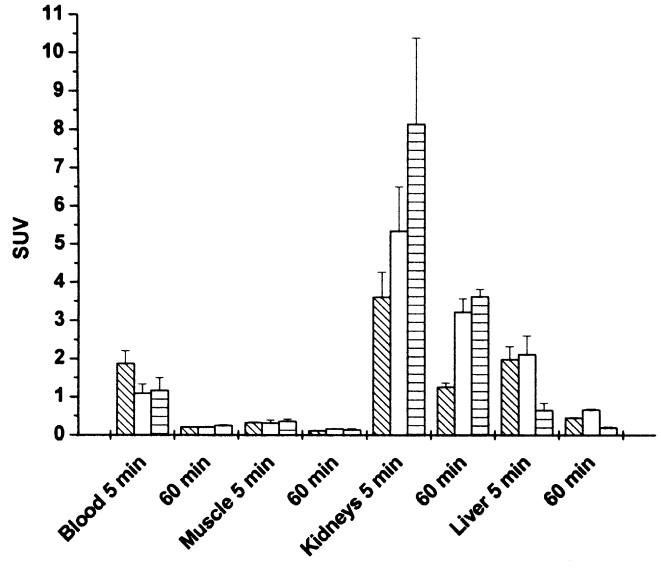


Fig. 3. Biodistribution of [^{99m}Tc(NS$_3$crown)(L2-RGD)], diagonal stripes; [^{99m}Tc(NS$_3$en)(L2-RGD)], no stripes; [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-RGD)], horizontal stripes, in rats after single intravenous application in a tail vain. Data are SUV (mean ± SD of 4 animals per group).

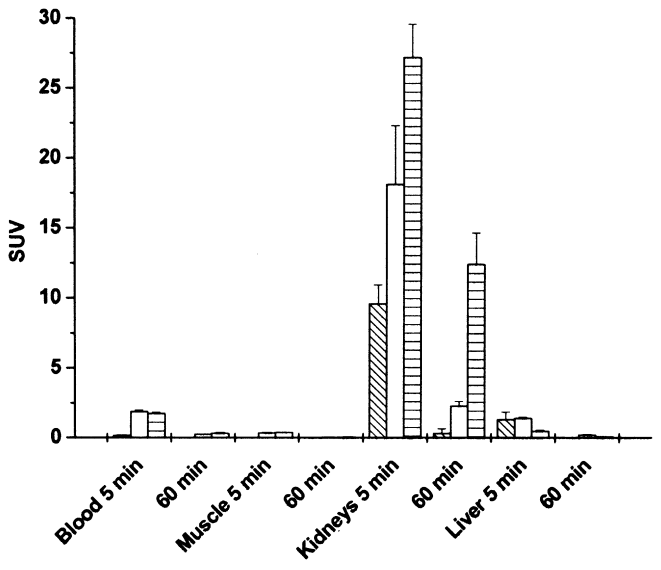


Fig. 5. Biodistribution of [^{99m}Tc(NS$_3$crown)(L2-Lys)], diagonal stripes; [^{99m}Tc(NS$_3$en)(L2-Lys)], no stripes; [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-Lys)], horizontal stripes, in rats after single intravenous application in a tail vain. Data are SUV (mean ± SD of 4 animals per group).

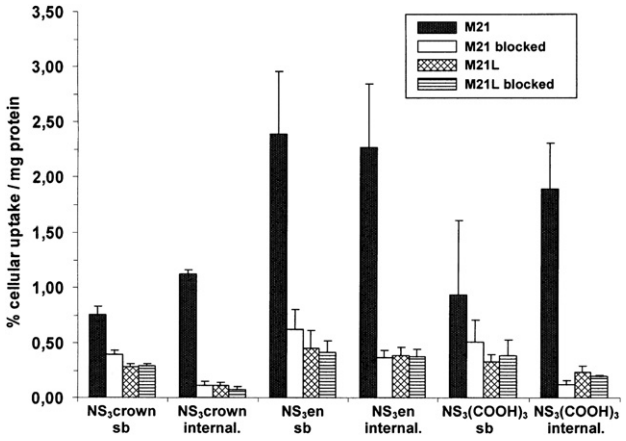


Fig. 6. Internalization and binding studies of [^{99m}Tc(NS$_3$crown)(L2-RGD)], [^{99m}Tc(NS$_3$en)(L2-RGD)] and [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-RGD)] in M21 and M21L cells (blocked: co-incubation with 10 μM c(RGDfV); sb: surface bound, internal.: internalized). Data are % of total activity added/mg protein (mean ± SD of 3).

2.2.4. In vivo stability

The metabolic stability of [^{99m}Tc(NS$_3$R)(L2-RGD)], NS$_3$R = NS$_3$crown, NS$_3$en, NS$_3$(COOH)$_3$ was assessed 60 min after intravenous application of the radioconjugates in rats. The blood samples were centrifuged to obtain the plasma and this was treated with acetonitrile and TFA to precipitate the proteins. The obtained supernatant was referred to as the plasma extract. The mean activity in the plasma was in the range of 83–92% of arterial blood activity; the activity of the plasma extract contained 20–50% of the plasma activity. The extracts and urine were analyzed by RP-HPLC for radiometabolites (Figs. 7–9). The relative activity in the plasma extracts assigned to the radiotracer 60 min after intravenous application was 87% for [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-RGD)], 75% for [^{99m}Tc(NS$_3$en)(L2-RGD)] and 78% for [^{99m}Tc(NS$_3$crown)(L2-RGD)]. In urine, 78% for [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-RGD)], 92% for [^{99m}Tc(NS$_3$en)(L2-RGD)] and 91% for [^{99m}Tc(NS$_3$crown)(L2-RGD)] of excreted radioactivity were intact radiotracer. The contents of the original tracer compared to the whole radioactivity in the excrement extracts were 81% for [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-RGD)], 85% for [^{99m}Tc(NS$_3$en)(L2-RGD)] and 81% for [^{99m}Tc(NS$_3$crown)(L2-RGD)].

