

# The $(\text{Tc}(\text{N})(\text{PNP}))^{2+}$ metal fragment labeled cholecystokinin-8 (CCK8) peptide for CCK-2 receptors imaging: *in vitro* and *in vivo* studies

STEFANIA AGOSTINI,<sup>a</sup> CRISTINA BOLZATI,<sup>a,b\*</sup> ELIANA DIDONÈ,<sup>a</sup> MARIO CAVAZZA-CECCATO,<sup>a</sup> FIORENZO REFOSCO,<sup>b</sup> LUIGI ALOJ,<sup>c</sup> CLAUDIO ARRA,<sup>c</sup> MICHELA AURILIO,<sup>c</sup> ANNA LUCIA TORNESELLO,<sup>d</sup> DIEGO TESAURO<sup>d</sup> and GIANCARLO MORELLI<sup>d\*</sup>

<sup>a</sup> Department of Pharmaceutical Sciences, University of Padua, Via Marzolo, 5, 35131 Padua, Italy

<sup>b</sup> ICIS – CNR, Corso Stati Uniti, 4, 35127 Padua, Italy

<sup>c</sup> Department of Nuclear Medicine, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione ‘G. Pascale’, Via M. Semmola, 80131, Naples, Italy

<sup>d</sup> Research Center on Bioactive Peptides, CIRPeB, University of Naples ‘Federico II’, Via Mezzocannone, 16, 80134 Naples, Italy

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**Abstract:** The radiolabeling of the natural octapeptide CCK8, derivatized with a cysteine residue (Cys-Gly-CCK8), by using the metal fragment  $[\text{Tc}(\text{N})(\text{PNP3})]^{2+}$  (PNP3 = *N,N*-bis(dimethoxypropylphosphinoethyl)methoxyethylamine) is reported. The  $[\text{Tc}(\text{N})(\text{NS-Cys-Gly-CCK8})(\text{PNP3})]^{2+}$  complex was obtained according to two methods (one-step or two-step procedure) that gave the desired compound in high yield. The complex is stable in aqueous solution and in phosphate buffer. *In vitro* challenge experiments with an excess of cysteine and glutathione indicate that no transchelation reactions occur, confirming the high thermodynamic stability and kinetic inertness of this compound. Stability studies carried out in human and mouse serum, as well as in mouse liver homogenates, show that the radiolabeled compound remains intact for prolonged incubation at 37 °C. Binding properties give  $K_d$  ( $19.0 \pm 4.6$  nmol/l) and  $B_{\text{max}}$  ( $\sim 10^6$  sites/cell) values in A431 cells overexpressing the CCK2-R. *In vivo* evaluation of the compound shows rapid and specific targeting to CCK2-R, a fourfold higher accumulation compared to nonreceptor expressing tumors. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** CCK8 peptide conjugate; technetium; Tc–nitrido complexes; nuclear medicine

## INTRODUCTION

Peptides are receptor-specific molecules that play an important role not only in the diagnosis and therapy of neoplastic diseases but also in the pathogenesis of other diseases [1–3]. This fact has generated high interest in the field of nuclear medicine and has stimulated the development of several radiolabeling procedures of these biomolecules with various radionuclides such as indium-111 ( $^{111}\text{In}$ ;  $t_{1/2} = 67.4$  h;  $\gamma = 171$ ; 245 keV) or iodine-131 ( $^{131}\text{I}$ ;  $t_{1/2} = 8.0$  days;  $\gamma = 364$  keV) either for imaging and/or therapy [4]. However, these radionuclides have less favorable imaging characteristics owing to their high gamma energies. In this respect, technetium-99m ( $^{99\text{m}}\text{Tc}$ ;  $t_{1/2} = 6.02$  h;  $\gamma = 142$  keV) represents, for diagnostic purposes, the ideal choice for single-photon emission computed tomography (SPECT).

Using this radionuclide, different peptide labeling procedures are available from the literature [5,6]. Basically, in all reported procedures the target

vector has to be: (i) derivatized with an appropriate chelating system for the  $^{99\text{m}}\text{Tc}$ ; (ii) labeled with high specific activity (low vector concentration) and (iii) capable of retaining its physico-chemical properties and affinity toward the corresponding receptor site. Moreover, for routine use the labeling procedure should be performed preferentially in a single step [5].

Recently, technetium mixed-ligand complexes displaying characteristic substitution-inert metal fragments ( $[\text{Tc}(\text{CO})_3]^+$  and  $[\text{Tc}(\text{N})(\text{PNP})]^{2+}$ ) have emerged as alternative platforms in the design of potential  $^{99\text{m}}\text{Tc}$  ‘target-specific’ radiopharmaceuticals [7–10]. In this context, attempts to develop Tc-99m target-specific agents using the  $[\text{Tc}(\text{N})(\text{PNP})]^{2+}$  synthon (PNP is an aminodiphosphine) have recently been undertaken [10–13]. As for the  $[\text{Tc}(\text{CO})_3]^+$  approach, one of the major advantages of the  $[\text{Tc}(\text{N})(\text{PNP})]^{2+}$  technology was the possibility to obtain well-defined complexes with very high yields (>90%) depending on the co-ligand [10–15]. Owing to its peculiar electronic structure, the  $[\text{Tc}(\text{N})(\text{PNP})]^{2+}$  fragment exhibits a strong electrophilic character and efficiently reacts with bifunctional ligands (L) carrying  $\pi$ -donors as coordinating atoms (S, O, N) to afford asymmetrical nitrido heterocomplexes of the type  $[\text{Tc}(\text{N})(\text{L})(\text{PNP})]^{0/+}$ .

\*Correspondence to: C. Bolzati, ICIS – CNR Corso Stati Uniti, 4, 35127 Padua, Italy; e-mail: bolzati@icis.cnr.it

G. Morelli, Research Center on Bioactive Peptides, CIRPeB, University of Naples ‘Federico II’, Via Mezzocannone, 16, 80134 Naples, Italy; e-mail: gmorelli@unina.it

Naturally occurring bidentate ligands such as cysteine display excellent coordinating properties toward the  $[\text{Tc}(\text{N})(\text{PNP})]^{2+}$ , providing a simple way of incorporating short peptide chains into a nitrido Tc-99m asymmetrical complex. Cysteine binds the  $[\text{Tc}(\text{N})(\text{PNP})]^{2+}$  moiety either through the  $[\text{NH}_2, \text{S}^-]$  pair or, alternatively, the  $[\text{O}^-, \text{S}^-]$  pair of donor atoms to yield the corresponding mixed compound with high specific activity (approximately 70 GBq/ $\mu\text{mol}$ ). Thus efficient bifunctional ligands can be easily obtained by coupling the selected target vector to the carboxylic or amino terminal group of a cysteine residue to give COO-functionalized  $\text{NH}_2$ , S-cysteine or N-functionalized O, S-cysteine ligands, respectively [10]. Asymmetrical  $[\text{Tc}(\text{N})(\text{cys} \sim)(\text{PNP})]^{0/+}$  complexes, characterized by remarkable *in vitro* and *in vivo* stability, were obtained by reacting the  $[\text{Tc}(\text{N})(\text{PNP})]^{2+}$  metal fragment with the bifunctional ligand (cys  $\sim$ ) [13,16]. The overall charge of the resulting mixed compound is dependent on the pair of donor atoms of cysteine. A  $[\text{NH}_2, \text{S}^-]$  chelating system yields monocationic complexes, while neutral complexes arise when cysteine coordinates through the  $[\text{O}^-, \text{S}^-]$  pair [10].

