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Comparison of *in vitro* and *in vivo* properties of [^{99m}Tc]cRGD peptides labeled using different novel Tc-cores

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Aim. **The $\alpha_v\beta_3$ integrin is involved in angiogenesis and tumor metastasis. Arginine-glycine-aspartic acid (RGD)-peptides bind with high affinity to this integrin. This study compares the influence of ^{99m}Tc-labeling applying novel Technetium-cores on imaging characteristics of the radiolabeled peptide.**

Methods. **Different peptide conjugates based on the cyclic pentapeptide c(RGDyK) (cRGD) were prepared and characterized (HYNIC-, Cys-, L2- and Pz1-cRGD). Radiolabeling experiments using different coligands for HYNIC-cRGD, the ^{99m}Tc(CO)$_3$ metal fragment for PZ-1-cRGD (pyrazolyl-derivative), the Tc-nitrido-core using a phosphine-coligand (PNP) for Cys-cRGD and an isonitrile-conjugate (L2-cRGD) together with a NS$_3$-coligand (4+1 concept) were performed and showed labeling yields >90% at high specific activities.**

Results. **A high *in vitro* stability was observed, plasma protein binding and lipophilicity varied considerably between different radiolabeled cRGD conjugates. Experiments on biological activity of the radiolabeled peptides using $\alpha_v\beta_3$ positive (M21) and negative (M21L) tumor cells did show specific uptake of various conjugates. Studies in tumor bearing animals revealed significant differences between different conjugates concerning pharmacokinetic behavior (predominant renal excretion to considerable hepatobiliary clearance) as well as tumor uptake (0.2-2.7%ID/g). Highest specific tumor uptake and tumor/background values were found for [^{99m}Tc]EDDA/HYNIC-c(RGDyK), [^{99m}Tc]Nitrido-PNP-Cys-c(RGDyK) and [^{99m}Tc(CO)$_3$]-Pz1-c(RGDyK).**

Conclusion. **Using novel Tc-cores such as the ^{99m}Tc(CO)$_3$ metal fragment, Tc-nitrido- and the 4+1 concept peptides could be labeled with [^{99m}Tc]technetium at high specific activities resulting in complexes with high stability, but binding moieties have to be optimized especially concerning hydrophilicity resulting in renal rather than hepatobiliary excretion. This comparative study underlines that peptide labeling strategies using ^{99m}Tc have to be properly selected and optimized. Different *in vitro* assays are necessary to predict targeting properties *in vivo*.**

KEY WORDS: Radiopharmaceuticals - Technetium-99m - $\alpha_v\beta_3$, peptides - Product labelling.

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Radiolabeled peptides have found increasing interest as radiopharmaceuticals especially in oncology both for diagnosis and therapy.[1] ^{99m}Tc-labeling is still an attractive approach for radiolabeling peptides for nuclear medicine imaging due to its ideal physical characteristics for SPECT and readily availability from a generator. A number of labeling approaches have been developed over the last years especially suitable for high specific targeting molecules, such as peptides,[2] providing high stability and high specific activity of the final product. Among them the derivatisation with 2-hydrazininicotinic acid (HYNIC) is one of the most often applied technologies and has been successfully used in clinical studies with [^{99m}Tc] Octreotide derivatives.[3] Especially Ethylendia-mine N,N'diacetic acid (EDDA) as coligand provides suitable pharmacokinetics for targeting peptide receptors.[4] Another very versatile approach is based on the use of the Tc-carbonyl core,[5] whereby tridentate ligand systems provide the required *in vivo* stability.[6] Recently also other ligand systems have been developed. One approach is based on the application of the Tc-nitrido-core using asymmetrically disubstituted complexes to specifically label peptides derivatized with bidentate ligands, such as cysteine or diphosphines.[7] Another approach is based on the use of a tetradentate tripodal chelator and a biomolecule derivatized with a monodentate isocyanido ligand. This so-called "4+1" mixed-ligand system consisting of technetium(III) allows high specific activity labeling of biomolecules.[8]

