U N I V E R S I T A' D E G L I S T U D I D I P A D O V A
SCUOLA DI DOTTORATO IN SCIENZE MOLECOLARI
INDIRIZZO SCIENZE FARMACEUTICHE
XXIV Ciclo

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Relazione sull'attività scientifica svolta presso Helmoltz-Zentrum Dresden-Rossendorf

Radiofarmaci "metal based" nella cardiologia nucleare e nell'*imaging* tumorale

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Radiofarmaci *"metal-based"* nella cardiologia nucleare e nell'*imaging* tumorale

Stato dell'Arte

Molti dei nuclidi impiegati nella preparazione di radiofarmaci sono metalli di transizione. Tra questi il ^{99m}Tc, radionuclide gamma emittente ($t_{1/2}$=6,02h; E=141 kev), prodotto attraverso l'uso di un generatore portatile di ^{99}Mo-^{99m}Tc, è considerato il nuclide di elezione nella diagnosi SPECT (*Single Photon Emission Tomography*). La recente scoperta di un nuovo ed efficiente metodo per la produzione di complessi del ^{99m}Tc contenenti il legame multiplo terminale tecnezio-azoto $[^{99m}Tc{\equiv}N]^{2+}$ ha aperto la possibilità di valutare l'attività biologica di diverse classi di azoturo complessi di ^{99m}Tc e di proporli come radiofarmaci di perfusione o recettori specifici[1].

Obbiettivi

Il presente progetto propone un'attività di ricerca che si estende a diversi settori della Medicina Nucleare, quali la Cardiologia, l'*Imaging* Molecolare, la sintesi di nuovi radiofarmaci *"metal-based"*.

Sintesi e valutazione biologica di una serie di nuovi ^{99m}Tc(N)-eterocomplessi e loro confronto con ^{99m}Tc(N)DBODC(PNP5)

Un primo obbiettivo consiste nel migliorare le caratteristiche biologiche di ^{99m}Tc(N)DBODC(PNP5), "lead compound" di una serie di azoturo complessi monocationici del tipo $[^{99m}Tc(N)DTC(PNP)]^{+}$ (DTC=ditiocarbammato alifatico a catena lineare; PNP=aminodifosfina), attualmente in fase di studio clinico come tracciante di perfusione cardiaca[2]. L'attività di ricerca sarà focalizzata al miglioramento di un parametro di elevata importanza clinica: l'estrazione di primo passaggio, mantenendo inalterate le favorevoli proprietà diagnostiche. Tale risultato potrà essere conseguito attraverso un attento studio di relazione struttura – attività che prevede una razionale modifica dei leganti ditiocarbammici, ottimizzando il volume molecolare e la lipofilia del complesso finale e, non da ultimo, il suo profilo farmacocinetico.

Saranno quindi sintetizzati leganti ditiocarbammici aliciclici, contenenti diversi eteroatomi opportunamente funzionalizzati: la sintesi sarà condotta in accordo a quanto riportato in letteratura, utilizzando ammine primarie o secondarie. I leganti ottenuti saranno quindi sottoposti a caratterizzazione chimico-fisica mediante analisi elementare e tecniche spettroscopiche.

I leganti ottenuti saranno utilizzati nella preparazione dei corrispondenti complessi a livello macroscopico con ^{99}Tc o $^{185/187}$Re ($10^{-3}/10^{-6}$ M) e a livello di tracciante ^{99m}Tc ($10^{-9}/10^{-11}$ M). I ^{99}Tc/$^{185/187}$Re complessi saranno quindi sottoposti a caratterizzazione chimico-fisica allo stato solido ed in soluzione. I complessi prodotti a livello di tracciante (^{99m}Tc) saranno invece caratterizzati per comparazione cromatografica TLC e HPLC con gli analoghi composti prodotti a livello millimolare.

Dalla cardiologia nucleare all'imaging tumorale

^{99m}Tc(N)DBODC(PNP5) si localizza irreversibilmente nelle strutture mitocondriali in forza di un gradiente elettrico transmembrana negativo. Inoltre, studi di metabolismo condotti al fine di chiarire il meccanismo di rapida rimozione di tale complesso dagli organi non bersaglio hanno dimostrato che ^{99m}Tc(N)DBODC(PNP5) è eliminato nella sua forma nativa e che l'escrezione è mediata dai trasportatori Pgp/MDR-Pgp (*multidrug resistant proteins*),aspetti questi che lasciano intravvedere la possibilità di estendere la applicabilità diagnostica di questo complesso e dei suoi derivati dal campo cardiologico a quello oncologico.

In questa parte del progetto si intende quindi valutare la possibilità di estendere l'applicabilità diagnostica di questo nuovo tracciante al monitoraggio e visualizzazione di alcune forme neoplastiche caratterizzate da elevata densità mitocondriale, nonché alla valutazione della refrattarietà delle cellule al suo accumulo.

Applicabilità della tecnologia $[Tc(N)(PNP)]^{2+}$ alla preparazione di radiofarmaci target-specifici

Un terzo obbiettivo consiste nel verificare la possibilità di applicare il frammento molecolare $[Tc(N)(PNP)]^{2+}$ alla marcatura di molecole biologicamente attive mantenendo inalterate le proprietà biologiche di queste ultime. $[Tc(N)(PNP)]^{2+}$ può essere coordinato in modo efficiente e versatile dall'amminoacido cisteina a formare complessi neutri o carichi, caratterizzati da una elevata attività specifica. Nel caso di peptidi, l'aggiunta di un residuo cisteinico terminale alla sequenza amminoacidica prescelta consente di ottenere un chelante bifunzionale in grado di reagire selettivamente con il frammento molecolare dando così origine al complesso finale[3].

Molecole di interesse nella selezione di sonde molecolari con cisteina possono essere: *i*) peptidi contenenti la sequenza RGD (Arg-Gly-Asp) selettiva verso l'integrina alfa-V beta-3 coinvolta nell'angiogenesi ed in numerose metastasi tumorali; *ii*) derivati dell'ormone alfa-MSH in grado di legare con elevata selettività recettori della melanocortina-1 (MC-1) sovra espressi sulla superficie di cellule melanomatose.

Alla preparazione dei corrispondenti complessi seguiranno lo studio di stabilità in vitro e di transchelazione con cisteina e glutatione, nonché la valutazione biologica in vitro ed in vivo.

Analogamente ed in parallelo sarà valutata anche l'applicabilità del frammento $[Tc(N)(R_2PS)]^{+}$ (R_2PS=fosfinotiolo variamente sostituito), nella preparazione di radiofarmaci target-specifici.

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3. 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 $[^{99m}Tc(N)(PNP)]^{2+}$ metal fragment, Nuclear Medicine and Biology 34 (2206) 511 – 522.

Introduction

^{99m}Tc-technetium (^{99m}Tc) and ^{188}Re-rhenium (^{188}Re) represent an attractive pair of radionuclides for biomedical use because of their favorable decay properties for diagnosis (^{99m}Tc: 6 h half-life, 140-keV γ-radiation) and therapy (^{188}Re: 17 h half-life, 2.12-MeV β^- max-radiation) and of their onsite availability, thanks to corresponding ^{99}Mo/^{99m}Tc- and ^{188}W/^{188}Re generator systems [1]. In addition, for ^{188}Re the emission of γ rays (E_γ = 152 keV) associated with the β^- decay mode can be conveniently utilized, using a conventional γ-camera, for imaging in monitoring the course of therapy and for calculations of radiation dose [2].