We report here the first application of  $[\text{Tc}(\text{N})(\text{PNP})]^{2+}$  technology to the labeling of a small bioactive peptide. In this study PNP3 (*N,N*-bis(dimethoxypropylphosphinoethyl)methoxyethylamine) was selected as the aminodiphosphine ligand and CCK8 was chosen as carrier bioactive peptide. This natural octapeptide is able to recognize the CCK receptors (CCK1-R and CCK2-R). Both receptors, localized in the cell membrane, belong to the G-protein-coupled receptor (GPCR) superfamily and are overexpressed in many malignant tumors. CCK1-R is expressed rarely in tumors except in gastroenteropancreatic tumors (38%), meningiomas (30%) and some neuroblastomas (19%). CCK2-R is found frequently in medullary thyroid carcinomas (92%), small cell lung cancer (57%), astrocytomas (65%) and stromal ovarian tumors (100%), while it is found occasionally in gastroenteropancreatic tumors and breast endometrial and ovarian adenocarcinomas [17–20].

In the past years the receptor interaction with the CCK peptide conjugates, modified on N-terminal-aa sequence with chelating groups to coordinate several radioactive metals such as In, Y or Re, was chemically and biologically investigated by several researchers [21–28]. In this context, the search for a  $^{99\text{m}}\text{Tc}$ -based radiotracer for targeted tumor imaging has involved hydrazino-nicotinamide (HYNIC)/co-ligand, tricarbonyl/ $\text{N}_2\text{O}$ ,  $\text{N}_4$  derivatized CCK or gastrin analogs [29–31].

All conjugates preserve their receptor affinity, but the stability and the biodistribution *in vivo* are highly dependent on the chelating moiety. These results stimulate to improve the inertness of the metal complexes and the biodistribution properties. Some issues can benefit from further studies; therefore,

it is worthwhile to assess the efficacies of different compounds and different labeling approaches.

Here, we describe the preparation and the chromatographic characterization of the  $[\text{Tc}(\text{N})(\text{NS-Cys-Gly-CCK8})(\text{PNP3})]^{+}$  peptide conjugate complex. Its *in vitro* stability was investigated in phosphate buffer solution (PBS), cysteine and glutathione solutions, as well as in human serum, mouse serum and mouse liver homogenates. Receptor binding specificity and affinity were evaluated in a cell culture system of CCK2-R over-expression. Preliminary *in vivo* receptor targeting and biodistribution studies were performed in nude mice bearing xenografts of A431-CCK2-R cells and A431 control cells.

## MATERIALS AND METHODS

### General

All chemicals and reagents were purchased from Sigma Aldrich (Milan, Italy). All solvents were of reagent grade and were used without further purification. The aminodiphosphine ligand *N,N*-bis(dimethoxypropylphosphinoethyl)methoxyethylamine [PNP3 =  $(\text{CH}_3\text{OC}_3\text{H}_6)_2\text{P}(\text{CH}_2)_2\text{N}(\text{C}_2\text{H}_4\text{OCH}_3)(\text{CH}_2)_2\text{P}(\text{C}_3\text{H}_6\text{OCH}_3)_2$ ] was purchased from Argus Chemicals (Prato, Italy). Owing to the tendency of the diphosphine ligands to oxidize, all the solvents used in the reactions with PNP3 were previously degassed to remove traces of dissolved dioxygen. Fmoc-amino acids and the other reagents for peptide synthesis were purchased from INBIOS (Pozzuoli, Italy).

Technetium-99m as  $\text{Na}^{99\text{m}}\text{TcO}_4$  was eluted from a  $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$  generator provided by Nycomed Amersham-Sorin (Saluggia, Italy).

Sep-Pak RP-C18 and OASIS HLB extraction cartridges were purchased from Waters Corporation (Milford, MA, USA).

### Analysis

TLC ( $\text{SiO}_2$  F<sub>254S</sub> and C18 F<sub>254S</sub>, Merck) and HPLC analyses were used to evaluate the radiochemical yield (RCY) and the stability as radiochemical purity (RCP) of the compounds. The radioactivity on TLC plates was detected and measured using a Cyclone instrument equipped with a phosphorus imaging screen and the OptiQuant image analysis software (Packard, Meridian, CT). HPLC was performed on a Beckman System Gold instrument equipped with a programmable solvent Model 126, a scanning detector Module 166 ( $\lambda = 215 \mu\text{m}$ ) and a radioisotope detector Model 3200 Bioscan.

HPLC analysis was performed with a reverse-phase Beckman C18 precolumn (5  $\mu\text{m}$ , 4.6  $\times$  45 mm) and a Beckman C18 column (5  $\mu\text{m}$ , 4.6  $\times$  250 mm). The flow rate was 1 ml/min. The results are summarized in Table 1 and compared with the chromatographic data of the analogous (*syn/anti*)  $[\text{Tc}(\text{N})(\text{NS-Cys-OEt})(\text{PNP3})]^{+}$  (where NS-Cys-OEt was L-cysteine ethylester) compound, prepared as previously described [9,10].

### Peptide Synthesis

The NS-Cys-Gly-CCK8 peptide was obtained by solid-phase peptide synthesis, performed under standard conditions using

**Table 1** Chromatographic data of <sup>99m</sup>Tc-complexes

Complex	TLC R <sub>F</sub> C18	TLC R <sub>F</sub> SiO <sub>2</sub>	HPLC R <sub>T</sub> (min)	%RCY a + b
a – [ <sup>99m</sup> TcN(NS-Cys-OEt)(PNP3)] <sup>+</sup>	<sup>a</sup> 0.33	—	<sup>d</sup> 26.03	91.37
b – [ <sup>99m</sup> TcN(NS-Cys-OEt)(PNP3)] <sup>+</sup>	<sup>a</sup> 0.21	—	<sup>d</sup> 29.03	
a – [ <sup>99m</sup> Tc(N)(NS-Cys-Gly-CCK8)(PNP3)] <sup>+</sup>	<sup>b</sup> 0.65; <sup>c</sup> 0.85	<sup>a</sup> 0.67	<sup>d</sup> 25.45; <sup>e</sup> 29.89	90.0
b – [ <sup>99m</sup> Tc(N)(NS-Cys-Gly-CCK8)(PNP3)] <sup>+</sup>	<sup>b</sup> 0.65; <sup>c</sup> 0.85	<sup>a</sup> 0.61	<sup>d</sup> 25.45; <sup>e</sup> 29.89	

**TLC:** C18: <sup>a</sup> MeCN/Et<sub>3</sub>N 0.01 M (pH 3, H<sub>3</sub>PO<sub>4</sub> 1 M) (60/40); <sup>b</sup> Sal/MeOH/THF/HAc<sub>(g)</sub>, (2/8/1/1); <sup>c</sup> MeOH/MeCN/THF/HAc<sub>(g)</sub> (3/3/2/2). SiO<sub>2</sub>: <sup>1</sup> EtOH/CHCl<sub>3</sub>/Tol/NH<sub>4</sub>Ac (0.5 M) (6/2/0.5/1.5).