Peptides binding to integrins have been radiolabeled for *in vivo* tumor targeting.[9] Especially the integrin $\alpha_v\beta_3$, which mediates binding to the extracellular matrix *via* a variety of different proteins (*e.g.* vitronectin, fibronectin and van Willebrand factor), is a target structure for the development of this new class of radiopharmaceuticals.[10] This integrin is involved in different pathological processes, such as tumor metastasis and tumor-induced angiogenesis, restenosis, osteoporosis and inflammatory processes.[11] For example, several studies have shown that there is a correlation between $\alpha_v\beta_3$ expression and the metastatic potential of the corresponding tumor.[12] Moreover, this integrin is expressed on activated endothelial cells during migration in the basement membrane in the angiogenic process. Thus, several approaches are focused on the inhibition of the tumor-induced angiogenesis *via* targeting this receptor. Development of inhibitors is based on peptides including the amino acid sequence arginine-glycine-aspartic acid (Arg-Gly-Asp) (single letter code: RGD), which is found to be essential for the binding of a variety of extracellular matrix proteins. The cyclic pentapeptide c(RGDfV)[13] has proven to be selective for the integrin $\alpha_v\beta_3$ and, thus, is used as a lead structure for tracer development.[14] Meanwhile, RGD-peptides labeled with ^{111}In, ^{90}Y, ^{99m}Tc, ^{123}I, ^{64}Cu and ^{18}F have been introduced.[11] Most of them show high $\alpha_v\beta_3$ affinity *in vitro* and allow targeting of receptor positive tumors *in vivo*. The most intensively studied compound yet is [^{18}F]Galacto-RGD, which allows non-invasive determination of $\alpha_v\beta_3$ expression not only in murine tumor models, but also in tumor patients.[15]

Herein, we report on the comparison of four different ^{99m}Tc-labeling strategies based on HYNIC, the Tc-carbonyl and Tc-nitrido-core, as well as the 4+1 mixed ligand system on the same integrin $\alpha_v\beta_3$ binding RGD-peptide. The cyclic pentapeptide cyclo(Arg-Gly-Asp-DTyr-Lys) was chosen for this purpose. This peptide has been shown to target $\alpha_v\beta_3$ integrins with high affinity and can easily be derivatized with chelating moieties at the Lys-residue.[16] Due to its small size the influence of the radioconjugate, depending upon the type of the labeling approach that is utilized, should be pronounced.

Materials and methods

Materials

Reagents were purchased from Aldrich-Sigma Chemical Co., except otherwise stated and used as received.

The chelators NS_3-COOH and Pz1 (3,5-Me_2-$pz(CH_2)_2N((CH2)_3COOH)(CH_2)_2NH_2]$) have been described previously.[8, 17]

Cyclo(Arg-Gly-Asp-DTyr-Lys), cyclo(Arg-Gly-Asp-DTyr-Lys(HYNIC)) (HYNIC-cRGD; HYNIC=2-Hydrazinonicotinic acid), cyclo(Arg-Gly-Asp-DTyr-Lys(Cys)) (Cys-cRGD), and cyclo(Arg-Gly-Asp-DTyr-Lys(Pz1)) (Pz1-cRGD) were synthesized by Biosynthan (Berlin, Germany) with a purity of >95% as analysed by reversed-phase high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS).

L2-BFCA (4-Isocyano-butanoic-acid-tetrafluorophenyl-ester) was prepared by formylation of 4-aminobutyric acid followed by esterification with 2,3,5,6-tetrafluorphenol, and conversion of the formylamino group into the isocyanide group.

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PNP {bis[(dimethoxypropylphosphanyl)ethyl]methoxyethylamine} was kindly provided by Prof. Duatti, Ferrara, Italy.

Na $^{99m}TcO_4^-$ was obtained from a commercial $^{99}Mo/^{99m}Tc$ generator (Ultratechnekow, Mallinckrodt, The Netherlands).

Analytical methods

High performance liquid chromatography

A Dionex P680 low pressure gradient pump with Spectra Physics, Spectra Chrom 100, variable UV detector and Bioscan, radiometric detection was used for RP-HPLC analysis. A Macherey and Nagel 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: ACN/0.1% TFA/H_2O: t: 0-1.5 min 0%ACN, 1.5-16.5 min 0-18% ACN, 16.5-21.5 min 18-60% ACN, 21.5-23 min 60% ACN.

Purification by solid phase extraction

For stability studies the radiolabeled peptides were purified using, a solid phase extraction (SPE) method. The radiolabeling mixture was passed through a C18-SEPPAK-Mini cartridge (Waters, Milford, MA, USA). The cartridge was washed with 5 mL of physiological sodium chloride solution and the radiolabeled peptide was eluted with 70% ethanol. This method efficiently removed all hydrophilic, non-peptide bound impurities (mainly $^{99m}TcO_4^-$, ^{99m}Tc coligands).