From the chemical point of view there is a wide analogy between ^{99m}Tc and ^{188}Re, making the chemistry of the two elements same or nearly identical in many cases. This circumstance offers the possibility to develop ^{99m}Tc-agent useful as the matched pair of the corresponding ^{188}Re-agents, making it feasible to obtain excellent diagnostic imaging in patients allowing for pre- and post- assessment of patients treated with therapeutic ^{188}Re analogues [3-5].

Differences are observed in the kinetics of ligand exchange reactions and in the red-ox behavior. As a consequence, the methods utilized for the preparation of ^{188}Re-radiopharmaceuticals cannot simply follow routes employed for obtaining ^{99m}Tc-complexes [3].

In analogy to ^{99m}Tc, the currently apply ^{188}Re-labeling methods use precursor in high oxidation state, $Na^{188}Re^{VII}O_4$. It is a fact that rhenium requires harsher reaction conditions to be reduced from its original oxidation state VII to lower oxidation states typically V/III with respect to technetium. Normally milligram amounts of tin chloride or other reducing agents are required for the complete reduction of $Na^{188}Re^{VII}O_4$, and the complex formation occurs in limited volumes, at high temperature and under strongly acidic conditions.

Moreover rhenium complexes are thermodynamically more stable in their higher oxidation states than the corresponding ^{99m}Tc-analogs; as a consequence, once formed ^{188}Re-complexes have a higher tendency to re-oxidize back to perrhenate than the corresponding ^{99m}Tc-agents [3].

All these facts constitute fundamental obstacles for the development of new ^{188}Re-radiopharmaceuticals especially target specific compounds which classically requires the labeling of sensitive biomolecules under mild reaction conditions.

However, in the last few years, there is an ongoing interest in the development of target specific ^{188}Re-radiopharmaceuticals, where ^{188}Re-containing chelate units are attached to biomolecules such as peptides or monoclonal antibodies [4-5].

The pharmacologically acceptable integration of the transition-metal rhenium, into a biomolecule is a fundamental challenge, and in this the chelate plays an important role. The usefulness of these chelates is dependent on a number of factors such as the stability of radionuclide binding within the chelate and the reactivity of the chelate with the considered biomolecule. The efficiency of radiolabeling of the chelating compound to produce the required radionuclide metal chelate is also important.

Various chelate systems coordinating the metal at different oxidation states are currently under investigation for stable labeling of biomolecules according to the bifunctional approach [6]. At oxidation state V, ligands of the "N_2S_2" or "N_3S" type are used. They rapidly bind the $[ReO]^{3+}$core to form stable five-coordinate complexes. Unfortunately, chelate ^{188}Re(O) -complexes frequently degrade much more rapidly than the technetium analogues, which are limiting the further development of rhenium-based therapeutic agents.

Technetium and rhenium mixed ligand compounds displaying characteristic of substitution-inert molecular-fragments have been proposed as important strategy in the development of target specific radiopharmaceuticals. Most representative examples of this technology are the $[M(CO)_3]^+$ system [7], the $[Tc(N)(PNP)]^{2+}$ system (PNP= diphosphinoamine) [8] and the so-called '4+1' mixed ligand system [9-11].

Among these, $[Tc(N)(PNP)]^{2+}$ based complexes, characterized by a remarkable *in vitro* and *in vivo* stability, have received attention in radiopharmaceutical design [12-25]. However, while the application of the $[Tc(N)(PNP)]^{2+}$ technology to the labeling of biomolecule and/or small peptide has only recently been investigated [16-22], the possibility to extend this technology to the preparation of ^{188}Re-labeled target vectors useful in therapy remains to be explored.

Based on these considerations, in this paper we evaluated the possibility to expand $[Tc(N)(PNP)]^{2+}$technology to the preparation of [^{188}Re(N)(PNP)]-labeled biomolecule.

Recently simple and efficient procedures for the preparation, in mild conditions, of ^{188}Re-complexes in intermediate/high oxidation states III/V from generator produced $Na^{188}Re^{VII}O_4$ have been proposed. These procedure differ from the commonly applied methods for the reduction of the $Na^{188}Re^{VII}O_4$ by the presence of additional reagents such as EDTA or oxalate anion [23-25]. The 'EDTA' method was based on the formation of $^{188}Re^{III}$-EDTA precursor, which easily undergo to an oxidative process. This behavior make possible to prepare different ^{188}Re-complexes in high oxidation

states through a combined ligand exchange/re-oxidation reaction of $^{188}Re^{III}$-EDTA with suitable ligands [23, 24, 26,27].

The 'oxalate' method, inspired by a basic principle of inorganic chemistry called 'expansion of the coordinating sphere', was based on the addition of oxalate ions to a radiopharmaceutical preparation which inducing a significant decrease in the standard reduction potential of the $[^{188}ReO_4]^-$ ion, changes the mechanism of the reaction [25]. This approach was also utilized to develop the first efficient procedure for producing the $[^{188}Re{\equiv}N]^{2+}$ core from $[^{188}ReO_4]^-$, under physiological conditions, and successfully used also in preparation of stable symmetrical bis-substitute ^{188}Re(N)-complexes [28]. In spite of this, the $[^{188}Re{\equiv}N]^{2+}$ core has yet to be applied for synthesizing ^{188}Re(N)-labeled receptor targeting radiopharmaceuticals.

Previous attempts to apply this synthetic pathway to the preparation of $[^{188}Re(N)(PNP)]$-complexes, in high yield, failed (*vide infra*).

Hence, using the 'EDTA' method, in this work is reported the production of the first series of $[^{188}Re(N)(PNP)]$-based compounds: the ligands and the complexes are reported in Figure 1. The bulky aminodiphosphine ligands N,N-bis[(dimethoxypropyl) phosphinoethyl]methoxyethylamine (PNP3) and N,N-bis[(dimethoxypropyl) phosphinoethyl]ethoxyethylamine (PNP5) were used as co-ligand, for their recognized ability in stabilization of the $[M{\equiv}N]^{2+}$ core (M = $^{99m/99}$Tc, Re) [29-30].

Cysteine derivative ligands were utilized as chelate for their well established capability to coordinate the $[^{99m}Tc(N)(PXP)]^{2+}$ moiety, either through the $[NH_2,S^-]$ pair of donor atoms or, alternatively, through the $[O^-, S^-]$ pair, yielding the corresponding asymmetrical complexes in very high specific activity [12,17,18], as well as for their easy conjugation to target vector through SPPS (solid phase peptide synthesis) at position that is not involved in receptor binding. The cysteamine and N-cysteinylmorpholin, which do not carry functional ester group, were used as references in order to explore the ability of the PPSN coordinating system in stabilizing the $[Re^V{\equiv}N]$ chelate toward reoxidation to perrhenate and ligand challenge reactions. N-cysteinylmorpholin ligand was also employed to mimic a bioconjugate cysteine molecule, because of the presence of a carbamidic bond.

Within is reported the synthesis, characterization and the biological evaluation of different $[^{188}Re(N)(cys{\sim})(PNP)]^{0/+}$complexes as prototype for development of target specific ^{188}Re(N)-based agents. The formation of the Re(N)-complex and the reactions of cysteine derivative ligands were studied in detail.

To establish the chemical identity of the ^{188}Re-agents the corresponding well characterized ^{99m}Tc and cold Re complexes were prepared.

The resulting ^{188}Re(N) mixed compounds have been evaluated with regard to the stability toward transchelation with GSH and degradation by serum enzymes.

The clearance of selected radiolabeled compounds from normal tissues and their *in vivo* stability were evaluated in rats by biodistribution and imaging studies.

The percentage of activity accumulated in the different tissues/organs was assessed as %ID by biodistribution studies and as %SUV by SPECT imaging and compared.