**HPLC:** A = NEt<sub>3</sub> 0.01 M pH 3 for H<sub>3</sub>PO<sub>4</sub> 1 M, B = CH<sub>3</sub>CN; <sup>d</sup> gradient: 0–5 min, B, 0%; 5–15 min, B 30%; 15–20 min, B 45%; 20–35 min, B, 45%; 35–37 min, B, 0%. <sup>e</sup> gradient: 0–5 min, B, 0%; 5–15 min, B 30%; 15–20 min, B, 39%; 20–40 min, B, 39%; 40–42 min, B, 0%.

the Fmoc strategy. The amino acid sequence of the Cys-Gly-CCK8 peptide, with the amidated C-terminus as in the natural peptide, was Cys<sup>24</sup>-Gly<sup>25</sup>-Asp<sup>26</sup>-Tyr<sup>27</sup>-Met<sup>28</sup>-Gly<sup>29</sup>-Trp<sup>30</sup>-Met<sup>31</sup>-Asp<sup>32</sup>-Phe<sup>33</sup>-NH<sub>2</sub>, according to the numbering of CCK-33. The fully automated peptide synthesizer Applied Biosystem model ABI 433 was used, adopting the 0.250 mmol scale. The Rink-amide 4-methylbenzhydrylamine (MBHA) resin, loading 0.5 mmol/g, was used. Double couplings were performed, adding four equivalents of protected amino acids activated by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HBTU) and HOBt, and eight equivalents of DIPEA in *N*-methylpyrrolidone (NMP); the stirring time was 60 min for each coupling. For deprotection and cleavage, the fully protected Cys-Gly-CCK8 peptide resin was treated with TFA containing tri-isopropylsilane (1.0%), ethanedithiol (2.5%) and water (2.5%). The crude peptide was precipitated at 0 °C by adding diethyl ether dropwise. Purification of the crude mixtures was carried out by RP-HPLC. Analytical RP-HPLC was carried out on a Shimadzu 10ADvp instrument using a Vydac C18 column, 4.6–250 mm, eluted with H<sub>2</sub>O/0.1% TFA (A) and CH<sub>3</sub>CN/0.1% TFA (B) with linear gradient from 5 to 65% B over 20 min at 1 ml/min flow rate. Preparative RP-HPLC was carried out on a Shimadzu 8A instrument equipped with a UV-vis Shimadzu detector using a Vydac C18 column, 22–250 mm, with the same eluents as used on the analytical scale and a gradient from 20 min at 25 ml/min flow rate. R<sub>f</sub> was 19.96 min. Mass spectra recorded on a MALDI-TOF Voyager-DE (PerSeptive Biosystems) system confirmed the product identity: M<sub>w</sub> (NS-Cys-Gly-CCK8) = 1220.

**Preparation of [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup>**

Method 1 (two-step procedure): Na<sup>99m</sup>TcO<sub>4</sub> (0.5 ml, 50.0 MBq-3.0 GBq) was added to a vial containing succinic dihydrazide (SDH) (5.0 mg), SnCl<sub>2</sub> (0.1 mg suspended in 0.1 ml of saline) and ethanol (0.5 ml). The vial was kept at room temperature for 30 min giving a mixture of <sup>99m</sup>Tc-nitrido precursors [<sup>99m</sup>Tc ≡ N]<sup>2+</sup>. Then the PNP3 ligand (1.0 mg dissolved in 0.1 ml of EtOH) and NS-Cys-Gly-CCK8 (0.10 mg suspended in 0.250 ml of phosphate buffer 0.1 M pH 7.4) were simultaneously added, and the reaction mixture was heated at 80 °C for 60 min. The pH of the reaction mixture, measured at the end of the reaction, was 7. RCY as determined by TLC and HPLC chromatography was 90.0%.

Method 2 (one-step procedure): Na<sup>99m</sup>TcO<sub>4</sub> (0.5 ml, 50.0 MBq-3 GBq) was added to a vial containing SDH (5.0 mg),

SnCl<sub>2</sub> (0.1 mg suspended in 0.1 ml of saline), ethanol (0.5 ml), NS-Cys-Gly-CCK8 (0.1 mg suspended in 0.250 ml of phosphate buffer 0.1 M pH 7.4) and PNP3 (1 mg dissolved in 0.1 ml of EtOH). RCY determined by TLC and HPLC, after 60 min at 80 °C, was 90.9%.

The [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> isolated by HPLC was concentrated on a Sep pack C18 column rinsed with H<sub>2</sub>O (10 ml) and eluted using a mixture of EtOH/phosphate buffer 0.2 M, pH 7.4, 80/20 (1 × 0.25 ml, 1 × 0.75 ml). The second fraction containing all the activity was utilized for *in vitro* and *in vivo* studies. After purification, the RCP evaluated by TLC and HPLC was >95%.

**Radiolabeling Efficiency of NS-Cys-Gly-CCK8 Ligand**

The radiolabeling efficiency of the NS-Cys-Gly-CCK8 ligand was determined following the standardized labeling condition reported in method 2.

The amount of PNP3 was fixed at 1 mg (2.06 × 10<sup>-3</sup> mmol), while the concentration of the NS-Cys-Gly-CCK8 ligand was progressively decreased in the range 200 μg (1.64 × 10<sup>-4</sup> mmol) to 5 μg (4.08 × 10<sup>-6</sup> mmol). The mixture was incubated at different temperatures (100, 80, 50 °C and RT) for 60 min. The RCY at 15, 30 and 60 min was determined by TLC and HPLC.

**In vitro Studies**

**Cysteine (Cys) and glutathione (GSH) challenge.** Challenge experiments were carried out on the purified complex using an excess of Cys or GSH. An aliquot (50 μl) of an aqueous stock solution of cysteine hydrochloride (10 mM or 1 mM) was added to a propylene test tube containing phosphate buffer (250 μl, 0.2 M; pH 7.4), water (100 μl) and the [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> complex (100 μl). The mixture was vortexed and incubated at 37 °C for 24 h. A control reaction containing an equal volume of water, instead of cysteine hydrochloride, was studied in parallel. At 30 min, 1, 2, 3 and 24 h, aliquots of the reaction mixture were withdrawn and analyzed by TLC and HPLC. A similar procedure was applied using GSH (50 μL, 10 mM) as the challenge ligand. The experiments were performed in duplicate.

**In vitro stability.** The *in vitro* stability of the complex was evaluated by monitoring the RCP at different time points using the following procedures: in a propylene test tube, 50 μl of purified Tc-99m compound was added to: (a) 950 μl of saline,

(b) 450  $\mu$ l of human serum, (c) 450  $\mu$ l of mouse serum and (d) 450  $\mu$ l of homogenate of mouse liver. The resulting mixture was incubated at 37 °C for 24 h.

At 15 min, 1, 2, 3 and 24 h, aliquots (50  $\mu$ l) of each solution were withdrawn, and diluted with 950  $\mu$ l of phosphate buffer (0.02 M, pH 7.4).