^{99m}Tc-labeling

Labeling HYNIC-RGD with EDDA as coligand ([^{99m}Tc]EDDA/HYNIC-cRGD)

In a rubber sealed vial, 10 µg of HYNIC-cRGD were incubated with 1 mL of EDDA/Tricine solution (20 mg/mL Tricine, 10 mg/mL EDDA in pH 6-7), 1 mL of $^{99m}TcO_4^-$ solution (800 MBq), and 20 µL of tin-(II)-solution (10 mg of $SnCl_2 \cdot 2H_2O$ in 10 mL of nitrogen purged 0.1 N HCl) for 10 min in a boiling water bath.

Labeling Pz1 RGD with $[^{99m}Tc(OH_2)_3(CO)_3]^+$ ([^{99m}Tc]Pz1-cRGD)

The carbonyl aquaion $[^{99m}Tc(OH_2)_3(CO)_3]^+$ was prepared as described previously from a commercial kit (Isolink, Mallinckrodt, Petten) according to manufacturer's instructions (1 mL volume, 200-1 000 MBq ^{99m}Tc). Five or 20 µg Pz1-cRGD were incubated with 100 µL carbonyl precursor, specific activity ranging between 18.5 and 74 GBq/µmol.

Labeling L2-cRGD with the 4+1 Approach: ([^{99m}Tc]NS_3/L2-cRGD)

One milligram of Na_2EDTA, 5 mg of Mannitol, and 100 µg of $SnCl_2$ were incubated with 500 MBq of $^{99m}TcO_4^-$ solution in a total volume of 0.8 mL at RT and incubated for 15 min; 0.3 mg coligand (NS_3-COOH) freshly dissolved in 300 µL of methanol were added and this solution was transferred to a vial containing the isocyanide derivatized peptide (L2) and incubated at 75 °C for 1 h.

Labeling of Cys-cRGD with the [^{99m}Tc]nitrido fragment ([^{99m}Tc]PNP-Cys-cRGD)

To 5 mg of succinic hydrazide and 0.05 mg of $SnCl_2$ 1 500-2 000 MBq $^{99m}TcO_4^-$ solution was added resulting in a total volume of 1.1 mL. After 15 min at RT 0.125 mg of PNP3 ligand ($N(CH_2CH_2OCH_3)(CH_2CH_2P(CH_2CH_2CH_2OCH_3)_2)_2$) and 0.05 mg of Cys-cRGD in 0.5 mL was added and incubated at 100 °C for 1 h.

Radiolabeled peptides were characterized and labeling yields were determined by HPLC.

In vitro *evaluation of radiolabeled peptides*

Stability

The stability of the radiolabeled peptides in aqueous solution was tested by incubation of the reaction mixture purified by SPE at a concentration of 200-1 000 pmol peptide/mL in a phosphate buffer as well as in a solution containing 10 000-fold molar excess of cysteine or histidine with respect to the peptide concentration at pH 7.4 at 37 °C up to 24 h. In addition, stability in fresh human plasma, as well as in kidney and liver homogenates, was measured. After incubation, plasma samples were precipitated with acetonitrile and centrifuged (1 750 g, 5 min). Degradation of the ^{99m}Tc complexes was assessed by HPLC.

For incubation in kidney and liver homogenates, kidneys or liver freshly excised from rat were rapidly rinsed and homogenized in 20 mM HEPES buffer pH 7.3 with an Ultra-Turrax T25 homogenator for 1 min at RT. The radiopeptides were incubated with fresh 30% homogenates at a concentration of 250-500 pmol peptide/mL at 37 °C up to 2 h. Samples were

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precipitated with acetonitrile, centrifuged (1 750 g, 5 min) and analyzed by HPLC method 1.

Protein binding

For protein binding assessment the SPE purified complexes were incubated at a concentration of 20-100 pmol peptide/mL in fresh human plasma at 37 °C and analysed up to 6 h by size-exclusion chromatography (MicroSpin™ G-50 Columns; Sephadex G-50). Protein binding of the ^{99m}Tc complex was determined by measuring activity partitioned between the chromatographic column and eluate in a γ-counter.

LogP-values

The ^{99m}Tc-labeled RGD conjugate in 0.5 mL phosphate-buffered saline (PBS) was added to 0.5 mL octanol in an Eppendorf tube. The tube was vigorously vortexed over a period of 15 min. An aliquot of both the aqueous and the octanol layers was collected, counted in the γ-counter and log P value was calculated.