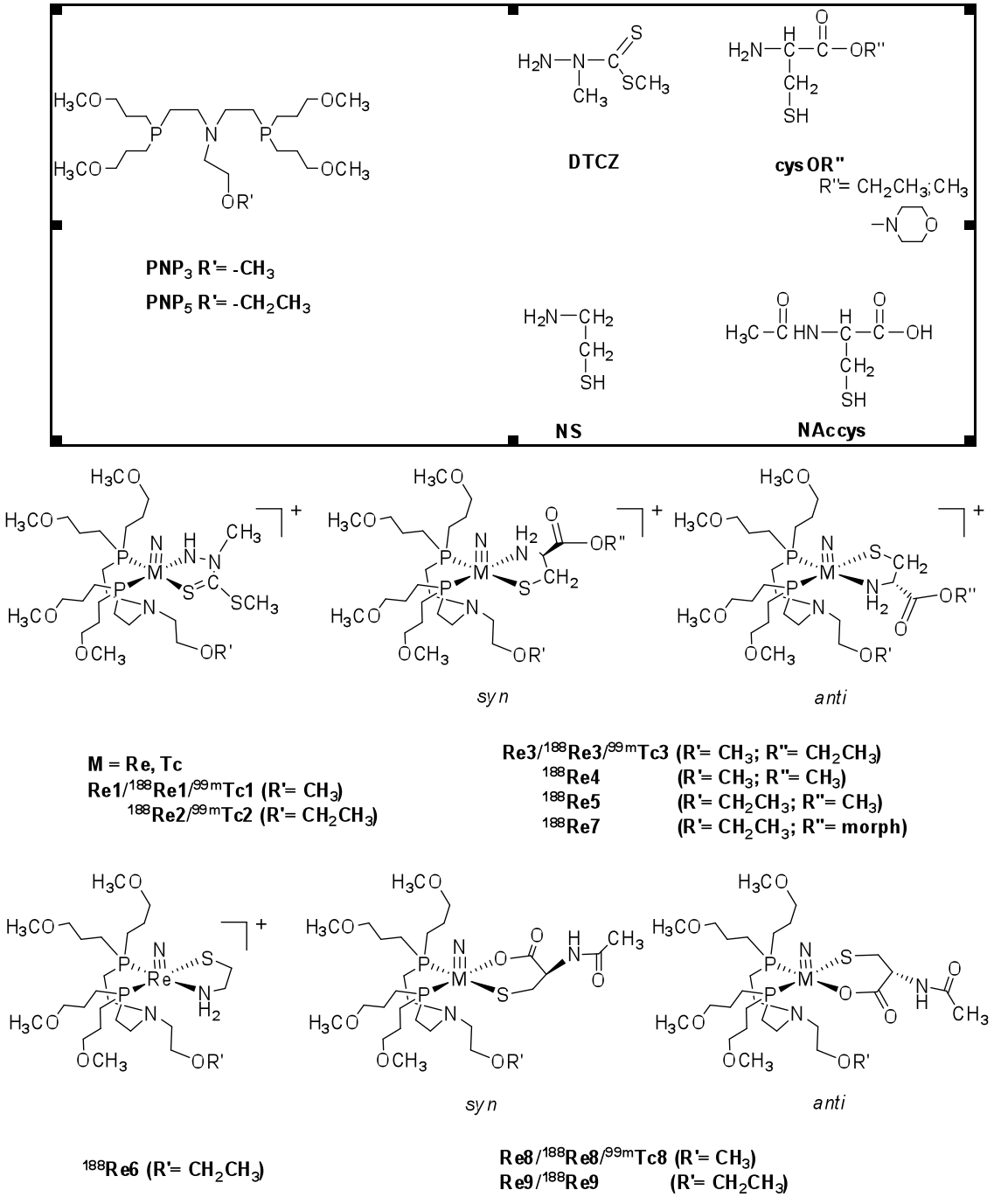


Figure 1: ^{99m}Tc and ^{188}Re complexes used in this study.

Materials and Methods

All chemicals and reagents were purchased from Aldrich Chemicals (Milano, Italy). All solvents were reagent grade and were used without further purification. PNP3 and PNP5 were prepared as previously described [31]. To avoid oxidation, all the solvents used in reactions with PNP3 and PNP5 were previously degassed. L-cysteine ethyl ester hydrochloride (HcysOEt·HCl), L-cysteine methyl ester hydrochloride (HcysOMe·HCl), N-acetyl-L-cysteine (NAccysH_2) and cysteamine hydrochloride (NH_2SH·HCl) were purchased from Aldrich Chemicals (Milano, Italy). N-cysteinyl-morpholine hydrochloride was synthesized as detailed below. S-methyldithiocarbazate (DTCZ) and [Re(N)$Cl_2(PPh_3)_2$] were prepared according to the literature methods [32-34].

^{99m}Tc as Na[$^{99m}TcO_4$] was eluted from a ^{99}Mo/^{99m}Tc generator (Elumatic III, IBA CIS bio, France) using 0.9% saline. ^{188}Re as Na[$^{188}ReO_4$] was eluted from a ^{188}W/^{188}Re generator (POLATOM, Otwock-Świerk, Poland) using 0.9% saline.

Elemental analyses (C, H, N, S) were performed on a Carlo Erba 1106 elemental analyzer. ^{1}H, ^{13}C, and ^{31}P NMR spectra were acquired at room temperature in $CDCl_3$ on a Brucker 300 instrument, using $SiMe_4$ as internal reference (^{1}H and ^{13}C) and 85% aqueous H_3PO_4 as external reference (^{31}P).

Mass spectrometric measurements (ESI-MS) were performed with ESI-TOF Mariner Biospectrometry Workstation (PerSeptive Biosystem, Stafford, TX, USA).

Chromatographic separations were accomplished on SiO_2 column (30 cm, ϕ 1.4 cm, 70-230 mesh, Aldrich)

Thin-layer chromatography (TLC) (SiO_2 F_{254S} and C_{18} F_{254S}, Merck) and high-performance liquid chromatography (HPLC) analysis were used to evaluate the radiochemical purity (RCP) and stability of the compounds. Radioactivity on TLC plates was detected and measured using a Cyclone instrument equipped with a phosphorus imaging screen and OptiQuant image analysis software (Packard, Meridian, CT, USA).

HPLC of the radioactive complexes was performed on a Perkin Elmer system equipped with a quaternary pump (Series 200 LC Pump), a UV/VIS-detector (759A, Applied Biosystems, λ=254 nm) and a home-made γ-ray detector (well-type NaI(Tl) crystal). Analysis were carried out on a reverse-phase C18 precolumn Knauer Eurospher (5 μm; 45×4.1 mm) and a reversed-phase Eurospher-100 C18 column Knauer (5 μm; 250×4.0 mm) or alternatively on a PRP-3 column Hamilton (10 μm; 150×4.1 mm) using the chromatographic condition reported in Table 1; flow rate: 1 mL/min.

Table 1. TLC (C18 F_{254S}, Merck), Mobile phase: [i]MeOH/MeCN/THF/NH_4OAc 0.5 M (2/2/1/2); [ii]MeCN/NEt_3 0.01 M pH 3 for H_3PO_4 6/4. HPLC: Eurospher-100 C18, mobile phases: A=NEt_3 0.01 M, pH=3 for H_3PO_4 1M, B=MeCN; gradient: 0 min, %B=0; 0-15 min, %B=45; 15-30 min, %B=45; 30-40 min, %B=0. 25 μL were injected. PRP3, mobile phases: A=NEt_3 0.01 M, pH=3 for H_3PO_4 1M, B=MeCN; gradient: 0 min, %B=0; 0-17 min, %B=28; 17-30 min, %B=28; 30-31 min, %B=0. 25 μL were injected.