One hundred microlitres of the sample a, b and c were withdrawn and directly analyzed by HPLC using the following elution conditions: reverse-phase Symmetry 300 C4 precolumn (5  $\mu$ m, 3.9  $\times$  20 mm) and a reverse-phase Symmetry 300 C4 column (5  $\mu$ m, 4.6  $\times$  150 mm). Solvents: A = DMGA (0.05 M, pH 8, for NaOH 0.1 M; DMGA = 3,3 dimethylglutaric acid), B = CH<sub>3</sub>CN; Gradient: 0 min, B, 25%; 0–25 min, B, 33%; 25–27 min, B, 25%. Flow rate 1 ml/min.

At the same time, the sample d was treated using an OASIS HLB extraction cartridge before HPLC injection. The sample was loaded on the cartridge, conditioned with MeOH (1 ml), equilibrated with water (1 ml) and rinsed with MeOH 5% (3 ml). The activity was eluted with a mixture of EtOH/phosphate buffer 0.2 M, pH 7.4, 80/20 (1 ml). Eighty percent of the initial activity was collected in the elution fraction and analyzed by TLC and HPLC.

The experiments were performed in duplicate.

**Binding experiments.** Saturation binding experiments were performed on A431 cells overexpressing the CCK2-R following the procedure previously described [27]. Briefly, the cells were plated at a density of 1–200 000 cells/well in 12 multiwell plates 2–3 days prior to the experiments. Duplicate wells were incubated with serial dilutions of the labeled compound in the culture medium at 4 °C. Bound radioactivity was recovered by trypsinizing the cells after two rapid washes in ice-cold PBS following 1 h incubation and measured by gamma counting. Binding curves were generated after normalizing for the number of cells and subtracting nonspecific binding using the Kaleidagraph software (Abelbeck Software, Version 3.5, distributed by Synergy Software, Reading, PA). The dissociation constant ( $K_d$ ) and the apparent number of binding sites per cell ( $B_{max}$ ) were derived by fitting to a standard saturation binding model as described [27].

### Biodistribution Studies

Xenografts of A-431 cells overexpressing the CCK2-R and control cells were generated in the opposite flanks of 6-week-old CD-1 nude mice (weight 17–23 g). The tumors were allowed to grow for 10–14 days following subcutaneous injection. The tissue and tumor distribution of [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> was determined 1 h after injecting ~50  $\mu$ Ci/mouse of labeled peptide in the lateral tail vein in five animals. Blood, lung, liver, spleen, kidney, stomach, gastrointestinal tract, muscle and xenograft samples were weighed, and the radioactivity determined by gamma counting. Dilutions of the injected compound were simultaneously counted for accurate determination of the injected dose. The relative amount of radioactivity in the organs was calculated and expressed as percentage of the injected dose/gram of tissue (%ID/g), normalized to a 20-g mouse.

## RESULTS AND DISCUSSION

Evidence on the CCK-2/gastrin-R expression in several malignant tumors has provided the molecular basis

for the development of radiolabeled CCK and gastrin analogs for targeted tumor imaging and radionuclide therapy [17–20]. Several research groups have been actively engaged in this effort using either CCK or gastrin motifs [21–28]. In this context, the search for a <sup>99m</sup>Tc-based radiotracer for targeted tumor imaging has begun with recent studies involving various labeling approaches with different chelating systems derivatized with CCK or gastrin analogs [29–31].

Recently, we have described the synthesis of a new class of asymmetrical nitrido complexes, based on the chemical properties of the substitution labile complex [Tc(N)X<sub>2</sub>(PNP)], which represents an interesting opportunity in designing receptor-specific Tc-99m agents [11–13]. The effective applicability of this method to the labeling of pharmacophore groups has been reported recently [13]. Using the avidin–biotin system as a model, a series of <sup>99m</sup>Tc-nitrido complexes containing functionalized biotin ligands were prepared and their biological profiles determined. In these compounds, the steric and the electronic influence of the Tc-carrying complex on the biotin–avidin receptor interaction was evaluated, evidencing that a careful selection of the spacer (at least a five-term spacer) may prevent the perturbation of the pharmacophore group operated by the inorganic moiety, thereby leaving the biomolecule free to interact with the receptor.

On the basis of these results, we decided to extend the applicability of [Tc(N)(PNP3)]<sup>2+</sup> technology to the preparation of target-specific radiolabeled peptides.

Among several biologically active forms of CCK, the nonsulfated octapeptide CCK8 was selected as the target vector. The peptide was combined through the terminal carboxylic group of the cysteine residue, to afford a COO-functionalised NH<sub>2</sub>, S-cysteine ligand. A glycine residue was introduced as the spacer group between the chelating agent and the biologically active peptide. The choice of cysteine position on the peptide skeleton sequence was based on previous results of molecular modeling studies (quantum mechanics and molecular mechanics) [32] which indicated that modifications on the N-terminal side of CCK8 obtained by introducing chelating moieties and their metal complexes should not affect the interaction with CCK receptors and should allow the CCK8 peptide to adopt the appropriate conformation for receptor binding. In addition, molecular mechanics calculations showed that the stabilization involved in the conjugate–CCK8/CCK1-R complex formation was about of the same order of magnitude as the CCK8/CCK1-R complex formation [32].

The NS-Cys-Gly-CCK8 peptide was synthesized according to the standard Fmoc chemistry procedure. The ligand was purified by HPLC and the identity established by mass spectroscopy.

Application of the ‘metal fragment’ approach is outlined as follows: the bifunctional ligand was reacted with the metal fragment to afford the monopositive

[Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> asymmetrical complex. The resulting <sup>99m</sup>Tc(N) mixed compound has been evaluated with regard to the following criteria: (i) ease of radiosynthesis and labeling efficiency; (ii) stability toward transchelation with Cys and GSH; and (iii) stability toward degradation with human serum, mouse serum and mouse liver homogenates. The capability of the native peptide sequence to preserve the biological properties was investigated both *in vitro* and *in vivo*. In particular, A431 cells overexpressing the CCK2-R were used to evaluate the *in vitro* affinity of the compound. Nude mice bearing control and CCK2-R-overexpressing A431 xenografts were used as the *in vivo* model.

**Preparation of (<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3))<sup>+</sup>**

The labeling procedures employed in preparation of the monocationic [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> are sketched in Scheme 1.

The preparation of the asymmetrical complex was conducted following two different methods. A two-step procedure (method 1) involved the preliminary production of a mixture of <sup>99m</sup>Tc-nitrido precursors, all containing the [Tc ≡ N]<sup>2+</sup> core, through the reduction of pertechnetate with tin(II) chloride in the presence of SDH as donor of the nitrido nitrogen atom. In the

second step, the diphosphine and the bidentate NS-Cys-Gly-CCK8 ligand were simultaneously added to the reaction vial to afford, after 30 min at 80 °C, the final mixed compound in high yield.