Binding affinity

The *in vitro* binding affinity of HYNIC-cRGD was determined in comparison with c(RGDfV) using [^{125}I]Echistatin as radioligand. [^{125}I]Echistatin was purchased from Amersham-Pharmacia Biotech (Vienna, Austria) and $\alpha_v\beta_3$ integrin was obtained from Chemicon (Temecula, CA, USA). Briefly, 96 well plates were coated with the $\alpha_v\beta_3$ integrin and incubated with a mixture of [^{125}I]Echistatin at varying concentrations of the peptide. Unbound radioligand was removed and wells were washed. Bound radioligand was removed with 2-M NaOH. The IC_{50} was calculated by fitting the percent inhibition values using Origin software (Northhampton, MA, USA).

Internalization and binding studies using $\alpha_v\beta_3$-positive and $\alpha_v\beta_3$-negative cells

$\alpha_v\beta_3$-positive M21 and -negative M21L cells were grown in culture until sufficient numbers of cells were available. For internalization experiments cells were counted to a concentration of 2 million cells/mL in RPMI1640 containing 1% Glutamine and 1% PBS. One-milliliter cell suspension was pipetted into an Eppendorf 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 10 µM c(RGDyK) (Figure 1) in PBS/0.5%BSA buffer (150 µL, non-specific series). Incubation was interrupted by centrifugation, followed by removal of medium and twice rapid rinsing with ice-cold TRIS buffered saline. Thereafter, the cells were incubated twice at ambient temperature in acid wash buffer (50 mM acetate buffer pH 4.2) for 15 min at 37 °C, a period sufficient to remove membrane bound radioligand. The supernatant was collected (membrane bound radioligand fraction) and the cells were washed with acid wash buffer. Cells were lysed by treatment in 1N NaOH and cell radioactivity collected (internalized radioligand fraction). The internalized fraction was determined by measuring radioactivity in the cells and was expressed in percentage of total added activity.

In vivo *evaluation of radiolabeled peptides*

All animal experiments were conducted in compliance with the Austrian animal protection laws and with approval of the Austrian Ministry of Science. Tumor uptake studies were performed in ν/ν 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. On the day of experiment, each 6 mice with M21 and 3 with M21L tumor were injected with ^{99m}Tc-labeled peptide (1 MBq/mouse, corresponding to 1 µg peptide) intravenously into the tail vein. They were sacrificed by cervical dislocation at 1 h (M21 and M21L) and 4 h postinjection (M21).

Tumors and other tissues (blood, lung, heart, stomach, spleen, liver, pancreas, kidneys, muscle, and intestines) were removed. The amount of radioactivity was determined with a γ-counter. Results were expressed as percentage of injected dose per gram of tissue (%ID/g) and tumor-to-organ ratios were calculated.

Results

All peptides were labeled at high specific activities. For HYNIC-cRGD, Pz1-cRGD and Cys-cRGD quantitative labeling yields (>95%) could be achieved using EDDA as coligand (Rt=15 min), the $Tc(CO)_3$-core (Rt=23 min) and PNP as coligand (Rt=23.8 min), respectively. [^{99m}Tc]PNP-nitrido-CYS-cRGD showed two diastereomers when analysed using an optimized