Compounds	**number**	**TLC**	**HPLC (C18)**				**HPLC (PRP3)**		**^{188}Re-RCY %**	**^{188}Re-RCY %**
		R_f	R_t, min				R_t, min			
			UV	Re	^{188}Re	^{99m}Tc	UV	^{188}Re	Method A	Method B
DTCZ			15.60				18.30			
$[MO_4]^-$		[i]0.90; [ii]0.90			8.80			15.50		
[M-EDTA]		[i]0.90; [ii]0.95			6.60			3.20		-----
[M(N)] int		[i]0.65; [ii]0.15			17.30			18.5		
$[M(N)(DTCZ)(PNP3)]^+$	**1**	[i]0.35; [ii]0.10		22.40	22.40	22.40			99	98
$[M(N)(DTCZ)(PNP5)]^+$	**2**	[i]0.40; [ii]0.10			24.80	24.75		21.70	99	
$[M(N)(cysOEt)(PNP3)]^+$ *a*	**3a**	[i]0.50; [ii]0.25		20.05		20.05			78	83
$[M(N)(cysOEt)(PNP3)]^+$ *b*	**3b**	[i]0.40; [ii] 0.15		22.30		22.30				
$[M(N)(cysOMe)(PNP3)]^+$ *a*	**4a**	[i]0.50; [ii]0.15			18.75				79	85
$[M(N)(cysOMe)(PNP3)]^+$ *b*	**4b**	[i]0.40; [ii]0.10			20.60					
$[M(N)(cysOMe)(PNP5)]^+$ *a*	**5a**	[i]0.40; [ii]0.10			20.10			18.40	66	
$[M(N)(cysOMe)(PNP5)]^+$ *b*	**5b**	[i]0.50; [ii]0.15			22.40			19.90		
$[M(N)(NS)(PNP5)]^+$	**6**				18.70			18.50	83	88
$[M(N)(cys\text{-}morph)(PNP5)]^+$ *a*	**7a**	[i]0.40			20.80				56	
$[M(N)(cys\text{-}morph)(PNP5)]^+$ *b*	**7b**				21.50					
[M(N)(NAccys)(PNP3)] *a*+*b*	**8**	[i]0.65; [ii]0.40		18.75	18.70	18.75			78	94
[M(N)(NAccys)(PNP5)] *a*	**9a**	[i]0.65; [ii]0.45			20.30			18.60	88	89
[M(N)(NAccys)(PNP5)] *b*	**9b**				21.00					

Synthesis of $^{185/187}$Re-complexes

[Re(N)(DTCZ)(PNP3)]$^{+}$ (Re1). To 25.1 mg (0.032 mmol) of [Re(N)Cl_2(PPh_3)$_2$] suspended in CH_2Cl_2 (7 mL), were added 20 mg (0.041 mmol) of PNP3 ligand dissolved in EtOH (3 mL). The mixture was stirred, under di-nitrogen atmosphere, at reflux for 90 min during which the solution became clear and the color changed to yellow. After then 5.59 mg (0.041 mmol) of DTCZ dissolved in EtOH (2 mL) were added. The reaction mixture was stirred at reflux for 12 additional hours. No apparent color change from yellow was observed during the reaction. The solvent was removed, under gentle di-nitrogen stream, and the residue was triturated and washed with n-hexane (3 x 3 mL) and Et_2O (3 x 5 mL). The resulting yellow oily residue was dissolved in the minimum amount of $CHCl_3$ and chromatographed on SiO_2 column preconditioned with $CHCl_3$, and eluted with a mixture of $CHCl_3$/MeOH 95/5 followed by $CHCl_3$/MeOH 93/7. A yellow band was separated and collected. The eluate was evaporated, and a yellow oil residue was obtained to yield the pure [Re(N)(DTCZ)(PNP3)]$^{+}$ compound (yield 91.4 %). Complex (**Re1**) was soluble in chlorinate solvents and in alcohols, and insoluble in Et_2O and *n*-hexane.

Anal. Calcd for $C_{26}H_{58}ClN_4O_5P_2ReS_2$ = 854.50: C, 36.54; H, 6.84; N, 6.56; S, 7.50. Found: C, 37.02; H, 7.60; N, 6.72; S, 7.90.

^{1}H NMR ($CDCl_3$) δ: 9.03 (s, 1H, N*H*), 4.29 (s, 3H, N*CH*$_3$), 3.7 (m, 1H, SC*H*N), 3.48 (m, 8H, PCH$_2$CH$_2$*CH*$_2$OCH$_3$), 3.33-3.38 (4s, 12H, PCH$_2$CH$_2$CH$_2$O*CH*$_3$), 3.17 (s, 3H, NCH$_2$CH$_2$*OCH*$_3$), 2.85-3.15 (m, 2H, NCH$_2$*CH*$_2$OCH$_3$), 2.80 (s, 3H, S*CH*$_3$), 2.48-2.70 (m, 4H, N*CH*$_2$CH$_2$P), 2.10-2.40 (m, 8H, P*CH*$_2$CH$_2$CH$_2$OCH$_3$), 2.01-2.30 (m, 4H, NCH$_2$*CH*$_2$P), 2.01-2.15, 2.30-2.50 (m, 2H, N*CH*$_2$CH$_2$OCH$_3$), 1.75 (m, 8H, PCH$_2$*CH*$_2$CH$_2$OCH$_3$).

^{13}C NMR ($CDCl_3$) δ: 76.2 (s, N*C*S), 72.29-72.70 (4d, PCH$_2$CH$_2$*CH*$_2$OCH$_3$), 68.04 (s, NCH$_2$*CH*$_2$OCH$_3$), 58.72 (s, NCH$_2$CH$_2$*OCH*$_3$), 58.52-58.73 (4s, PCH$_2$CH$_2$CH$_2$O*CH*$_3$), 51.99 (s, N*CH*$_2$CH$_2$OCH$_3$), 47.92 (s, N*CH*$_2$CH$_2$P), 46.62 (s, S*CH*$_3$), 45.77 (s, N*CH*$_3$), 23.87-27.2 (3d, 2s, P*CH*$_2$CH$_2$CH$_2$OCH$_3$, PCH$_2$*CH*$_2$CH$_2$OCH$_3$, NCH$_2$*CH*$_2$P).

^{31}P NMR ($CDCl_3$) δ: 15.65 (d, $^{2}J_{PP}$=12.1 Hz); 7.69 (d, $^{2}J_{PP}$=12.1 Hz).

ESI-MS(+) (*m/z*): 819.23 [M]$^{+}$ (100%).

syn,anti-[Re(N)(cysOEt)(PNP3)]$^{+}$ (Re3). To 26.3 mg (0.033 mmol) of [Re(N)Cl_2(PPh_3)$_2$] suspended in CH_2Cl_2 (7 mL), were added 20 mg (0.041 mmol) of PNP3 ligand dissolved in EtOH (3 mL). The mixture was stirred, under di-nitrogen atmosphere, at reflux for 2h during which the solution became clear and the color

changed to yellow. After then 0.02 mL of Et_3N followed by 9.5 mg (0.051 mmol) of HcysOEt·HCl dissolved in EtOH (2 mL) were added. The reaction mixture was stirred at reflux for 12 additional hours. No apparent color change from yellow was observed during the reaction. The solvent was removed, under gentle di-nitrogen stream, and the residue was washed with *n*-hexane (3 x 3 mL), Et_2O (3 x 5 mL) and EtOH (3 mL). The resulting yellow oily residue was dissolved in $CHCl_3$ (0.5 mL) and chromatographed on SiO_2 column preconditioned with $CHCl_3$, and eluted with a mixture of $CHCl_3$/MeOH 93/7 followed by $CHCl_3$/MeOH 90/10. A yellow band was separated and collected. The eluate was evaporated, and a separated yellow oil residue was obtained to yield the pure *anti/syn* $[Re(N)(\mathbf{cysOEt})(PNP3)]^+$ (yield 91 %). Complexes (*anti/syn*-**Re3**) were soluble in chlorinate solvents and in alcohols, and insoluble in Et_2O and *n*-hexane.