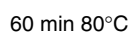
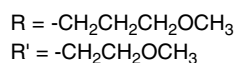
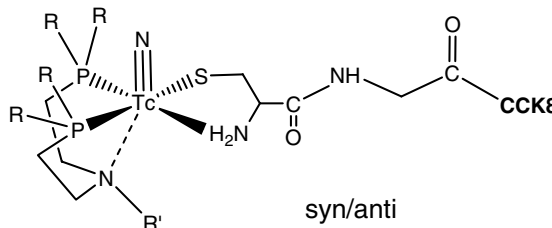
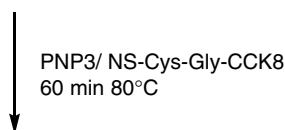
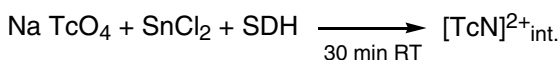
Alternatively, a one-step procedure (method 2) can be used to obtain the final asymmetrical compound. This procedure was carried out through the addition of a fresh pertechnetate solution to the vial containing the following reagents: SnCl<sub>2</sub>, SDH, PNP3 and NS-Cys-Gly-CCK8.

The [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> complex exhibited the same chromatographic profile as that obtained by method 1 (Figure 1).

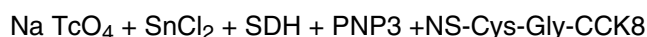
Interestingly, both procedures gave almost the same final RCYs for the asymmetrical product, and conversion to the corresponding symmetrical complexes comprising two identical bidentate ligands was never detected.

The high yield of the final mixed compound obtained in the one-pot procedure proves that the formation of the [Tc ≡ N]<sup>2+</sup> core was accompanied with the rapid coordination of PNP3 to form the [<sup>99m</sup>Tc(N)(PNP3)]<sup>2+</sup> metal fragment. These results demonstrated that the PNP ligand plays a fundamental role both in stabilizing the [<sup>99m</sup>Tc<sup>V</sup>(N)]<sup>2+</sup> core and in promoting the reactivity of the intermediate complex containing the [<sup>99m</sup>Tc(N)(PNP)]<sup>2+</sup> moiety, which immediately and

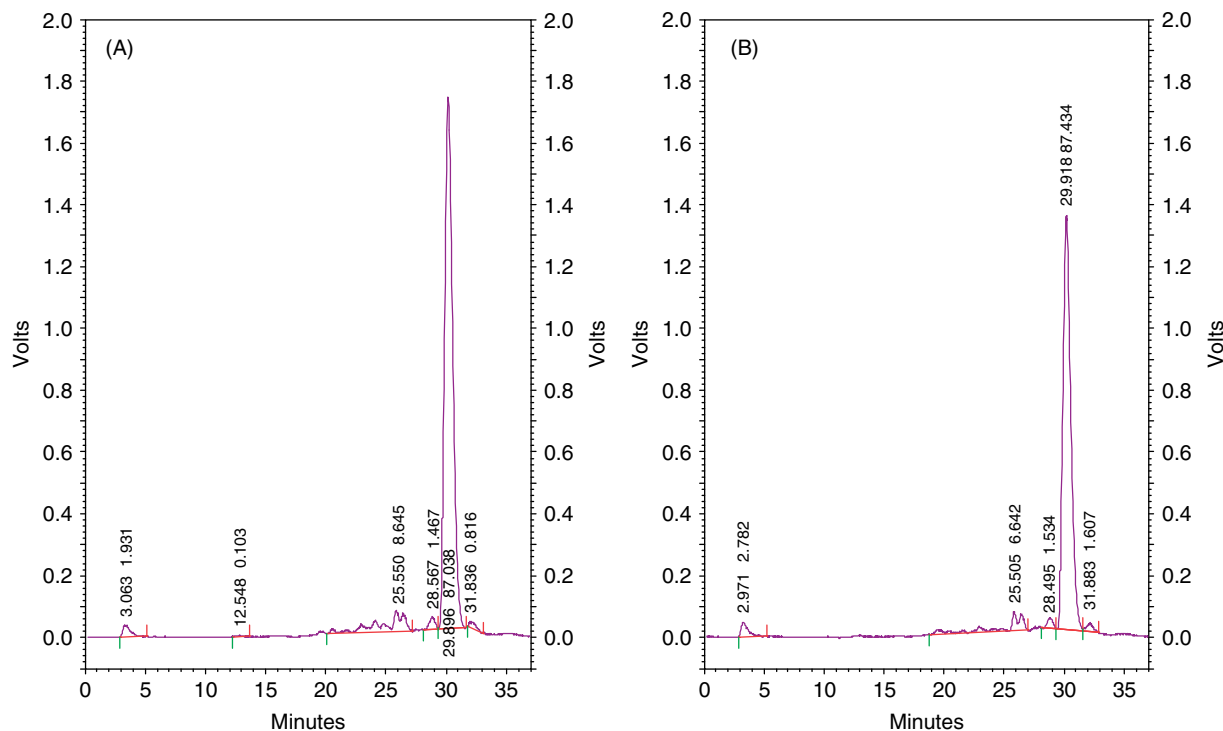
**Method 1**



**Method 2**



**Scheme 1** The labeling procedures employed in preparation of the monocationic [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> complex.



**Figure 1** HPLC comparison of the (*syn/anti*) [ $^{99m}\text{Tc}(\text{N})(\text{NS-Cys-Gly-CCK8})(\text{PNP3})\text{]}^+$  complex prepared (A) by method 1 (two-step reaction), 60 min 80 °C and (B) by method 2 (one-step reaction), 60 min 80 °C.

efficiently reacted with the bidentate ligand to afford the final compound.

TLC and HPLC data of the complexes and their RCY are summarized in Table 1.

TLC techniques revealed, analogously to what was observed for the [ $^{99m}\text{Tc}(\text{N})(\text{NS-Cys-OEt})(\text{PNP3})\text{]}^+$  complex [10], the existence of two different compounds in about the ratio 60 : 40, as the *syn/anti* isomeric forms of the asymmetrical [ $^{99m}\text{Tc}(\text{N})(\text{NS-Cys-Gly-CCK8})(\text{PNP3})\text{]}^+$  complex, depending on the orientation of the COO-substituted cysteine pendant group with respect to the central  $\text{Tc} \equiv \text{N}$ -terminal core. Unfortunately, it was not possible by HPLC to achieve isomeric resolution under a wide range of chromatographic conditions. Therefore, the mixture of the two isomeric forms was directly utilized for *in vitro* and *in vivo* studies.

### Labeling Efficiency of the NS-Cys-Gly-CCK8 Ligand

For a receptor-based peptide radiopharmaceutical, the use of a large amount of the bifunctional chelating agent (BFCA)-peptide may result in receptor site saturation, blocking the docking of the  $^{99m}\text{Tc}$ -labeled BFCA-conjugate, as well as in unwanted pharmacological side effects. Consequently, the concentration of the BFCA-conjugate in each preparation has to be very low, usually in the  $10^{-5}$ – $10^{-6}$  M range [6]. An estimation of the labeling efficiency of the *N*-cysteine-modified CCK analog was reported. In particular, we investigated the influence of concentration of

the NS-Cys-Gly-CCK8 ligand on the RCY of the [ $^{99m}\text{Tc}(\text{N})(\text{NS-Cys-Gly-CCK8})(\text{PNP3})\text{]}^+$  heterocomplex. In detail, following the one-step procedure (method 2), the bidentate ligand was labeled at different concentrations under standardized conditions – concentration of PNP3 ligand (1.0 mg), temperature (100, 80, 50 °C and RT) and reaction times (15, 30 and 60 min). The dependence of the RCY of the heterocomplex formation on the NS-Cys-Gly-CCK8 concentration (200–5  $\mu\text{g}$ ) is reported in Table 2.