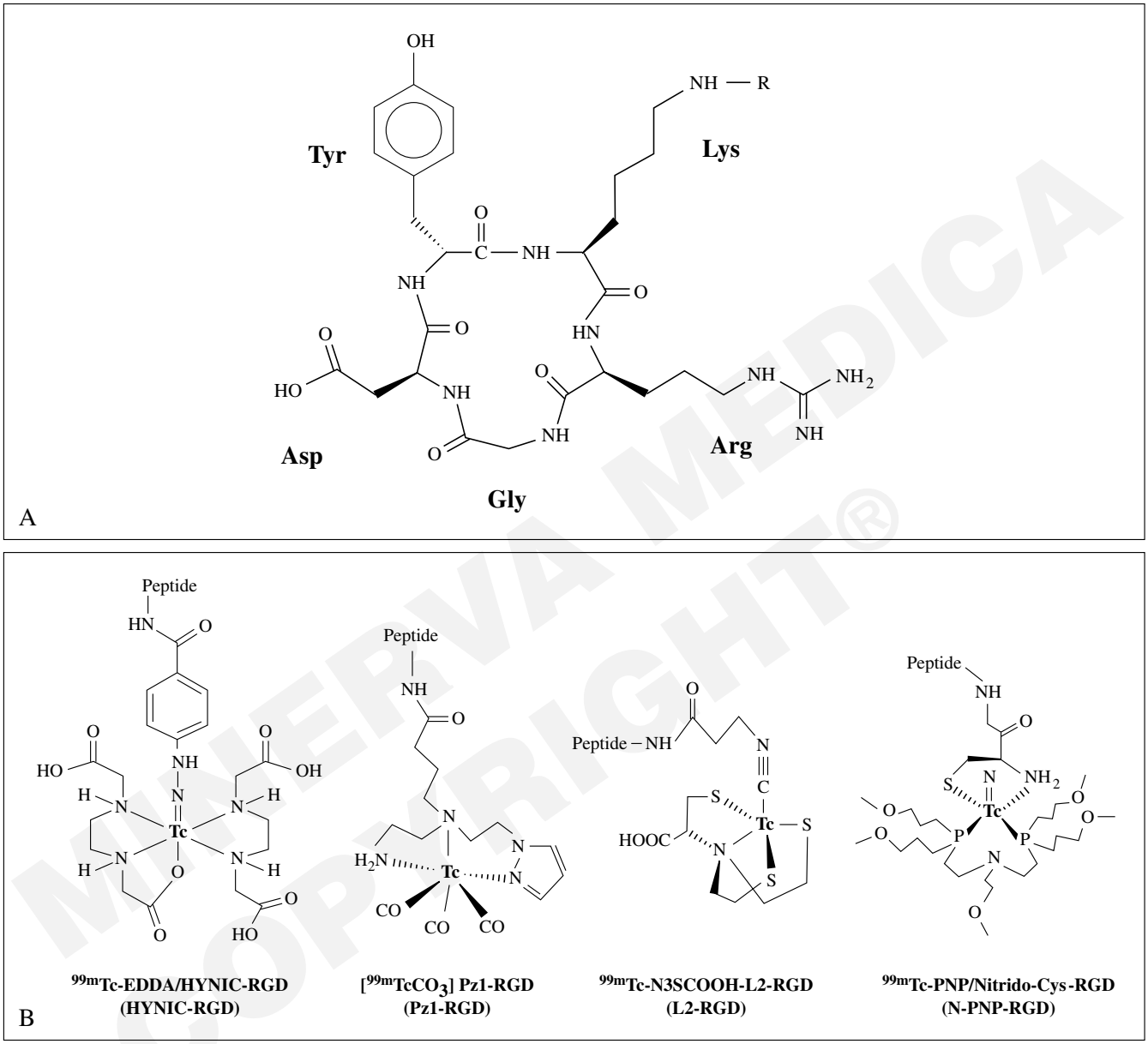

Figure 1.—Structures of the c(RGDyK) (A) and the resulting ^{99m}Tc-labeled peptides (B).

HPLC gradient system (data not shown), which is in accordance with previous findings of pure Tc-complexes of this kind. For L2-cRGD labeling yields were <80% using NS_3-COOH as coligand (Rt=23.1 min), thus requiring HPLC purification before further characterization. Typical radiochromatograms of the radiolabeling solutions are shown in Figure 2.

All ^{99m}Tc-labeled peptides showed high stability in solution with a radiochemical purity >95% up to 6 h after preparation. Also challenge with excess cysteine and incubation in serum or tissue homogenates revealed no significant decomposition or degradation of the ^{99m}Tc-labeled peptides.