Formation of [^{99m}Tc(NS$_3$R)(L2-Lys)], NS$_3$R = NS$_3$crown, NS$_3$en, NS$_3$(COOH)$_3$ as metabolites in the plasma extract, urine or excrement extract could be excluded by comparing with their chromatograms.

Table 2
Biodistribution of [^{99m}Tc(NS$_3$en)(L2-RGD)] in ν/ν mice bearing M21 or M21L tumor xenografts after single intravenous application in a tail vain. Data are %ID/g (mean ± SD of 3 or 4 animals per group).

Xenograft	M21		M21/M21L		M21	
Time p.i. (min)	60		60		240	
%ID/g	Mean	SD	Mean	SD	Mean	SD
Blood	0.96	0.15	1.17	0.14	0.17	0.01
Pancreas	0.35	0.07	0.42	0.02	0.13	0.01
Spleen	0.74	0.14	0.95	0.09	0.25	0.01
Kidneys	2.68	0.64	3.36	0.21	0.98	0.10
Muscle	0.46	0.08	0.51	0.12	0.12	0.02
Heart	0.64	0.12	0.75	0.09	0.17	0.04
Lung	1.77	0.38	2.02	0.02	0.59	0.18
Liver	2.22	0.62	0.75	0.09	0.61	0.50
Femur	0.52	0.31	0.42	0.10	0.17	0.06
Intestine	19.3	3.6	19.5	3.1	13.4	5.6
Stomach	1.57	0.29	1.71	0.04	0.69	0.20
M21 tumor	1.50	0.37	1.70	0.13	0.40	0.09
M21L tumor	–	–	0.52	0.23	–	–

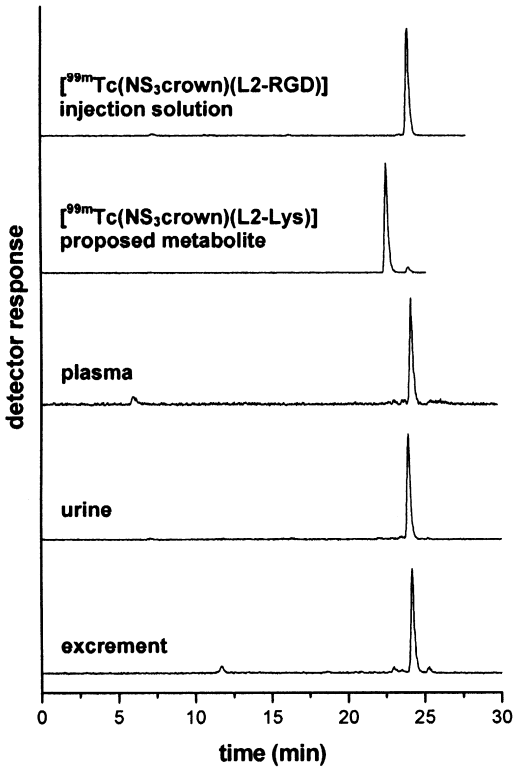


Fig. 7. Radiochromatograms (HPLC system 5) of [^{99m}Tc(NS$_3$crown)(L2-RGD)], [^{99m}Tc(NS$_3$crown)(L2-Lys)] and rat plasma extract, urine, excrement extract 60 min after i.v. injection of [^{99m}Tc(NS$_3$crown)(L2-RGD)].

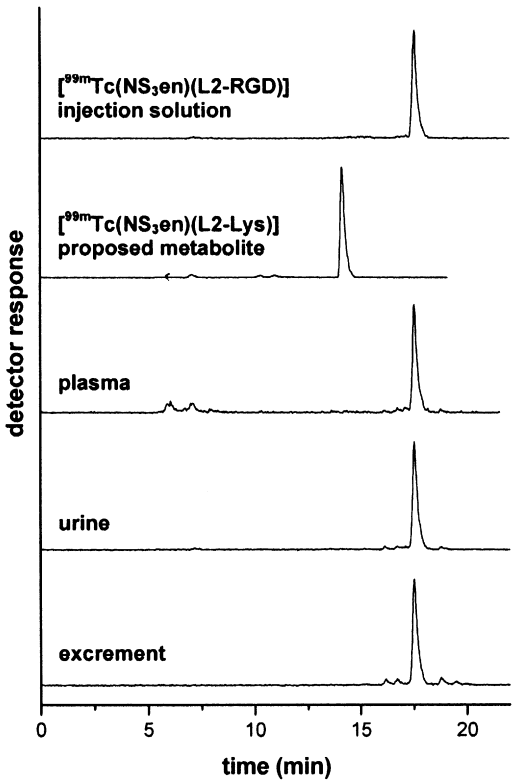


Fig. 8. Radiochromatograms (HPLC system 5) of [^{99m}Tc(NS$_3$en)(L2-RGD)], [^{99m}Tc(NS$_3$en)(Lys-L2)] and rat plasma extract, urine, excrement extract 60 min after i.v. injection of [^{99m}Tc(NS$_3$en)(L2-RGD)].