Data revealed that the highest RCY was achieved with an amount of NS-Cys-Gly-CCK8 in the range of 200–50  $\mu\text{g}$ , corresponding to a molar amount in the range  $1.13 \times 10^{-4}$ – $2.81 \times 10^{-5}$  M. In this concentration interval, no significant variation of the %RCY was observed by increasing the temperature to 100 °C. Shorter reaction time (15 min) and lower temperature (50 °C and RT) reduced the RCY.

### *In vitro* Studies

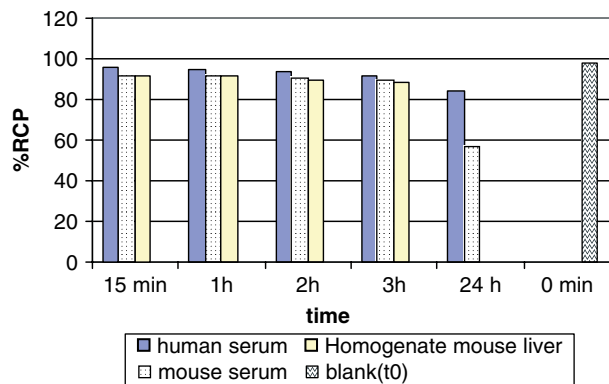
The labeled product was stable in the saline and phosphate buffer (0.2 M; pH 7.4) for 24 h, and it was found to be highly inert toward transchelation by an excess of free cysteine or glutathione, confirming the high thermodynamic stability and kinetic inertness of this compound.

The *in vitro* stability of the compound was evaluated after incubation with human serum, mouse serum and homogenate of mouse liver at 37 °C for 24 h. The

**Table 2** Effect of the amount of ligands and temperature on the %RCY formation of the complex [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup>. The experiments were performed in triplicate

NS-Cys-Gly-CCK8		Temperature (°C)	Time (min)	%RCY
(μg)	(mmol)			
200	1.6 × 10 <sup>-4</sup>	80	60	91.0 ± 3.4
100	8.2 × 10 <sup>-5</sup>	80	60	90.0 ± 2.01
50	4.1 × 10 <sup>-5</sup>	80	60	86.6 ± 1.09
25	2.1 × 10 <sup>-5</sup>	80	60	75.6 ± 2.00
10	8.2 × 10 <sup>-6</sup>	80	60	63.1 ± 2.89
5	4.1 × 10 <sup>-6</sup>	80	60	30.6 ± 1.99
200	1.6 × 10 <sup>-4</sup>	80	30	87.4 ± 1.91
100	8.2 × 10 <sup>-5</sup>	80	30	86.1 ± 2.01
50	4.1 × 10 <sup>-5</sup>	80	30	77.5 ± 1.09
25	2.1 × 10 <sup>-5</sup>	80	30	63.1 ± 1.00
10	8.2 × 10 <sup>-6</sup>	80	30	49.8 ± 2.89
5	4.1 × 10 <sup>-6</sup>	80	30	21.6 ± 3.99
200	1.6 × 10 <sup>-4</sup>	80	15	76.3 ± 6.40
100	8.2 × 10 <sup>-5</sup>	80	15	75.2 ± 5.00
50	4.1 × 10 <sup>-5</sup>	80	15	64.4 ± 3.09
200	1.6 × 10 <sup>-4</sup>	100	60	92.0 ± 4.90
100	8.2 × 10 <sup>-5</sup>	100	60	89.9 ± 3.21
50	4.1 × 10 <sup>-5</sup>	100	60	83.6 ± 2.09
200	1.6 × 10 <sup>-4</sup>	50	60	71.0 ± 1.4
100	8.2 × 10 <sup>-5</sup>	50	60	69.0 ± 1.01
50	4.1 × 10 <sup>-5</sup>	50	60	66.6 ± 2.09
200	1.6 × 10 <sup>-4</sup>	RT	60	62.1 ± 3.6
100	8.2 × 10 <sup>-5</sup>	RT	60	53.0 ± 1.98
50	4.1 × 10 <sup>-5</sup>	RT	60	39.1 ± 4.20

complex was found to be stable in all conditions within the first 3 h (Figure 2). At 24 h, 80% of the activity was associated with the peptide after incubation in human serum, and about 50% after incubation in mouse serum. This fact suggests that the complex was stable to the enzymatic actions.



**Figure 2** *In vitro* stability studies of the (syn/anti) [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> complex.

**Cell Binding**

Experiments were performed at 4 °C to block receptor internalization and thereby to measure only the ligand-receptor interaction.

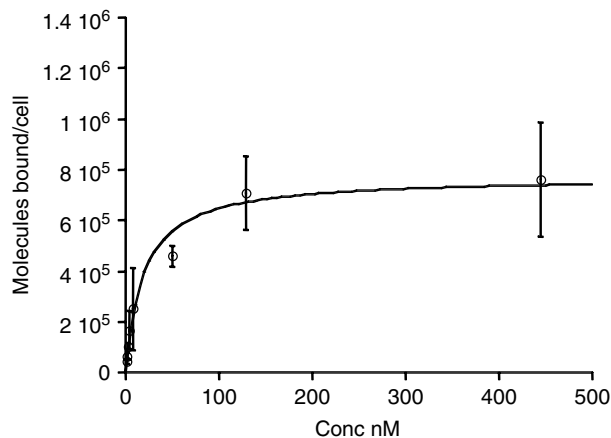
[<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> showed high specific binding to A431-CCK2-R cells, with the typical saturation curve (Figure 3). The apparent *K<sub>d</sub>* for the receptor was estimated to be 19.0 ± 4.6 nmol/l (mean ± SE), and the number of binding sites was in the order of 10<sup>6</sup> per cell.

The level of nonspecific binding, evaluated by incubation of the complex with nonreceptor expressing cells or with receptor expressing cells in presence of 100-fold excess of unlabeled peptide, was very low. These values were similar to those found in other CCK8 derivatives previously described [27].

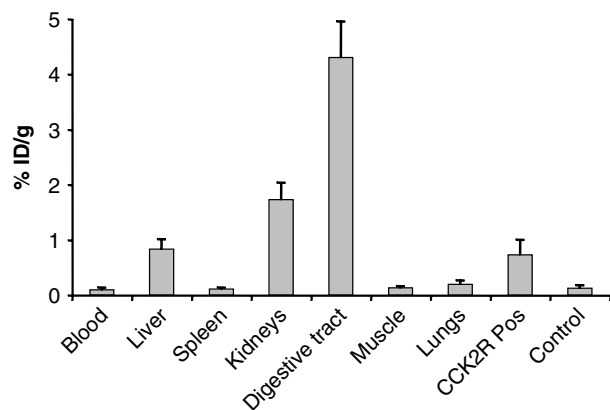
**Biodistribution Experiments**

The preliminary tissue distribution studies of the radiolabeled peptide evaluated in nude mice at 1 h p.i. are reported in Figure 4. The complex exhibits rapid and specific uptake in the CCK2-R positive A431 xenografts compared to the receptor-negative tumor, with a target-to-nontarget ratio (receptor positive/receptor-negative tumor) of 4 : 1. The activity was rapidly cleared from blood and normal tissues (lungs, spleen and muscle). Relatively high concentrations of the compound were also observed in liver and kidneys. Clearance of the unbound radioactivity appeared to be mainly through the hepato-biliary system, as most activity at 1 h was found in the intestinal tract.