Both lipophilicity, determined by HPLC retention

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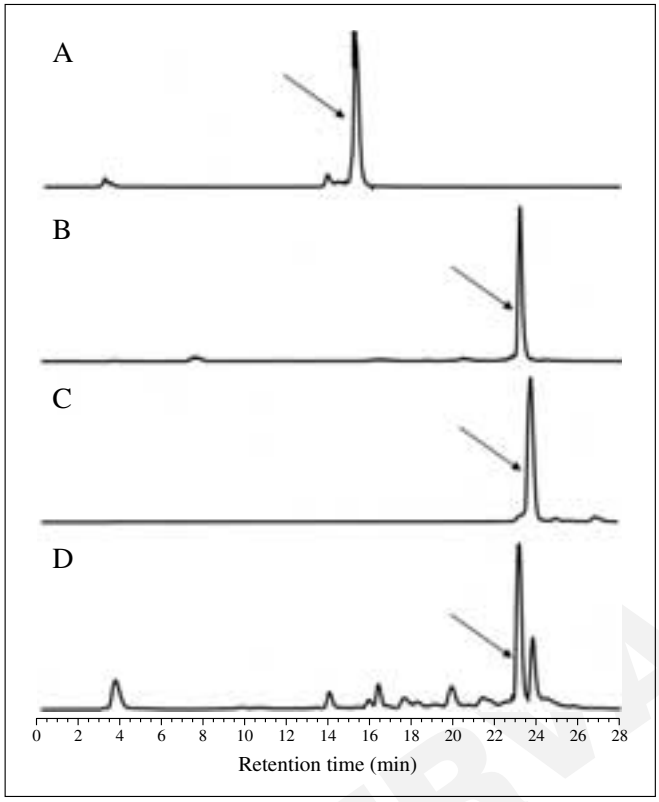

Figure 2.—Radio-high performance liquid chromatograms of the ^{99m}Tc-labeled peptides. A) ^{99m}Tc-EDDA/HYNIC-cRGD; B) [^{99m}Tc(CO)$_3$]Pz1-cRGD; C) [^{99m}Tc]PNP/nitrido-Cys-cRGD; D) [^{99m}Tc]NS$_3$-COOH-L2-cRGD, arrows indicate the ^{99m}Tc-labeled peptide.

TABLE I.—In vitro *parameters as determined for all four ^{99m}Tc-labeled RGD peptides*.

Compound	tR (min)	Log P O/W	Plasma protein binding 2 h (%)
[^{99m}Tc]EDDA/HYNIC-cRGD	15	-3.57	1.8
[^{99m}Tc(CO)$_3$]Pz1-cRGD	23	-0.92	20.1
[^{99m}Tc]PNP/Nitrido-Cys-cRGD	23.8	-1.72	14.3
[^{99m}Tc]NS$_3$-COOH-L2-cRGD	23.1	-2.3	18.8

times or by log P values (-0.92 to -3.57), and binding to serum proteins (2-32%) varied considerably, however not correlating for all compounds. These data are summarized in Table I. [^{99m}Tc]EDDA/HYNIC-cRGD showed lowest HPLC retention times, lowest log P-values (-3.57) and lowest values of plasma protein binding. [^{99m}TcCO$_3$]Pz1-cRGD and [^{99m}Tc]PNP/nitrido-Cys-RGD were considerably more lipophilic with log P-values of -0.91 and -1.72, respectively, correlating with higher protein binding values. Although [^{99m}Tc]NS$_3$-COOH-L2-cRGD was determined to be rather hydrophilic with a log P of -2.3, protein-binding values (18.8%) were considerably higher than for the HYNIC-compound.

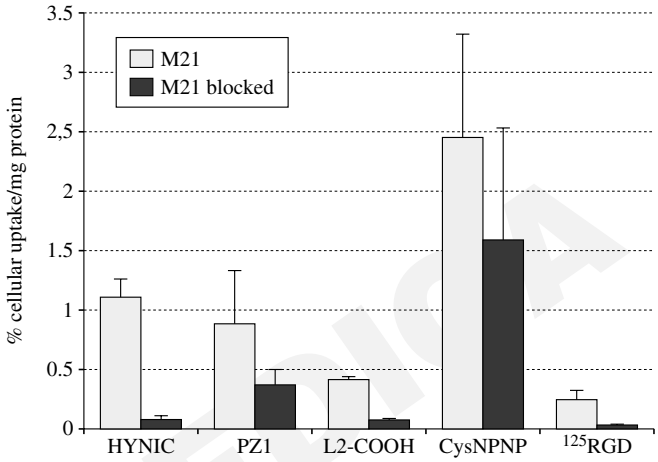

Figure 3.—Uptake of ^{99m}Tc-labeled cyclic Arginine-glycine-aspartic acid (cRGD) peptides in $\alpha_v\beta_3$ positive M21 tumor cells *in vitro*.

The cold peptide conjugates showed high affinity to the $\alpha_v\beta_3$ integrin with IC_{50} values in the low nanomolar range (HYNIC-cRGD=6 nM, Pz1-cRGD=3 nM, Cys-cRGD=6.6 nM, L2-cRGD=11.8 nM). In comparison for c(RGDfV) an IC_{50} value of 3.7 nM was found. All ^{99m}Tc-labeled peptides showed specific internalization in $\alpha_v\beta_3$-positive M21 cells with values from approx. 0.5-2% of total/mg protein, compared to values for [^{125}I]c(RGDyK) of only 0.24%. Uptake values were significantly lower when $\alpha_v\beta_3$ receptors were blocked with excess c(RGDyK). Cellular uptake values are summarized in Figure 3.