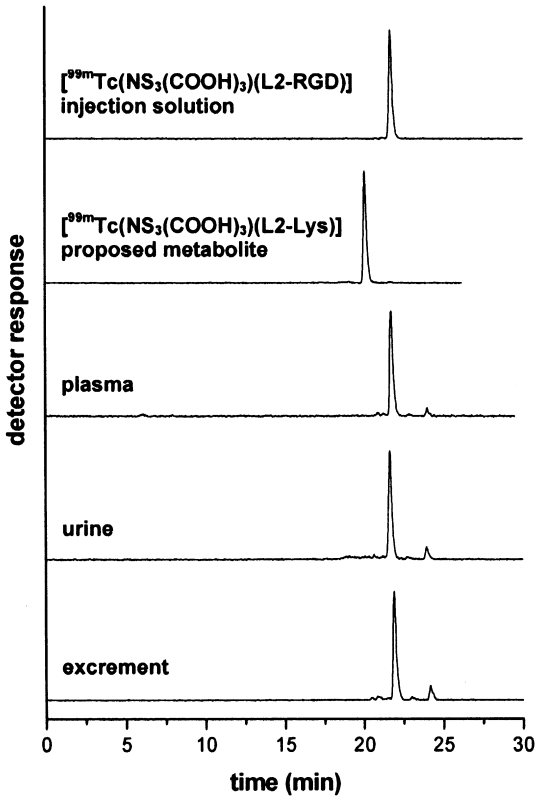


Fig. 9. Radiochromatograms (HPLC system 5) of [^{99m}Tc(NS_3(COOH)$_3$)(L2-RGD)], [^{99m}Tc(NS_3(COOH)$_3$)(L2-Lys)] and rat plasma extract, urine, excrement extract 60 min after i.v. injection of [^{99m}Tc(NS_3(COOH)$_3$)(L2-RGD)].

In vivo metabolism studies of radiolabeled RGD-derivatives containing cyclo(Arg-Gly-Asp-D-Phe-Lys) in mice have been reported [21,22]. Accordingly, 24% intact radiotracer was found in the plasma extract 30 min p.i. of ^{99m}Tc-DKCK-RGD and one radiometabolite was detected [21]. For [^{18}F]Galacto-RGD, 86% intact radiotracer was found in the plasma extract 120 min p.i. and also only one radiometabolite was detected [22]. The differences in the stability data suggested that the instability of ^{99m}Tc-DKCK-RGD is mainly caused at the site of the radiolabel and not by metabolism of cyclo(Arg-Gly-Asp-D-Phe-Lys). In contrast to ^{99m}Tc-DKCK-RGD and [^{18}F]Galacto-RGD, there was no main metabolite detected for [^{99m}Tc(NS_3crown)(L2-RGD)] and [^{99m}Tc(NS_3en)(L2-RGD)] (Figs. 7 and 8). [^{99m}Tc(NS_3(COOH)$_3$)(L2-RGD)] caused a main metabolite at a retention time of about 24.3 min (Fig. 9).

The observed metabolic stability of tracers in the series [^{99m}Tc(NS_3R)(L2-RGD)] is roughly in the same range as reported for [^{18}F]Galacto-RGD. These investigations confirm a suitable metabolic stability of the used cyclic RGD-peptide, the ^{99m}Tc '4 + 1' complex unit as also shown previously [2,11], and further of its novel versions grafted with the described pharmacokinetic modifiers.

3. Conclusion

The concept of applying '4 + 1' mixed-ligand complexes for ^{99m}Tc-labeling of radiopharmaceutically interesting peptides is challenged by the lipophilic nature of the '4 + 1' complex unit resulting in an unwanted extensive hepatobiliary excretion. The mixed-ligand framework carries the possibility that hydrophilic coligands may have a compensatory effect. The presented novel NS_3-chelators grafted with pharmacokinetic modifiers – a crown ether, an amine and a tricarboxylic acid (NS_3crown, NS_3en, NS_3(COOH)$_3$) were intended to decrease the lipophilicity under physiological conditions by a neutral hydrophilic, a positively charged and a negatively charged group. Complexes containing the new coligands could be synthesized and were assessed as conjugated to an RGD-peptide. The lipophilicity (distribution coefficient log $D_{O/W}$, pH = 7.4) of the RDG-containing radiotracers decreased in the order of the coligands NS_3crown, NS_3en and NS_3(COOH)$_3$. The new coligands shift the excretion pathway to a roughly similar rate of renal and hepatobiliary excretion for the tracers containing NS_3crown and NS_3en, and further to a predominant renal excretion for compounds containing NS_3(COOH)$_3$. This marks a considerable improvement of *in vivo* properties of ^{99m}Tc '4 + 1' peptide conjugates, since those tracers prepared up to now contained unmodified NS_3 or only a single free carboxylic group as modifier and showed an extensive hepatobiliary excretion [12,13]. The effect of the coligands was confirmed by a comparable grading between the biodistribution data of smaller tracers bearing only Lys in place of the RGD-peptide with the exception of [^{99m}Tc(NS_3crown)(L2-Lys)] which was quickly excreted by the kidney and the liver at about the same rate. The biodistribution of [^{99m}Tc(NS_3en)(L2-RGD)] in mice bearing M21 and M21L (control) tumor xenografts exhibited a specific tumor uptake. However, the target-background ratios were low which demands more comprehensive structural changes when aiming to develop a radiopharmaceutical based on a '4 + 1' complex containing RGD-peptide. The stability studies in normal rats indicated a suitable metabolic stability of cyclo(Arg-Gly-Asp-D-Tyr-Lys) and the novel modified ^{99m}Tc '4 + 1' complex units. The presented coligand strategy offers new properties of ^{99m}Tc '4 + 1' mixed-ligand conjugates, making them more attractive for application to radiopharmaceutically relevant biomolecules.