A number of other CCK-R binding peptides, labeled with different approaches, have been evaluated [22–29]. Receptor-specific targeting has been easily achieved, and much of the development efforts has focused



**Figure 3** Receptor binding of the (syn/anti) [<sup>99m</sup>Tc(N)(NS-Cys-Gly-CCK8)(PNP3)]<sup>+</sup> on A431-CCK2-R cells at 4 °C. The cell line showed saturable binding of the peptide conjugate with equivalent *K<sub>d</sub>* = 19.0 ± 4.6 nmol/l. The *B<sub>max</sub>* value was 7.7 × 10<sup>5</sup> per cell.



**Figure 4** Biodistribution data at 1 h after intravenous injection. Organ-associated radioactivity was expressed as the %ID per gram of tissue normalized to a 20-g mouse.

on reducing nontarget tissue accumulation. Most compounds described appear to be preferentially cleared through the urinary system, although in some cases high retention level in the kidneys for prolonged periods can be observed. This drawback may sometimes require corrective measures such as co-injection of competing compounds to reduce kidney uptake [33].

The high intestinal activity of  $[^{99m}\text{Tc}(\text{N})(\text{NS-Cys-Gly-CCK8})(\text{PNP3})]^{2+}$  is indeed less desirable; however, the remarkably rapid clearance with the resulting low liver as well as kidney retention warrants further development of such labeling schemes in order to improve the overall distribution properties of compounds labeled with this approach. In order to promote the renal excretion pathway, more hydrophilic labeling systems should be used.

## CONCLUSIONS

Stable  $^{99m}\text{Tc}(\text{N})$  technetium complex incorporating a bioactive peptide was successfully obtained in high yield through the application of the labeling procedure based on  $[^{99m}\text{Tc}(\text{N})(\text{PNP})]^{2+}$  technology. The mixed compound was efficiently prepared using a simple one-step procedure, which met the basic requirements for the safe and rapid synthesis of a pure radiochemical agent.

Despite the linkage with the sterically demanding  $[^{99m}\text{Tc}(\text{N})(\text{PNP3})]^{2+}$  molecular fragment, the labeling procedure did not modify the receptor binding affinity of the peptide. Actually, the binding constant to CCK2 receptors was in the same order as other peptide radioisotope conjugates and was found to be adequate for further evaluation. *In vivo* studies showed the capability of the compound to target tumor cells.

These results are promising, but the pharmacokinetics properties of the compound need to be improved.

Therefore, in view of the application of this system in nuclear imaging, modification of the

hydrophilic/lipophilic properties of this compound will be necessary to reduce its intestinal accumulation and to increase its renal clearance as well as its CCK2-R positive A431 xenografts uptake. Modification of the complex can be achieved by increasing the hydrophilic properties of the  $[\text{Tc}(\text{N})(\text{PNP})]^{2+}$  moiety through the introduction of more hydrophilic or easily metabolizable groups on the two phosphorus atoms, or alternatively, by introducing an appropriate linker group between the peptide and the BFCS capable of modulating the hydrophilic or lipophilic properties of the final hetero-complex.

## REFERENCES

- Boerman OC, Oyen WJ, Corstens FH. Radio-labeled receptor-binding peptides: a new class of radiopharmaceuticals. *Semin. Nucl. Med.* 2000; **30**: 195–208.
- Reubi JC, Mäecke HR, Krenning EP. Candidate for peptide receptor radiotherapy today and in the future. *J. Nucl. Med.* 2005; **46**: 67S–75S.
- Weiner RE, Thakur ML. Radiolabeled peptides in diagnosis and therapy of oncological diseases. *Appl. Radiat. Isot.* 2002; **57**: 749–763.
- Fichna J, Janecka A. Synthesis of target specific radiolabeled peptides for diagnostic imaging. *Bioconjugate Chem.* 2003; **14**: 3–17.
- Liu S, Edwards DS, Barrett JA.  $^{99m}\text{Tc}$  labeling of highly potent small peptides. *Bioconjugate Chem.* 1997; **8**: 621–636.
- Liu S, Edwards DS.  $^{99m}\text{Tc}$ -labeled small peptides as diagnostic radiopharmaceutical. *Chem. Rev.* 1999; **99**: 2235–2268.
- Alberto R, Schibli R, Waibel R, Abram U, Schubiger AP. Basic aqueous chemistry of  $[\text{M}(\text{OH})_2(\text{CO})_3]^{+}$  ( $\text{M} = \text{Re}, \text{Tc}$ ) directed towards radiopharmaceutical application. *Coord. Chem. Rev.* 1999; **190–192**: 901–919.
- Bolzati C, Boschi A, Duatti A, Prakash S, Uccelli L, Refosco F, Tisato F, Bandoli G. Geometrically controlled selective formation of nitrido technetium(V) asymmetrical heterocomplexes with bidentate ligands. *J. Am. Chem. Soc.* 2000; **122**: 4510–4511.
- Bolzati C, Boschi A, Uccelli L, Tisato F, Refosco F, Cagnolini A, Duatti A, Prakash S, Bandoli G, Vittadini A. Chemistry of the strong electrophilic metal fragment  $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$  (PXP = Diphosphine ligand). A novel tool for the selective labeling of small molecules. *J. Am. Chem. Soc.* 2002; **124**: 11468–11479.
- Boschi A, Bolzati C, Benini E, Malagò E, Uccelli L, Duatti A, Piffanelli A, Refosco F, Tisato F. A novel approach to the high-specificity-activity labeling of small peptides with the technetium-99m fragment  $[^{99m}\text{Tc}(\text{N})(\text{PXP})]^{2+}$  (PXP = Diphosphine ligand). *Bioconjugate Chem.* 2001; **12**: 1035–1042.
- Bolzati C, Mahmood A, Malagò E, Uccelli L, Boschi A, Jones AJ, Refosco F, Duatti A, Tisato F. The  $[^{99m}\text{Tc}(\text{N})(\text{PNP})]^{2+}$  metal fragment: a technetium-Nitrido Synthron for use with biologically active molecules. The N-(2-Methoxyphenyl)piperazyl-cysteine analogues as examples. *Bioconjugate Chem.* 2003; **14**: 1231–1242.
- Boschi A, Uccelli L, Duatti A, Bolzati C, Refosco F, Tisato F, Romagnoli R, Baraldi PG, Varani K, Borea PA. Asymmetrical nitrido Tc-99m heterocomplexes as potential imaging agents for benzodiazepine receptors. *Bioconjugate Chem.* 2003; **14**: 1279–1288.
- Bolzati C, Caporale A, Agostini S, Carta D, Cavazza-Ceccato M, Refosco F, Tisato F, Schievano E, Bandoli G. Avidin-biotin system: a small library of cysteine biotinylated derivatives designed for the  $[^{99m}\text{Tc}(\text{N})(\text{PNP})]^{2+}$  metal fragment. *J. Nucl. Med. Biol.* 2007 (in press).