Anal. Calcd for $C_{28}H_{61}ClN_3O_7P_2ReS$ = 867.47: C, 38.77; H, 7.09; N, 4.84; S, 3.70. Found: C, 39.10; H, 7.69; N, 5.00; S, 3.90.

***anti/syn*-Re3.** ^{1}H NMR ($CDCl_3$) δ: 8.17 and 5.36 (2d, 2H, $^2J_{HH}$=12Hz, NH_{2anti}), 6.00 and 6.58 (2d, 2H, $^2J_{HH}$=12 Hz NH_{2syn}), 4.34 (m, 1H, C(O)*CH*NH_{anti}), 3.8 (m, 1H C(O)*CH*NH_{syn}), 4.28 (m, 2H, C(O)O*CH*$_2$CH$_3$), 3.49 (m, 8H, PCH$_2$CH$_2$*CH*$_2$OCH$_3$), 3.40-3.50, (m, 2H, NCH$_2$*CH*$_2$OCH$_3$), 3.35-3.36 (2s, 12H, PCH$_2$CH$_2$CH$_2$O*CH*$_3$), 3.22 (s, 3H, NCH$_2$CH$_2$*OCH*$_3$), 3.18, 2.78-2.80 (2m, 2H, S*CH*$_2$CH), 2.61, 2.85, (m, 2H, N*CH*$_2$CH$_2$OCH$_3$), 2.56-2.92(m, 4H, N*CH*$_2$CH$_2$P), 2.37-1.95 (m, 4H, NCH$_2$*CH*$_2$P), 1.98-2.20 (m, 8H, P*CH*$_2$CH$_2$CH$_2$OCH$_3$), 2.20-1.55 (m, 8H, PCH$_2$*CH*$_2$CH$_2$OCH$_3$), 1.30 (t, 3H, C(O)OCH$_2$*CH*$_3$).

^{13}C NMR ($CDCl_3$) δ: 170.55 (s, *C(O)*OCH$_2$CH$_3$), 77.2 (s, C(O)*CH*NH), 72.16-72.58 (3d, PCH$_2$CH$_2$*CH*$_2$OCH$_3$), 68.13 (s, NCH$_2$*CH*$_2$OCH$_3$), 62.2 (s, C(O)O*CH*$_2$CH$_3$), 58.55-58.90 (4 s, PCH$_2$CH$_2$CH$_2$O*CH*$_3$, NCH$_2$CH$_2$*OCH*$_3$), 51.82 (s, N*CH*$_2$CH$_2$OCH$_3$), 48.25 (s, N*CH*$_2$CH$_2$P), 47.31 (s, S*CH*$_2$CH), 27.25-23.77 (5s, 3d, P*CH*$_2$CH$_2$CH$_2$OCH$_3$, PCH$_2$*CH*$_2$CH$_2$OCH$_3$, NCH$_2$*CH*$_2$P), 13.79 (s, C(O)OCH$_2$*CH*$_3$).

^{31}P NMR ($CDCl_3$) δ: 13.31 (d, $^2J_{PP}$= 9.72 Hz, *syn*), 9.93 (d, $^2J_{PP}$= 9.72 Hz, *syn*); δ: 11.90 (d, $^2J_{PP}$= 9.72 Hz, *anti*), 11.61 (d, $^2J_{PP}$= 9.72 Hz, *anti*).

ESI-MS(+) (*m/z*): 832.25 $[M]^+$ (100%)

syn, anti-[Re(N)(cysOMe)(PNP3)]$^+$ (Re4). Complexes were prepared using the procedure specified above for **Re3**. MeOH was utilized as reaction solvent instead of EtOH in order to preserve the OMe group in the cysteine derivative ligand. The solvent was removed and the residue was treated washed with *n*-hexane (3 x 3 mL) and Et_2O (4 x 5 mL). The resulting yellow-orange oily residue was dissolved in $CHCl_3$ (0.5 mL) and chromatographed on SiO_2 column preconditioned with $CHCl_3$, and eluted with a

mixture of $CHCl_3$/MeOH 93/7 followed by $CHCl_3$/MeOH 90/10. A yellow band was separated and collected. The eluate was evaporated, and a yellow oil residues was obtained to yield the pure anti /syn [Re(N)(**cysOMe**)(PNP3)]$^+$ compound (yield 87 %). anti/syn- **Re4** were soluble in chlorinate solvents and in alcohols, and insoluble in Et_2O and *n*-hexane.

Anal. Calcd for $C_{27}H_{59}N_3O_7P_2ReS$ = 853.45: C, 38.00; H, 6.97; N, 4.92; S, 3.76. Found: 38.45; H, 7.28; N, 4.85; S, 3.96.-

anti-synRe4: ^{1}H NMR ($CDCl_3$) δ: 8.33, 5.31 (2m, 2H, NH_{2anti}), 6.63, 6.00 (2m, 2H, NH_{2syn}), 4.32 (m, 1H, C(O)*CH*NH), 3.82 (s, 3H, C(O)OCH$_{3anti}$), 3.71 (m, 1H, C(O)*CH*NH$_{syn}$) 3.46 (m, 8H, PCH$_2$CH$_2$*CH$_2$*OCH$_3$), 3.44-3.42 (m, 2H, NCH$_2$*CH$_2$*OCH$_3$), 3.40-3.33 (4s, 12H, PCH$_2$CH$_2$CH$_2$O*CH$_3$*), 3.21 (s, 3H, NCH$_2$CH$_2$*OCH$_3$*), 3.16, 2.76 (2m, 2H, S*CH$_2$*CH), 2.85 (m, 4H, N*CH$_2$*CH$_2$P), 2.74, 2.85 (m, 2H, N*CH$_2$*CH$_2$OCH$_3$), 2.13 (m, 4H, NCH$_2$*CH$_2$*P), 2.00 (m, 8H, P*CH$_2$*CH$_2$CH$_2$OCH$_3$), 2.00-1.70 (m, 8H, PCH$_2$*CH$_2$*CH$_2$OCH$_3$).

^{31}P NMR ($CDCl_3$) δ: 12.83 (d, $^2J_{PP}$=11.66 Hz, *anti*), 11.43 (d, $^2J_{PP}$=11.66 Hz, *anti*); δ: 13.23 (d, $^2J_{PP}$=9.36 Hz, *syn*), 9.81 (d, $^2J_{PP}$=9.36 Hz, *syn*).

ESI-MS(+) (*m/z*): 818.31 [M]$^+$ (100%)

syn,anti-[Re(N)(cysNAc)(PNP3)]$^+$ (Re8). The complex was prepared using the procedure indicated for Re2. The crude product was purified by column chromatography. Thus, the resulting orange oily residue was dissolved in $CHCl_3$ (0.5 mL) and loaded on SiO_2 column preconditioned with $CHCl_3$, and eluted with a mixture of EtOH/$CHCl_3$/Tol 1.5/2/1. A yellow band was collected. The eluate was evaporated, and the separated yellow oil residues was obtained to yield the pure *syn/anti* [Re(N)(**NAccys**)(PNP3)]$^+$ compounds (yield 61.5 %). Complex (**Re8**) was soluble in chlorinate solvents and in alcohols, and insoluble in Et_2O and *n*-hexane.