4. Experimental

4.1. Materials

The RGD-peptide cyclo(Arg-Gly-Asp-D-Tyr-Lys) × 2 TFA, c(RGDyK), was obtained by customized synthesis from Biosyntan GmbH (Germany). Fmoc-Lys was obtained from Bachem. Solvents for HPLC were of HPLC grade and purchased from Merck or Fisher Scientific. All other reagents and solvents were of analytical grade unless stated otherwise and purchases from Merck, Sigma–Aldrich or Fisher Scientific and used without further purification. Na^{99m}TcO$_4$ was eluted from a commercial ^{99}Mo/^{99m}Tc generator (Covidien). The coupling agent 2,3,5,6-tetrafluorophenyl 4-isocyanobutanoate (L2-BFCA) was synthesized according to a previously published method [13]. 3-(Benzylthio)-2-(bis(2-(benzylthio)ethyl)amino)propanoic acid (N(Sbz)$_3$COOH) was synthesized according to the previously published procedure [11]. Tris(2-methoxycarbonylethoxymethyl)aminomethane (H_2N–C(–CH_2–O–CH_2–CH_2–COO–Me)$_3$) was synthezised according to Newkome et al. [23]. The synthesis of the hexathiourea Re(III) precursor complex [Re(tu)$_6$]Cl$_3$ × 4H_2O (tu = SC(NH_2)$_2$) was carried out according to Gambino et al. [26]. The Re(III)-dimethyl phenyl phosphino complex [Re(NS_3)(PMe_2Ph)] was prepared as published by Spies et al. [27].

4.2. Instrumentation

Electrospray ionization mass spectrometry (ESI–MS) was performed using a Micromass Tandem Quadrupole Mass Spectrometer (Quattro LC). Nuclear magnetic resonance spectra were recorded on a 400 MHz Varian Inova 400 spectrometer. Chemical shifts are reported as δ in ppm relative to the residual solvent signal or TMS (^{1}H: 400 MHz, ^{13}C: 101 MHz). TLC strips were scanned using a Rita linear radioanalyser (Raytest). Shown chromatograms were normalized to the peak height. Lyophilization was performed with

a freeze dryer Alpha 1–4 (Christ). Submilligram peptide amounts were weighted using a micro balance M2P (Sartorius). Distribution coefficients were radiometrically determined using the NaI(Tl) scintillation counter Isomed (Nuklearmedizintechnik Dresden).

RP-HPLC analyses and purification were performed on a Smartline system (Knauer) equipped with a pump 1000, a manager 5000, a PDA detector 2800 and a homemade γ-ray detector (Bohrloch, NaI (Tl) crystal). Shown chromatograms were normalized to the peak height. The gradient and HPLC conditions were as follows: eluent A: NH_3 (0.01 M) in acetonitrile, eluent B: NH_3 (0.01 M) in water, eluent C: 0.1% (v/v) trifluoroacetic acid (TFA) in acetonitrile; eluent D: 0.1% (v/v) TFA in water, eluent E: 0.1% (v/v) TFA in ethanol, eluent F: 0.04% (v/v) TFA in acetonitrile, eluent G: 0.05% (v/v) TFA in water.

System 1: Gemini 5 μ C18 110 Å (Phenomenex), 250 × 10 mm, gradient elution using eluents A and B: 5–65% A in 40 min, 2 mL/min; 220 nm.

System 2: Jupiter 4 μ Proteo 90 Å (Phenomenex), 250 × 10 mm, gradient elution using eluents C and D: 20–80% C in 20 min, 2 mL/min; 220 nm.

System 3: Jupiter 4 μ Proteo 90 Å (Phenomenex), 250 × 4.6 mm, gradient elution using eluents C and D: 20–80% C in 20 min, 1 mL/min; 220 nm and/or γ-detection.

System 4: Jupiter 4 μ Proteo 90 Å (Phenomenex), 250 × 10 mm, gradient elution using eluents D and E: 20–80% E in 20 min, 2 mL/min; γ-detection.

System 5: Zorbax 300SB-C18 5 μm (Agilent), 250 × 9.4 mm, gradient elution using eluents F and G: 10–70% F in 40 min, 2 mL/min; γ-detection.

4.3. Methods

4.3.1. Synthesis of NS_3crown, NS_3en and $NS_3(COOH)_3$

4.3.1.1. Coupling. Synthesis of 3-(benzylthio)-2-(bis(2-(benzylthio) ethyl)amino)-1-(1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl) propan-1-one ($N(Sbz)_3$crown): $N(Sbz)_3COOH$ (0.2 mmol) and hydroxybenzotriazole (HOBt, 0.2 mmol) were dissolved in tetrahydrofurane (THF, 3 mL) and cooled at approximately 0 °C. The solution of N,N′-dicyclohexylcarbodiimide (DCC, 0.2 mmol) dissolved in THF (2 mL) was added dropwise and the reaction mixture was stirred at 0 °C for 15 min. Afterwards, 1-aza-18-crown-6 (0.2 mmol) dissolved in THF (2 mL) were added and the reaction mixture was stirred over night at r.t. The residue was filtered off and the solvent of the supernatant was removed under vacuum. The residual yellow oil was purified by column chromatography (silica gel 60, eluent: trichloromethane/ethanol, 19/1, v/v). A pale yellow oil was obtained. Yied: 50%; TLC (silica gel 60 F_{254s}, eluent: trichloromethane/ethanol, 19/1, v/v): $R_f \sim 0.3$; ^{1}H NMR ($CDCl_3$): 2.26–2.30 (t, 4H, CH_2, J = 8.2 Hz), 2.33–2.50 (m, 3H, NCH_2) 2.51–2.59 (m, 3H, SCH_2CH), 2.65–2.72 (m, 2H, SCH_2CH), 2.94–3.00 (m, 1H, NCHCO), 3.30–3.37 (m, 1H, SCH_2CH), 3.53–3.70 (m, 26H, $CON(CH_2)_2$, SCH_2Ph), 3.81–3.88 (m, 1H), 7.17–7.28 (m, 15H, phenyl); ^{13}C NMR ($CDCl_3$): 27.6, 30.8 (SCH_2C), 36.5 (SCH_2C), 37.4 (SCH_2Ph), 47.7, 48.7 ($CON(CH_2)_2$), 51.3, 62.5 (NCHCO), 69.7, 70.6, 70.7, 70.8, 70.9, 71.0, 127.2, 128.7, 129.0, 129.1, 138.6, 138.9, 170.9. ESI–MS (m/z) found (calcd): 779.5 (779.3) for $[N(Sbz)_3crown + Na]^+$ ($=[C_{40}H_{56}N_2NaO_6S_3]^+$).