14. Bolzati C, Refosco F, Cagnolini A, Tisato F, Boschi A, Duatti A, Uccelli L, Dolmella A, Marotta E, Tubaro M. Synthesis, solution-state and solid-state structural characterization of monocationic nitrido heterocomplexes [M(N)(DTC)(PNP)]<sup>+</sup> (M = <sup>99m</sup>Tc, Re ~; DTC = Dithiocarbamate; PNP = Heterodiphosphine). *Eur. J. Inorg. Chem.* 2004; **9**: 1902–1913.
15. Bolzati C, Benini E, Cazzola E, Jung C, Tisato F, Refosco F, Pietzsch HJ, Spies H, Uccelli L, Duatti A. Synthesis, characterization and biological evaluation of neutral nitrido technetium(V) mixed ligand complexes containing dithiolates and aminodiphosphanes. A novel system for linking technetium to biomolecules. *Bioconjugate Chem.* 2004; **15**: 628–637.
16. Bolzati C, Cavazza-Ceccato M, Agostini S, Refosco F, Tisato F, Bandoli G. In Vitro and in vivo stability studies of the potential myocardial imaging agent [<sup>99m</sup>Tc(N)(DBODC)(PNP5)]<sup>+</sup>. In *Technetium, Rhenium and Other Metals in Chemistry and Nuclear Medicine 7*, Mazzi U (ed.). Padova: Italy, 2006; 395–398.
17. Béhé M, Behr TM. Cholecystokinin-B (CCK-B)/gastrin receptor targeting peptides for staging and therapy of medullary thyroid cancer and other CCK-B receptor expressing malignancies. *Biopolymers* 2002; **66**: 399–418.
18. Reubi JC, Waser B. Unexpected high incidence of cholecystokinin B/gastrin receptors in human medullary thyroid carcinomas. *Int. J. Cancer* 1996; **67**: 644–647.
19. Reubi JC, Schaer JC, Waser B. Cholecystokinin (CCK)-A and CCK-B/gastrin receptors in human tumors. *Cancer Res.* 1997; **57**: 1377–1386.
20. Imdahl A, Mantamadiotis T, Eggstein S, Farthmann EH, Baldwin GS. Expression of gastrin, gastrin/CCK-B and gastrin CCK-C receptors in human colorectal carcinomas. *J. Cancer Res. Clin. Oncol.* 1995; **121**: 661–666.
21. Behr TM, Jenner N, Radetzky S, Béhe M, Gratz S, Yücekent S, Raue F, Becker W. Targeting of cholecystokinin-B/gastrin receptors in vivo: preclinical and initial clinical evaluation of the diagnostic and therapeutic potential of radiolabeled gastrin. *Eur. J. Nucl. Med.* 1998; **25**: 424–430.
22. Reubi JC, Waser B, Schaer JC, Laederch U, Erion J, Srinivasan A, Schmidt MA, Bugaj JE. Unsulfated DTPA- and DOTA-CCK analogs as specific high-affinity ligands for CCK-B receptor-expressing human and rat tissues in vitro and in vivo. *Eur. J. Nucl. Med.* 1998; **25**: 481–490.
23. de Jong M, Bakker WH, Bernard BF, Valkema R, Kwekkeboom DJ, Reubi JC, Srinivasan A, Schmidt M, Krenning EP. Preclinical and initial clinical evaluation of <sup>111</sup>In-labeled nonsulfated CCK8 analog: a peptide for CCK-B receptor-targeted scintigraphy and radionuclide therapy. *J. Nucl. Med.* 1999; **40**: 2081–2087.
24. Kwekkeboom DJ, Bakker WH, Kooij PPM, Erion J, Srinivasan A, de Jong M, Reubi JC, Krenning EP. Cholecystokinin receptor imaging using an octapeptide DTPA-CCK analogue in patients with medullary thyroid carcinoma. *Eur. J. Nucl. Med.* 2000; **27**: 1312–1317.
25. Behr TM, Jenner N, Béhé M, Angerstein C, Gratz S, Raue F, Becker W. Radiolabeled peptides for targeting cholecystokinin-B/gastrin receptor-expressing tumors. *J. Nucl. Med.* 1999; **40**: 1029–1044.
26. Béhé M, Becker W, Gotthardt M, Angerstein C, Behr TM. Improved kinetic stability of DTPA-DGlu as compared with conventional monofunctional DTPA in chelating indium and yttrium: preclinical and initial clinical evaluation of radiometal labeled minigastrin derivatives. *Eur. J. Nucl. Med. Mol. Imaging* 2003; **30**: 1140–1146.
27. Aloj L, Caracò C, Panico M, Zanetti A, Del Vecchio S, Tesaro D, De Luca S, Arra C, Pedone C, Morelli G, Salvatore M. In vitro and in vivo evaluation of <sup>111</sup>In-DTPAGlu-G-CCK8 for cholecystokinin-B receptor imaging. *J. Nucl. Med.* 2004; **45**: 485–494.
28. Behr TM, Béhé M. Cholecystokinin-B/gastrin receptor-targeting peptides for staging and therapy of medullary thyroid cancer and other cholecystokinin-B receptor-expressing malignancies. *Semin. Nucl. Med.* 2002; **32**: 97–109.
29. Laverman P, Béhé M, Oyen WJG, Willems PHGM, Corstens FHM, Behr TM, Boerman OC. Two technetium-99m-labeled cholecystokinin-8 (CCK8) peptides for scintigraphic imaging of CCK receptors. *Bioconjugate Chem.* 2004; **15**: 561–568.
30. von Guggenberg E, Béhé M, Behr TM, Saurer M, Seppi T, Decristoforo C. <sup>99m</sup>Tc-labeling and in vitro and in vivo evaluation of HYNIC- and (N $\alpha$ -His)acetic acid-modified [D-Glu1]minigastrin. *Bioconjugate Chem.* 2004; **15**: 864–871.
31. Nock BA, Maina T, Béhé M, Nikolopoulou A, Gotthardt M, Schmitt JS, Thomas M, Behr TM, Maecke HR. CCK-2/gastrin receptor-targeted tumor imaging with <sup>99m</sup>Tc-labeled minigastrin analogs. *J. Nucl. Med.* 2005; **46**: 1727–1736.
32. Morelli G, De Luca S, Tesaro D, Saviano M, Pedone C, Dolmella A, Visentin R, Mazzi U. CCK8 peptide derivatized with diphenylphosphine for rhenium labelling: synthesis, molecular mechanics and quantum mechanics calculations. *J. Pept. Sci.* 2002; **8**: 373–381.
33. Behr M, Kluge G, Becker W, Gotthardt M, Behr TM. Use of polyglutamic acids to reduce uptake of radiometal-labeled minigastrin in the kidneys. *J. Nucl. Med.* 2005; **46**: 1012–1015.