Anal. Calcd for $C_{28}H_{58}N_3O_8P_2ReS$= 845.29: C, 39.80; H, 6.92; N, 4.97; S, 3.79. Found: C 39.98, H 7.01, N 4.69, S 3.98.

syn-anti Re8. ^{1}H NMR ($CDCl_3$) δ: 7.90 (d, 1H $^3J_{HH}$=5.79 Hz, C(O)CH*NH$_{syn}$),* 6.88 (d, 1H, $^3J_{HH}$=5.79 Hz, C(O)CH*NH $_{anti}$*), 5.01 (m, 1H, C(O)*CH*NH$_{anti}$), 4.94 (m, 1H, C(O)*CH*NH$_{syn}$), 3.65-3.32 (m, 8H, PCH$_2$CH$_2$*CH$_2$*OCH$_3$), 3.57, 2.35 (2m, 2H, S*CH$_2$*CH), 3.40 (m, 2H, NCH$_2$*CH$_2$*OCH$_3$), 3.37-3.33 (4s, 12H, PCH$_2$CH$_2$CH$_2$O*CH$_3$*), 3.20 (s, 3H, NCH$_2$CH$_2$*OCH$_3$*), 2.98-2.60 (m, 4H, N*CH$_2$*CH$_2$P), 2.88, 2.47 (m, 2H, N*CH$_2$*CH$_2$OCH$_3$), 2.35-2.12 (m, 8H, P*CH$_2$*CH$_2$CH$_2$OCH$_3$), 2.25-1.85 (m, 4H, NCH$_2$*CH$_2$*P), 2.12-1.54 (m, 8H, PCH$_2$*CH$_2$*CH$_2$OCH$_3$), 2.01 (s, 3H, NHC(O)*CH$_3$*).

^{13}C NMR ($CDCl_3$) δ: 177.38 (s, O*C(O)*CH), 168.77 (s, NH*C(O)*CH$_3$), 77.2 (s,

C(O)*CH*NH) 73.06-72.29 (4d, $PCH_2CH_2\mathit{CH_2}OCH_3$), 68.16 (s, $NCH_2\mathit{CH_2}OCH_3$), 58.67-58.56 (3 s, $PCH_2CH_2CH_2O\mathit{CH_3}$), 55.92 (s, $NCH_2CH_2\mathit{OCH_3}$) 51.60 (s, $N\mathit{CH_2}CH_2OCH_3$), 48.08 (s, $N\mathit{CH_2}CH_2P$), 47.32 (s, $S\mathit{CH_2}CH$), 25.77-23.34 (4s, 3d, $P\mathit{CH_2}CH_2CH_2OCH_3$, $PCH_2\mathit{CH_2}CH_2OCH_3$, $NCH_2\mathit{CH_2}P$), 17.25, 16.84 (2s, $\mathit{CH_3}C(O)NH$);

^{31}P NMR ($CDCl_3$) δ: 18.61 (d, $^2J_{PP}$=9.48 Hz, *anti*), 17.74 (d, $^2J_{PP}$ =9.36 Hz, *anti*), 11.90 (d, $^2J_{PP}$=9.36 Hz, *syn*), 11.55 (d, $^2J_{PP}$=9.36 Hz, *syn*).

ESI-MS(+) (*m/z*): 845.29 $[M]^+$ (100%)

Synthesis of ^{188}Re-complexes

Method A

$[^{188}Re(N)(DTCZ)(PNP3/5)]^+$ (^{188}Re1; ^{188}Re2). To a purged di-nitrogen vial containing 5 mg EDTA (ethylenediaminotetraacetic acid), 5 mg mannitol and 1 mg $SnCl_2$ were added 0.6 mL HCl (0.1 M) and 1 mL fresh solution of $Na[^{188}ReO_4]$ (ca. 150 MBq). The vial was kept at room temperature for 10 minutes. Then 2 mg of DTCZ (dithiocarbazate) (dissolved in 0.2 mL EtOH) were added and the reaction mixture was further kept at room temperature for 45 min (*reaction a*). After this, 1 mg PNP3/5 (dissolved in 0.1 mL EtOH) was added. The reaction vial was incubated at 100°C for 30 minutes (*reaction b*).

Alternatively to a purged di-nitrogen vial containing 5 mg EDTA, 5 mg mannitol and 1 mg $SnCl_2$ were added 0.6 mL HCl (0.1 M) and 1 mL fresh solution of $Na[^{188}ReO_4]$ (ca. 150 MBq). The vial was kept at room temperature for 10 minutes. Then 2 mg of DTCZ (dissolved in 0.2 mL EtOH) and 1 mg PNP3/5 (dissolved in 0.1 mL EtOH) were added. The reaction vial was incubated at 100°C for 30 minutes (*reaction b′*)

RCYs of the complexes evaluated by HPLC are reported in Table 1.

HPLC profile of complexes **^{188}Re1** is reported in the Figure 2.

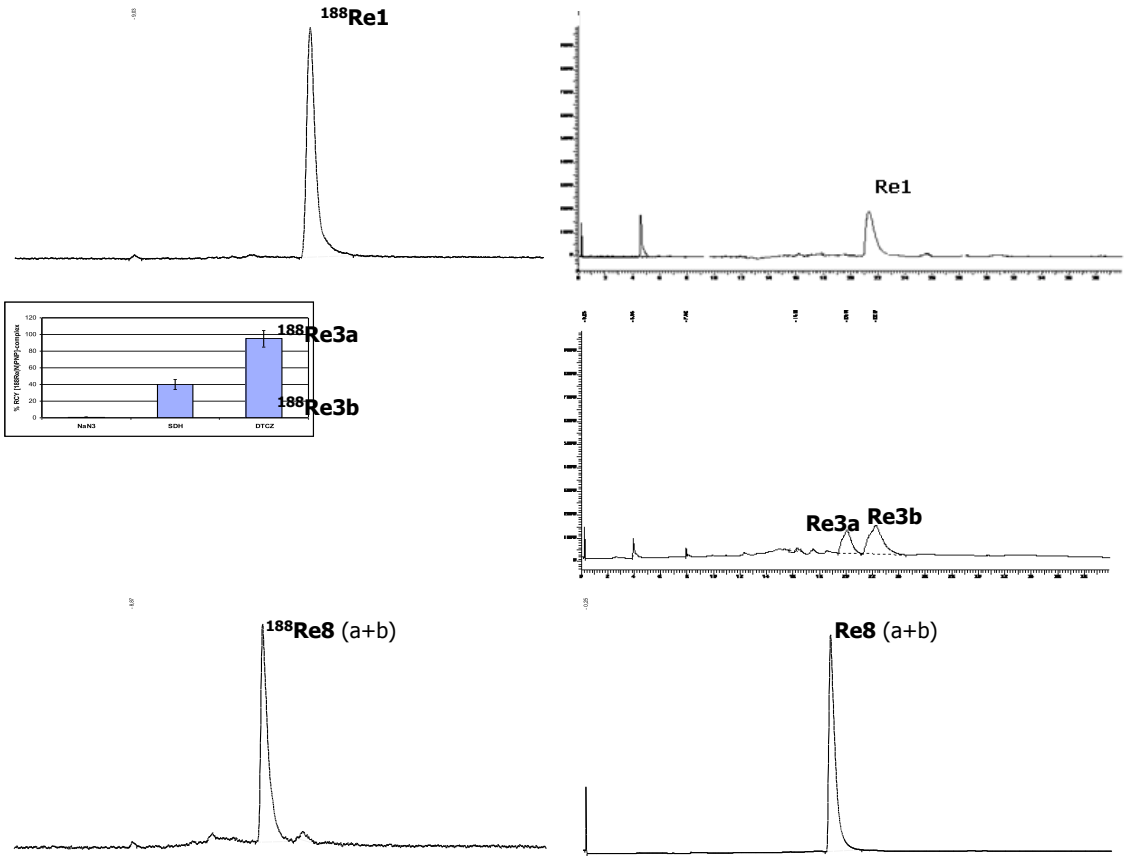


Figure 2: RP-HPLC profiles of the complexes ^{188}Re1, ^{188}Re3 and ^{188}Re 8 prepared according to method A, and comparison with the analogues compounds prepared at millimolar level with cold Re.