Synthesis of tert-butyl 2-(3-(benzylthio)-2-(bis(2-(benzylthio) ethyl)amino) propanamido) ethylcarbamate ($N(Sbz)_3$(boc)en): At 0 °C, a solution of DCC (0.5 mmol) in trichloromethane (2 mL) was added dropwise to a solution of $N(Sbz)_3COOH$ (0.5 mmol) dissolved in trichloromethane (6 mL) and stirred for 15 min. Afterwards, N-boc-ethylenediamine (0.55 mmol) dissolved in trichloromethane (2 mL) were added dropwise and the reaction mixture was stirred at r.t. for 12 h. The precipitated byproduct was filtered off and the solvent was removed in vacuum. The residual yellow oil was purified by column chromatography (silica gel 60, eluent: trichloromethane/ethylacetate, 5/1, v/v, R_f = 0.6). A colorless viscous oil was obtained. Yield: 35%; ^{1}H NMR ($CDCl_3$): 1.41 (s, 9H, C–CH_3), 2.29–2.38 (m, 4H, CH_2), 2.49–2.55 (m, 3H, NCH_2) 2.62–2.68 (m, 2H, SCH_2CH), 3.08–3.23 (m, 1H, NCHCO), 3.20–3.24 (m, 4H, SCH_2CH), 3.62 (s, 4H, SCH_2Ph), 3.66 (s, 2H, SCH_2Ph), 3.68 (m, 1H), 5.10 (s, 1H, NH–COO), 7.17–7.28 (m, 15H, phenyl), 7.83 (s, 1H, NH–CH_2); ^{13}C NMR ($CDCl_3$): 28.7, 29.1, 30.6 (SCH_2C), 36.5 (SCH_2C), 38.0 (SCH_2Ph), 39.7, 40.9, 50.2, 64.7 (NCHCO), 79.4, 127.3, 128.7, 129.0, 129.1, 138.3, 138.5, 156.2, 172.6; ESI–MS (m/z) found (calcd): 653.9 (654.3) for $[M + H]^+$ ($=[C_{35}H_{48}N_3O_3S_3]^+$).

Synthesis of N-(2-aminoethyl)-3-(benzylthio)-2-(bis(2-(benzylthio)ethyl)amino)pro-panamide ($N(Sbz)_3$en): Trifluoroacetic acid (0.8 mmol) was added to $N(Sbz)_3$(boc)en (0.05 mmol) dissolved in trichloromethane (2 mL) and stirred at r. t. for 12 h. Afterwards, the reaction mixture was neutralized using a saturated aqueous $NaHCO_3$-solution followed by 3-fold extraction with dichloromethane. The combined organic phases were dried with $MgSO_4$ and the solvent was removed in vacuum. A colorless oil was obtained. Yield: 90%; ^{1}H NMR ($CDCl_3$): 2.30–2.38 (m, 4H, CH_2), 2.49–2.56 (m, 3H, NCH_2) 2.62–2.73 (m, 4H, SCH_2CH), 3.09–3.14 (m, 2H), 3.25–3.27 (m, 2H, SCH_2CH), 3.61 (s, 4H, SCH_2Ph), 3.67 (s, 2H, SCH_2Ph), 3.68 (m, 1H), 7.16–7.29 (m, 15H, phenyl), 7.76 (s, 1H, NH–CH_2); ^{13}C NMR ($CDCl_3$): 29.1, 30.7 (SCH_2C), 36.5 (SCH_2C), 38.0 (SCH_2Ph), 41.9, 42.8, 50.3, 64.8 (NCHCO), 127.3, 128.7, 128.8, 129.0, 129.1, 138.3, 138.5, 172.3; ESI–MS (m/z) found (calcd): 553.8 (554.2) for $[M + H]^+$ ($=[C_{30}H_{40}N_3OS_3]^+$).

Synthesis of 5-(2-(benzylthio)ethyl)-6-(benzylthiomethyl)-9,9-bis ((2-carboxyethoxy)me-thyl)-7-oxo-1-phenyl-11-oxa-2-thia-5,8-diazatridecane-13-carboxylic acid ($N(Sbz)_3(COOH)_3$): $N(Sbz)_3COOH$ (2.17 mmol) were dissolved in trichloromethane (20 mL) and cooled at approximately 0 °C. The solution of N,N′-dicyclohexylcarbodiimide (DCC, 2.17 mmol) dissolved in trichloromethane (20 mL) were added dropwise and the reaction mixture was stirred at 0 °C for 15 min. Afterwards, H_2N–C($-CH_2-O-CH_2-CH_2-COO-Me)_3$ (2.17 mmol) dissolved in trichloromethane (20 mL) were added and the reaction mixture was stirred over night at 0 °C. The residue was filtered off and the solvent of the supernatant was removed under vacuum. For saponification, the obtained conjugate (1 mmol) was dissolved in dioxane (10 mL) and aqueous sodium hydroxide solution (1 M, 10 mL) were added. After stirring over night, the reaction mixture was acidified using glacial acetic acid. Subsequently, the reaction mixture was extracted with trichloromethane (3 × 20 mL). The combined organic phases were dried using $MgSO_4$ and then the solvent removed in vacuum. A yellow viscous oil was obtained. Yield: 60%; ^{1}H NMR ($CDCl_3$): 2.37 (m, 6H, CH_2–CO), 2.48 (m, 4H, CH_2) 2.58 (m, 4H, SCH_2CH), 2.99–3.04 (m, 1H), 3.17–3.19 (t, 1H, J = 8 Hz) 3.57–3.66 (m, 22H, SCH_2Ph, OCH_2), 7.18–7.27 (m, 15H, phenyl), 7.36 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): 29.1, 30.6 (SCH_2C), 35.0 (SCH_2C), 36.5 (SCH_2Ph), 37.9, 59.7, 65.2 (NCHCO), 66.9 (C–O–C), 69.1 (C–O–C), 127.2, 127.3, 128.7, 129.0, 138.6, 171.8, 176.2 (COOH); ESI–MS (m/z) found (calcd): 829.3 (829.3) for $[M–H]^-$ ($=[C_{41}H_{53}N_2O_{10}S_3]^-$).