In a mouse tumor model, significant variations in pharmacokinetic behavior were found ranging from predominant renal excretion up to predominant hepatobiliar elimination: data are summarized in Table II. The radiolabeled conjugate [^{99m}Tc]EDDA/HYNIC-cRGD showed values below 4%ID/g in all organs, whereas all other ^{99m}Tc-compounds revealed higher uptake values especially in liver (6.7-8.89%ID/g) and intestines (10.1-32.8%ID/g). Tumor uptake values were comparable for [^{99m}Tc]EDDA/HYNIC-cRGD, [^{99m}Tc(CO)$_3$]Pz1-cRGD and [^{99m}Tc]PNP/nitrido-Cys-cRGD ranging from 2.44-2.73% and were significant for lower (<1%ID/g) in $\alpha_v\beta_3$-negative M21L tumors.

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TABLE II.—*Biodistribution of all ^{99m}Tc-compounds under study in $\alpha_v\beta_3$ positive M21 tumor-bearing nude mice, in comparison tumor values in $\alpha_v\beta_3$ negative M21L tumors.*

	[^{99m}Tc]EDDA/HYNIC-cRGD	[^{99m}Tc(CO)$_3$]Pz1-cRGD	[^{99m}Tc]PNP/nitrido-Cys-cRGD	[^{99m}Tc]NS$_3$-COOH-L2-cRGD
Blood	0.98±0.04	0.96±0.06	0.48±0.15	0.59±0.03
Heart	1.01±0.05	0.97±0.13	0.7±0.15	0.3±0.02
Stomach	2.5±0.75	2.12±1.15	1.13±0.16	0.69±0.26
Liver	2.63±0.14	8.89±0.71	7.7±1.92	6.7±0.65
Kidneys	3.68±0.13	5.65±0.88	6.87±0.46	2.69±0.21
Muscle	0.76±0.69	1.04±0.68	0.44±0.06	0.14±0.02
Intestine	2.04±0.18	10.09±1.3	23.7±1.93	32.83±2.33
Tumor	2.73±0.26	2.5±0.29	2.44±0.61	0.2±0.04
Tumor M21L	0.85±0.2	0.71±0.08	0.85±0.07	0.23±0.02

[^{99m}Tc]NS$_3$-COOH-L2-cRGD showed only very low tumor uptake (0.20%ID/g) with no significant difference to receptor negative tumors.

Discussion

The introduction of a radiometal such as ^{99m}Tc in a highly specific biomolecule can be achieved *via* different approaches.[18] The so-called pendant approach, whereby a metal binding moiety is attached to the biomolecule, has so far been more successful than the integrated approach where the radiolabel is a major constituent of the biological recognition site. In particular, the derivatisation of small peptides with bifunctional chelators as part of the whole peptide synthesis is currently a well-established method, even special ^{99m}Tc-binding building blocks for peptide synthesis have already been described.[19]

The corresponding peptide conjugate can usually be radiolabeled in a rather straight forward way in aqueous solution and ideally used without further purification. This postlabeling strategy has shown major advantages over prelabeling approaches, whereby the ^{99m}Tc-chelate is formed first, followed by attachment to the peptide. Although the ^{99m}Tc binding moiety has to fulfil strict criteria, such as achievement of high specific activity labeling, high *in vitro* and *in vivo* stability and compatibility with the peptide chain, currently a variety of labeling approaches are available. It is known that the choice of the bifunctional chelator has a profound effect on the pharmacokinetics of the resulting ^{99m}Tc-labeled construct.[20] In a study on radiolabeled somatostatin analogs, we compared triamidomonothiol-(N_3S) with HYNIC-conjugates. In this study, the NS_3 ligands resulted in predominant hepatobiliar excretion whereas HYNIC labeling using a suitable coligand shifted excretion to almost exclusive renal pathways.[21] Pharmacokinetics of HYNIC-derivatized peptides are strongly dependent on the coligand used.[22] When using a suitable coligand, such as EDDA, the HYNIC technology has proven to be suitable for radiolabeling peptides for clinical application.[23]