syn,anti-[^{188}Re(N)(cys~)(PNP3,5)]$^{+}$ (^{188}Re 3-9). To a purged di-nitrogen vial containing 5 mg EDTA, 5 mg mannitol and 1 mg $SnCl_2$ were added 0.6 mL HCl (0.1 M) and 1 mL fresh solution of Na[$^{188}ReO_4$] (approx. 150 MBq). The vial was kept at room temperature for 10 minutes. After that 2 mg of DTCZ (dissolved in 0.2 mL EtOH) were added and the vial was further kept at room temperature for 45 min. The reaction mixture was buffered with 0.75 mL phosphate buffer (0.125 M; pH = 7.7) and 5 mg of cysteine derivative ligand (dissolved in 0.2 mL of saline) followed by 1 mg of PNP3/5 (dissolved in 0.1 mL EtOH) were rapidly added. The vial was heated at 100°C for 45 minutes. The pH of the reaction mixture, measured at the end of the reaction was 3.6. RCYs of the complexes evaluated by HPLC are reported in Table1. HPLC profiles of some representative complexes (**^{188}Re3** and **^{188}Re8**) are reported in the figure 2 (*reactions d, e, f*).

Method B

syn,anti-[^{188}Re(N)(cys~)(PNP3,5)]$^{+}$ (^{188}Re 3-9) To a purged di-nitrogen vial containing 5 mg EDTA, 1 mg $SnCl_2$ (dissolved in 0.1 mL of HCl 0.1 M), 0.1 mg of DTCZ (dissolved in 0.01 mL EtOH) and 0.6 mL HCl (0.1 M) 0.5 mL of fresh solution Na[^{188}ReO$_4$] (ca. 150 MBq) were added. The vial was kept at room temperature for 10 minutes. The reaction mixture was buffered with 0.75 mL phosphate buffer (0.125 M; pH = 7.7) and 5 mg of the appropriate cysteine derivatives ligand (dissolved in 0.2 mL of saline) followed by 1 mg of PNP3/5 (dissolved in 0.1 mL EtOH) were rapidly added. The vial was heated at 100°C for 30 minutes (*reactions g, h, i*). The pH of the reaction mixture, measured at the end of the reaction was in the range 3.2-3.6. For each compound RCY evaluated by HPLC was reported in table 1.

Synthesis of ^{99m}Tc-complexes

[^{99m}Tc(N)(DTCZ)(PNP3)]$^{+}$ (^{99m}Tc1). To a vial containing 2.0 mg of DTCZ, 5.0 mg of EDTA, 0.1 mg of $SnCl_2$ suspended in 0.1 mL of saline and 1 mL of EtOH was added Na[^{99m}TcO$_4$] (0.25 mL, 50.0 MBq – 2.2 GBq) followed by 1.0 mg of PNP3 (dissolved in 0.1 mL of EtOH). The reaction vial was heated at 80 °C for 30 minutes giving the final complex in high yield (96%).

syn,anti-[^{99m}Tc(N)(cysOR)(PNP3)]$^{+}$ (R= Et, ^{99m}Tc3; R= Me, ^{99m}Tc4) and syn,anti-[^{99m}Tc (N)(NAc-cys)(PNP3)]$^{+}$ (^{99m}Tc8). The complexes were prepared in high yields applying the procedure previously reported [12], which utilized SDH (succinic dihydrazide) as source of N^{3-} group, and the following method which utilized DTCZ as N^{3-} donor.

Na[^{99m}TcO$_4$] (0.25 mL, 50.0 MBq – 2.2 GBq) was added to a vial containing 2.0 mg of DTCZ, 5.0 mg of EDTA, 0.1 mg of $SnCl_2$ suspended in 0.1 mL of saline and 1 mL of EtOH. The vial was kept at room temperature for 30 minutes giving a mixture of ^{99m}Tc-nitrido precursors, $[Tc{\equiv}N]_{int}^{2+}$. Then, 5 mg of the appropriate cysteine derivatives ligand (dissolved in 0.2 mL of phosphate buffer 0.1 M, pH=7.4) were added and the reaction mixture was heated at 100 °C for 30 min. Finally, 1.0 mg of PNP3 (dissolved in 0.1 mL of EtOH) and was added and the solution was further heat at 100 °C for 30 min. The radiochemical yield (RCY), of each compound, determined by TLC and HPLC chromatography, was reported in Table 1.

Purification through solid phase extraction.

A Sep-Pak® C18 plus cartridge was equilibrated with 5 mL of EtOH and 5 mL of water, the reaction mixture was diluted with 8 mL of water and then loaded on it. The cartridge was rinsed with 20 mL of water and 3 mL of EtOH (25%). The complex was eluted with 2 mL of a mixture of EtOH/saline (9/1). 63% of the loaded activity was collected in this fraction.

After purification RCP of the complexes evaluated by HPLC was always >92%.

Stability Studies in Solution.

After purification, the ^{188}Re complexes were dissolved in EtOH/saline (9/1) and diluted with an equivalent volume of phosphate buffer (0.1 M, pH 7.4) 0.9% NaCl, or H_2O containing 25% propylene glycol and incubated at 37 °C for 24 h.

At different times, aliquots were withdrawn and analyzed by HPLC.

Challenge Experiments with Glutathione (GSH).

After purification 500 μL of the ^{188}Re complexes dissolved in EtOH/saline (9/1) were diluted with an equivalent volume of an aqueous solution of 20 mM GSH containing 25% of propylene glycol, resulting in a final glutathione concentration of 10 mM. The solution was incubated at 37 °C for 24 h. At different times, aliquots were withdraw and analyzed by HPLC as described above.

Stability Studies in Plasma.

100 μL of the selected ^{188}Re complexes solution (0.9% NaCl, 12% propylene glycol) was incubated in 400 μL of rat plasma at 37 °C for 24h. At different times, aliquots were withdrawn and analyzed by HPLC as described above. Analyses were carried out with a PRP-3 column.

Biodistribution Studies.

All animal experiments in rodent models were carried out according to the guidelines of the German Regulations for Animal Welfare. The protocol was approved by the local Ethical Committee for Animal Experiment. The animals were housed under standard conditions in controlled-airflow cabinets with free access to standard food and tap water. For biodistribution experiments a total of 500 µL of ^{188}Re complexes solution