4.3.1.2. Deprotection of the tristhiolate ligands. A solution of 0.05 mmol of $N(Sbz)_3$crown, $N(Sbz)_3$en or $N(Sbz)_3(COOH)_3$ in dry THF (1 mL) was added dropwise to liquid ammonia (about 5 mL, ~−50 to −30 °C). Sodium was added in small pieces under gentle stirring until the solution remained deep blue for 15 min. Afterwards the solvents were removed using an argon stream. Water (5 mL) was added to the residue and the solution was acidified with hydrochloric acid (1 M) to pH 3–4. The solution was extracted with ethyl acetate (3 × 5 mL) to remove organic side products. The solvent of the aqueous phase (water) was removed in vacuum. The

residue was resuspended in methanol and then filtered off to separate the product from sodium chloride. The solvent (methanol) of the filtrate was removed under reduced pressure. White powders or colorless oils were obtained. Yield: ~100% (partly contaminated with sodium chloride).

3-Mercapto-N,N-bis(2-mercaptoethyl)-1-oxo-1-(1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)propan-2-aminium-chlorid (NS_3crown): ^{1}H NMR (D_2O): 2.71–2.81 (m, 4H), 2.91–2.97 (m, 1H) 3.13–3.26 (m, 3H), 3.37–3.44 (m, 2H), 3.51–3.64 (m, 22H), 3.68–3.76 (m, 2H), 4.74–4.77 (m, 1H, CHCON(CH_2)$_2$); ^{13}C NMR (D_2O): 18.0, 22.2, 47.0, 49.4, 53.7, 63.2, 63.3, 68.4, 68.8, 69.8, 167.0.

1-(2-Ammonioethylamino)-3-mercapto-N,N-bis(2-mercaptoethyl)-1-oxopropan-2-aminium-dichlorid (NS_3en): ^{1}H NMR (D_2O): 2.87–3.07 (m, 4H), 3.10–3.35 (m, 4H), 3.44–3.74 (m, 6H), 4.33–4.36 (m, 1H, *CH*CONH); ^{13}C NMR (D_2O): 18.8, 22.0, 37.7, 38.9, 54.5, 66.5, 167.2.

1-(1,3-Bis(2-carboxyethoxy)-2-((2-carboxyethoxy)methyl)propan-2-ylamino)-3-mercapto-N,N-bis(2-mercaptoethyl)-1-oxopropan-2-aminium-chlorid (NS_3(COOH)$_3$): ^{1}H NMR (CD_3OD): 2.52 (m, 6H, CH_2COOH), 2.58–2.81 (m, 10H) 3.44 (t, 1H, J = 6.84 Hz, CHCONH), 3.69 (m, 12H, CH_2O), 7.56 (s, 1H, CONH); ^{13}C NMR (CD_3OD): 21.9, 22.7, 34.6, 54.1, 60.3, 66.9, 67.5, 68.7, 171.8, 174.2.

4.3.2. Synthesis of the isocyano-RGD derivative L2-RGD

The RGD-peptide cyclo(Arg-Gly-Asp-D-Tyr-Lys) × 2 TFA (10 μmol) was dissolved in 0.5 mL N,N-dimethylformamide (DMF). Triethylamin (35 μmol, reagent grade) and L2-BFCA (9 μmol) dissolved in DMF (0.1 mL) were added. After being stirred for 12 h at r.t., the solvent was removed under vacuum and the residue was purified by HPLC (system 1). A white powder was obtained after lyophilization. Yield: 40–50%; HPLC (system 1): t_R = 16.5 min; ESI–MS (m/z) found (calcd): 715.4 (715.5) for $[M + H]^+$ ($=[C_{32}H_{46}N_{10}O_9]^+$).

4.3.3. Synthesis of the isocyano-lysine derivative L2-Lys

Fmoc-Lys (40 μmol) was dissolved in a mixture of DMF (2 mL) and water (0.8 mL). Triethylamin (0.2 mmol, reagent grade) and L2-BFCA (40.5 μmol, dissolved in 0.2 mL DMF) were added. After being stirred for 12 h at r.t., the solvent was removed under vacuum. In order to deprotect the compound, the residue was dissolved in DMF (1 mL), piperidine (0.5 mL) was added and it was placed in an ultrasound bath for 30 min. Afterwards the solvent was removed under vacuum. The crude product was purified by HPLC (system 1). A white powder was obtained after lyophilization. Yield: 30–40%; HPLC (system 1): t_R = 8.6 min; ESI–MS (m/z) found (calcd): 242.2 (242.3) for $[M + H]^+$ ($=[C_{11}H_{20}N_3O_3]^+$).

4.3.4. Synthesis of [Re(NS_3R)(PMe_2Ph)]; NS_3R = NS_3crown, NS_3en, NS_3(COOMe)$_3$

The reaction mixture containing the precursor $[Re(tu)_6]Cl_3 \times H_2O$ (0.1 mmol), dissolved in methanol (5 mL), dimethyl phenyl phosphine (0.2 mmol) and 0.1 mmol of the respective tetradentate ligand NS_3crown, NS_3en or NS_3(COOH)$_3$ was heated under reflux for 3 h. The acidification during the reaction resulted in esterification of the carboxyl groups of NS_3(COOH)$_3$ whereby [Re(NS_3(COOMe)$_3$)(PMe_2Ph)] was formed. Afterwards the solvent was removed in vacuo and the crude product was purified by column chromatography (silica gel 60, THF/*n*-hexane: 4/3, R_f = 0.5–0.7). After removal of the solvent the products were obtained as green solid. Yield: 80–90%; ESI–MS (m/z) found (calcd): 807.4, 807.6 (807.2, 809.2) for $[Re(NS_3crown)(PMe_2Ph) + H]^+$ ($=[C_{27}H_{47}N_2O_6PReS_3]^+$), 604.2, 606.2 (604.1, 606.1) for $[Re(NS_3en)(PMe_2Ph) + H]^+$ ($=[C_{17}H_{30}N_3OPReS_3]^+$), 923.5, 925.6 (923.2, 925.2) for $[Re(NS_3(COOMe)_3)(PMe_2Ph) + H]^+$ ($=[C_{31}H_{51}N_2O_{10}PReS_3]^+$), two signals corresponding to ^{185}Re and ^{187}Re.