Since then, a number of new labeling approaches have been developed, thus increasing the chemical variability dramatically. The precursor complex $[^{99m}Tc(OH_2)_3(CO)_3]^+$ is one of these promising cores for the development of new ^{99m}Tc-based radiopharmaceuticals.[24] The exchange of the three labile water molecules by a great variety of chelators gives high flexibility in the labeling approach.[25] The 4+1 chemistry developed by Pietzsch *et al.* allows ^{99m}Tc-labeling at high specific activities simply by modification of a biomolecule with a monodentate binding moiety.[26] The nitrido chemistry provides a tool for selective targeting of an N-terminal cystein residue, opening the option to label a natural amino acid with high selectivity and high stability at high specific activities.[27]

This study provides comparative data on labeling a small peptide utilizing these different technetium-cores. The cyclic pentapeptide c(RGDyK) provided an excellent platform to compare these different labeling strategies, because it consists only of 5 amino acids, thus, the effects of the different chelator systems may have a greater impact on the tracer properties as opposed to what is observed for larger peptides or even proteins. All labeling strategies fulfilled main criteria for labeling small, potentially highly potent small peptides. Specifically, high specific activities where achieved, *i.e.* small amounts of peptide were

necessary to achieve high labeling yields, whereby specific optimizations such as using small reaction solvent volumes in the case of $Tc(CO)_3$-labeling had to be taken into account. The labeled peptides showed a suitable high stability and very low rates of ligand exchange when challenged with cysteine or histidine.

The decisive influence of the chelator on the targeting properties of a highly specific biomolecule was confirmed *in vitro*. The different labeling strategies resulted in tracers with very heterogeneous lipophilicity and protein binding behavior. Studies in cells revealed significant retention of binding affinity and specific internalization for all compounds of this study. Differences in both excretion patterns and tumor uptake values were found *in vivo*. For ^{99m}Tc-labeled HYNIC-, Pz1- and Cys-cRGD conjugates excretion patterns shifted from predominant renal excretion to considerable intestinal excretion with increasing lipophilicity and plasma protein binding, whereas tumor uptake values were comparable (2.44-2.73%ID/g). This proofs a suitable stability of the complexes and also indicates that by increasing hydro-philicity, *e.g.* by introduction of pharmacokinetic modifiers such as carbohydrates, improvement of pharmacokinetic behavior might be achievable (especially for Pz1- and Cys-cRGD labeled *via* the nitrido chemistry). L2-cRGD labeled using the 4+1 approach and an NS_3-COOH coligand showed different *in vivo* behavior. Although being comparably hydrophilic, the highest intestinal uptake indicating predominant hepatobiliar clearance was observed for L2-cRGD. In addition, tumor uptake was very low both in receptor positive as well as in receptor negative tumors. This could be explained by a specific metabolic behavior of this kind of complexes. Further investigations on the influence of the coligand on *in vivo* behavior could give more insight in this phenomenon.

It is interesting to note that only the combination of all *in vitro* parameters was predictive for the *in vivo* behavior. For instance the high stability, low lipophilicity, low protein binding and retained receptor mediated cell uptake of [^{99m}Tc]EDDA/HYNIC-RGD also resulted in suitable pharmacokinetics with high, receptor mediated tumor uptake.

Conclusions

In conclusion, our study compared novel technetium cores including HYNIC, $Tc(CO)_3$, Tc 4+1 and Tc-nitrido for the preparation of ^{99m}Tc-labeled peptides. Although all strategies allowed labeling at high specific activities with good *in vitro* stability, HPLC analysis and protein binding assays revealed considerable differences. In a tumor model, varying pharmacokinetics and tumor uptake values were observed with comparatively high uptake levels for [^{99m}Tc$(CO)_3$]-Pz1-RGD, [^{99m}Tc]Nitrido-PNP/Cys-RGDyK and [^{99m}Tc]EDDA/HYNIC-RGD. Retrospectively this study demonstrates that in the labeling of peptides with ^{99m}Tc, strategies have to be properly selected and optimized. Different *in vitro* assays are necessary to predict targeting properties *in vivo*. For peptide radiopharmaceuticals high stability, hydrophilic properties and low plasma protein binding conjoined with retention of biological activity usually allow to identify the most promising candidates for sensitive *in vivo* detection of receptors in oncology, but potentially also in other diseases. For central nervous diseases requiring brain uptake much different characteristics might be sought to develop a successful imaging agents.

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