4.3.5. Synthesis of [Re(NS_3R)X]; NS_3R = NS_3crown, NS_3en, NS_3(COOH)$_3$; X = L2-RGD, L2-Lys

The isocyanide L2-RGD or L2-Lys (2 μmol) was dissolved in a mixture of methanol (0.8 mL) and water (0.2 mL). An equimolar amount of either [Re(NS_3crown)(PMe_2Ph)], [Re(NS_3en)(PMe_2Ph)] or [Re(NS_3(COOMe)$_3$)(PMe_2Ph)] dissolved in MeOH (0.2 mL) was added. After stirring for 1–2 h at r.t., the solvent was removed under vacuum. In order to prepare [Re(NS_3(COOH)$_3$)X], aqueous NaOH (1 M, 1 mL) was added to the residue and the suspension was stirred for 1 h followed by removing the solvent under vacuum. The crude products were purified by HPLC (system 2). Green powders were obtained after lyophilization. Yield: 30–40%; HPLC (system 3): t_R = 10.8 min for [Re(NS_3crown)(L2-RGD)], t_R = 8.2 min for [Re(NS_3en)(L2-RGD)], t_R = 9.5 min for [Re(NS_3(COOH)$_3$)(L2-RGD)], t_R = 9.2 min for [Re(NS_3crown)(L2-Lys)], t_R = 4.5 min for [Re(NS_3en)(L2-Lys)], t_R = 10.2 min for [Re(NS_3(COOH)$_3$)(L2-Lys)]; ESI–MS (m/z) found (calcd): 1383.5, 1385.6 (1383.5, 1385.5) for [Re(NS_3crown)(L2-RGD)+H]$^+$ ($=[C_{51}H_{82}N_{12}O_{15}ReS_3]^+$), 1180.6, 1182.6 (1180.3, 1182.3) for [Re(NS_3en)(L2-RGD)+H]$^+$ ($=[C_{41}H_{65}N_{13}O_{10}ReS_3]^+$), 1457.8, 1459.8 (1457.4, 1459.4) for [Re(NS_3(COOH)$_3$)(L2-RGD)+H]$^+$ ($=[C_{52}H_{80}N_{12}O_{19}ReS_3]^+$), 909.6, 911.7 (909.3, 911.3) for [Re(NS_3crown)(L2-Lys)+H]$^+$ ($=[C_{30}H_{55}N_5O_9ReS_3]^+$), 706.5, 708.5 (706.2, 708.2) for [Re(NS_3en)(L2-Lys)+H]$^+$ ($=[C_{20}H_{38}N_6O_4ReS_3]^+$), 983.5, 985.5 (983.2, 985.2) for [Re(NS_3(COOH)$_3$)(L2-Lys)+H]$^+$ ($=[C_{31}H_{53}N_5O_{13}ReS_3]^+$), two signals corresponding to ^{185}Re and ^{187}Re.

4.3.6. Preparation of ^{99m}Tc '4 + 1' conjugates [^{99m}Tc(NS_3R)X]; NS_3R = NS_3crown, NS_3en, NS_3(COOH)$_3$; X = L2-RGD, L2-Lys

EDTA solution (100 μL, 10 mg Na_2EDTA in 1 mL water), mannitol solution (100 μL, 50 mg mannitol in 1 mL water), NS_3-solution (200 μL, 200–400 nmol NS_3en, NS_3crown or NS_3(COOH)$_3$ dissolved in a mixture of 100 μL ethanol and 100 μL water), and ascorbic acid solution (1 mg in 50 μL water) were transferred into a vial containing L2-RGD or Lys-L2 (100–200 nmol), respectively. $SnCl_2$ solution (0.1 mg in 20 μL ethanol) was added followed by the addition of $^{99m}TcO_4^-$ solution (1–2 mL, 0.5–1 GBq). Ethanol was added to adjust the content of it in the reaction mixture to approximately 25% of the mixture volume. After a reaction time of 30–60 min, the radiochemical yield was 60–80%. The radioconjugates were purified by HPLC (system 4) to get a radiochemical purity of higher than 95%. To prepare the injection solution for biodistribution studies, the HPLC-eluate was heated to about 50 °C and the volume was reduced using an argon gas stream. Afterwards the solution was diluted with phosphate buffered saline and the pH value was adjusted to 7.4, ethanol was added to adjust its content to about 10% yielding a radioconjugate solution of about 50 MBq in 0.5 mL. HPLC (system 3): t_R = 10.7 min for [^{99m}Tc(NS_3crown)(L2-RGD)], t_R = 8.0 min for [^{99m}Tc(NS_3en)(L2-RGD)], t_R = 9.6 min for [^{99m}Tc(NS_3(COOH)$_3$)(L2-RGD)], t_R = 9.1 min for [^{99m}Tc(NS_3crown)(L2-Lys)], t_R = 4.4 min for [^{99m}Tc(NS_3en)(L2-Lys)], t_R = 10.2 min for [^{99m}Tc(NS_3(COOH)$_3$)(L2-Lys)]; HPLC (System 5): t_R = 23.9 min for [^{99m}Tc(NS_3crown)(L2-RGD)], t_R = 17.4 min for [^{99m}Tc(NS_3en)(L2-RGD)], t_R = 21.6 min for [^{99m}Tc(NS_3(COOH)$_3$)(L2-RGD)], t_R = 22.5 min for [^{99m}Tc(NS_3crown)(L2-Lys)], t_R = 14.1 min for [^{99m}Tc(NS_3en)(L2-Lys)], t_R = 20.0 min for [^{99m}Tc(NS_3(COOH)$_3$)(L2-Lys)].

4.3.7. Determination of distribution coefficients

Distribution coefficients log $D_{o/w}$ were determined by a distribution experiment using an 1-octanol/buffer system. The buffer solution containing the ^{99m}Tc-labeled compound was prepared by using the HPLC-eluate containing the purified radioconjugate, removing of the solvent by heating the vial at about 50 °C when applying an argon gas steam, redissolving in phosphate buffer

(0.1 M) and adjustment of the pH value to 7.4. The buffer solution (0.5 mL) containing approximately 0.5 MBq ^{99m}Tc-tracer and 1-octanol (0.5 mL) were mixed and mechanical shaken in a microcentrifuge tube for 15 min and then centrifuged for phase separation. The activity concentrations of both phases were determined radiometrically using a NaI(Tl) scintillation counter and the distribution coefficients were calculated. The results are the average values of four independent experiments.

4.3.8. Internalization and binding studies in $\alpha_v\beta_3$-positive and $\alpha_v\beta_3$-negative cells

$\alpha_v\beta_3$-positive M21 and -negative M21L human melanoma cells were grown in culture until sufficient number of cells were available. For internalization experiments cells were collected to a concentration of 2×10^6 cells/mL in RPMI1640 containing 1% Glutamine and 1% PBS. Each 1 mL was pipetted into a separate tube. After addition of ^{99m}Tc-labeled peptide (>100 000 cpm, 1 nM) cells were incubated at 37 °C for 90 min in triplicates with either PBS/0.5%BSA buffer alone (150 μL, total series), or with c(RGDyK) (10 μM) in PBS/0.5%BSA buffer (150 μL, non-specific series). Incubation was interrupted by centrifugation, removal of medium and rapid rinsing twice with ice-cold TRIS buffered saline two times. Thereafter, the cells were incubated twice at room temperature in acid wash buffer (50 mM acetate buffer pH = 4.2) for 15 min at 37 °C. The supernatant was collected (membrane bound radioligand fraction) and the cells were washed with acid wash buffer. Cells were lyzed by treatment in NaOH (1 M) and cell radioactivity collected (internalized radioligand fraction). Protein concentration in the NaOH fraction was determined using spectrophotometric determination according to Bradford. The internalized and non-internalized fractions were determined by measuring radioactivity and the internalized fraction was expressed as % of total activity per mg protein.

4.3.9. Biodistribution

The animal studies were carried out according to the relevant national regulations.

The specified injected molar amounts of radioconjugates were estimated considering the specific activity of ^{99m}Tc, the total amount of Tc contained in the generator eluate [28], which was eluatetd 24 h after its last elution and a time period of one ^{99m}Tc half life between generator elution and application into the animal.

4.3.9.1. Distribution in normal rat. The Wistar rats (Harlan Winkelmann, Germany) were housed under standard conditions with free access to standard pellet feed and water. Animals were between 7 and 9 weeks of age.

Four animals (body weight 150–200 g) for each time point were intravenously injected into a tail vain with 0.3 MBq (roughly 1×10^{-13} mol) of the radioconjugate solution diluted in electrolyte solution E-153 (0.5 mL, Serumwerk Bernburg, Germany) containing 5% Tween 80. Animals were euthanized at 5 and 60 min post-injection. Blood and selected organs were collected, weighted and counted in a Wallac Wizard automatic gamma counter (Perkin Elmer). The percent injected dose (% ID) and % ID/g of each organ or tissue were calculated. The SUV (standardized uptake value) was calculated to normalize the biodistribution data and calculated as followed: SUV = tissue activity concentration (Bq/g) × body weight (g)/injected activity (Bq).

4.3.9.2. Distribution in tumor bearing mice. Tumor uptake studies were performed in v/v mice (Charles River, Germany). For the induction of tumor xenografts, M21 and M21L cells were subcutaneously injected at a concentration of 5×10^6 cells/mouse and allowed to grow until tumors of 0.3–0.6 mL were visible. To study the tumor uptake of [^{99m}Tc(NS$_3$crown)(L2-RGD)], each mouse was injected with 1 MBq (roughly 4×10^{-13} mol) of the radioconjugate into the tail vein and sacrificed by cervical dislocation at 1 h and 4 h p.i.

4.3.10. In vivo stability

The *in vivo* stability of [^{99m}Tc(NS$_3$crown)(L2-RGD)], [^{99m}Tc(NS$_3$en)(L2-RGD)] and [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-RGD)] was studied using rat arterial blood samples, urine and faeces at 1 h after i.v. injection of the radiotracer solution (0.5 mL) containing about 20 MBq (roughly 7×10^{-12} mol). The samples were centrifuged to precipitate cells and corpuscular parts. A solution consisting of acetonitrile (50 μL), water (45 μL) and trichloroacetic acid (5 μL) was added to the supernatant (200 mL) and then the mixture was cooled at −20 °C. After centrifugation, the supernatant was analyzed by HPLC (system 5). To identify potential radiometabolites, the obtained chromatograms were compared with chromatograms of the injection solution and the reference compounds [^{99m}Tc(NS$_3$crown)(L2-Lys)], [^{99m}Tc(NS$_3$en)(L2-Lys)] and [^{99m}Tc(NS$_3$(COOH)$_3$)(L2-Lys)].

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Appendix. Supporting Information

Supplementary data associated with this article can be found in the on-line version, at doi:10.1016/j.ejmech.2010.05.010.

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