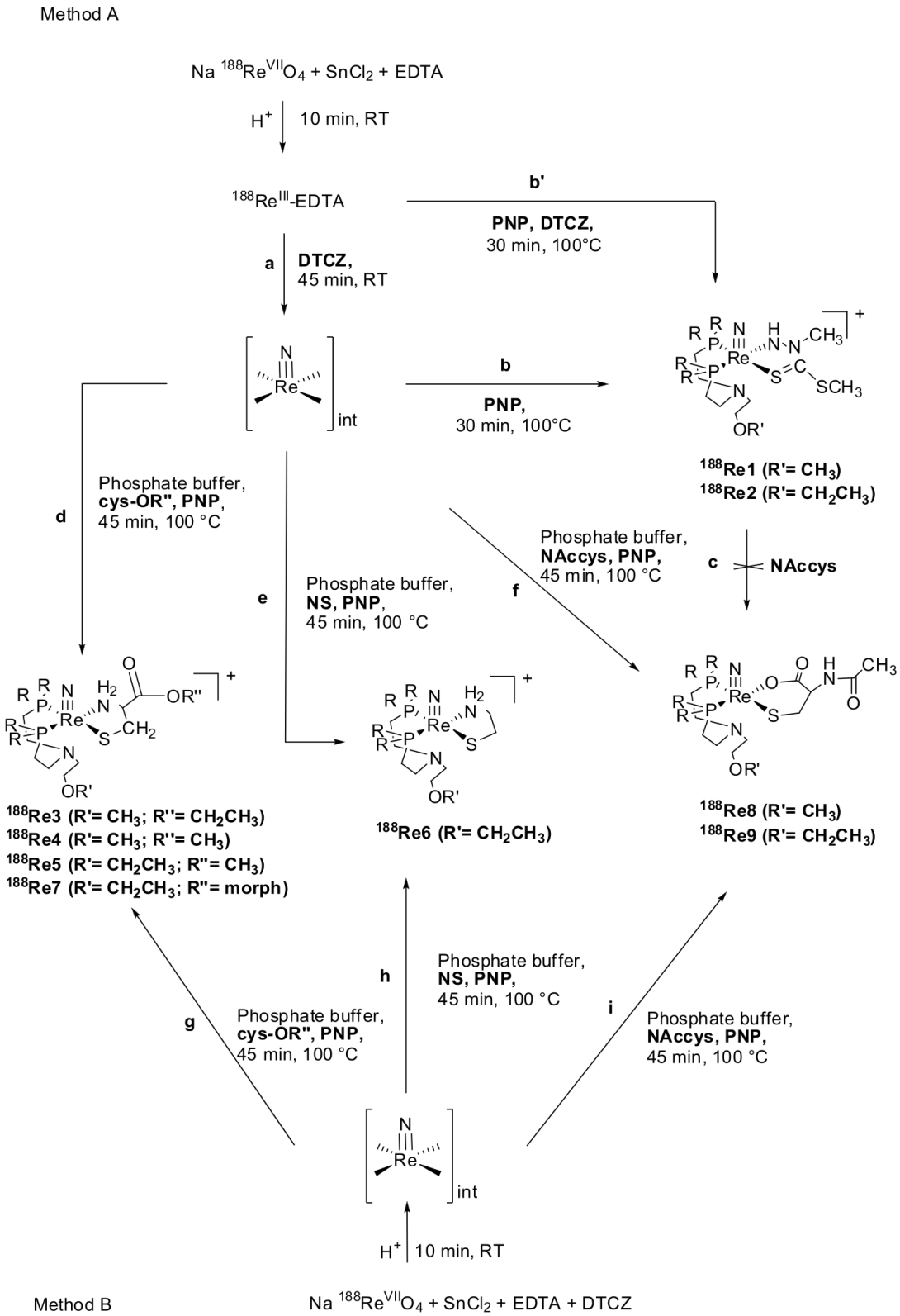
(0.2 MBq, saline, propylene glycol 25%) was injected into the lateral tail vein of male Wistar rats (165-195 g). After the injection, the rats were sacrificed by heart puncture under ether anesthesia 5, 60 and 360 min p.i. Selected organs were isolated for weighting and counting. The radioactivity in the tissues or organs was calculated in terms of percentage of injected dose per organ as well as percent injected dose per gram blood.

*Small animal SPECT imaging studies***.**

A dedicated small animal SPECT imaging system (NanoSPECT, Bioscan, Washington DC, USA) was used. This system employs a gantry of four NaI detectors with multi-pinhole collimators and provides a spatial resolution of <0.5 mm. Assessment of radioactivity distribution and calculation of radioactivity concentration in defined 3D regions of interest (ROIs) in animals was performed using ROVER software (ABX GmbH, Radeberg, Germany). Male NMRI *nu/nu* mice (30 - 35 g) mice were injected in the lateral tail vein with 25 - 35 MBq of **^{188}Re7** or **^{188}Re8**. The animals were imaged at 1 and 5 h post-injection of ^{188}Re complexes. General anesthesia of NMRI *nu/nu* mice was induced with inhalation of desflurane 10% (v/v) (Suprane, Baxter, Germany) in 40% oxygen/air (gas flow 1 L/min), and was maintained with desflurane 8% (v/v). The mice were transferred on the system bed kept at 35°C into the gantry for imaging: gas anesthesia was maintained for the whole duration of the scan. The full body length was set for scanning. The scanning protocol was carried out as inline SPECT with a minimum acquisition of 10.000 counts per projection. Before the study a 20 mL syringe with 20 MBq ^{188}Re-activity in 20 mL solution was used for cross calibration of the scanner with a gamma counter (LKB Wallac Compugamma 1282, Perkin Elmer, Rodgau, Germany). The absolute activity was corrected for the injected dose, and the results expressed as the percentage of the injected activity per gram of the tissue.

Results

Mixed Re(N)-complexes were prepared as detailed in Scheme 1.



The reaction require the *in situ* production of the [Re(N)Cl_2(PNP)] intermediate compounds, via ligand-exchange reaction of the labile precursor [Re(N)Cl_2(PPh_3)$_2$] with the appropriate diphosphine ligand in CH_2Cl_2 solution. The spectroscopic characterization of the complexes [Re(N)Cl_2(PNP)] was previously reported [20].

These intermediate compounds react with the selected bidentate ligand (L=DTCZ, cys~), in presence of an excess of Et_3N, yielding the final mixed compounds of the type [ReN(L)(PNP)]$^{+/0}$ (**Re1,3,4,8**). The overall charge of the resulting asymmetrical complexes was dependent on the nature of the donating atoms of the bidentate ligand.

Elemental analyses, as reported in the Experimental Section, are in good accord with the proposed formulation.

The complete characterization of the isolated compounds was obtained by multinuclear NMR spectroscopy. ^{31}P NMR spectra of all rhenium complexes display signals in agreement with the diphosphine coordination, the pertinent peaks moving downfield in the 7.69-15.65 ppm positive region from the -34.4 ppm value exhibited by uncoordinated PNP3 ligands.

Coordination of the bidentate ligand on the equatorial plane removes the magnetic equivalence of the facing diphosphine phosphorus. Consequently, the ^{31}P NMR spectrum of each nitrido heterocomplex has two distinct signals.

For **Re3, Re4** and **Re8**, two distinct isomeric forms have been observed and identified as *syn* or *anti* isomers depending on the orientation of the N- or COO-substituted cysteine pendant group with respect to the central Re≡N terminal core. The final isomeric ratio, evaluated by TLC and HPLC chromatography, is approximately 50/50. In all cases, the isomer separation was not achieved, thus the mixture of two isomers were purified by silica column chromatography and analyzed by common spectroscopic and analytical methods.

syn and *anti* isomers are distinguished in ^{1}H-NMR spectra by the different proton patterns of the coordinated cysteine framework, as previously determined in similar isomeric pairs, [Re(N)(PNP2)(NAccys)] (PNP2 = aryldiphosphine and NAccys = *N*-acetyl-L-cysteine) containing N,S carboxylic- substituted cysteine and O,S-amine-substituted cysteine, respectively [36], and [^{99g}TcN(2-MPP-cys-OS)(PNP3/4)] where 2-MPPcys-OS = 2-methoxyphenyl piperazine cysteine O,S; PNP4 bis(dimethylphosphinoethyl) methylamine [20].

The assignment of the *anti* isomer in complexes **Re3, Re4** and **Re8** is based on the assumption that the pertinent NH and methyne protons of cysteine are shifted upfield and downfield, respectively, relative to the corresponding proton resonances in the *syn* isomer (Figure 3).

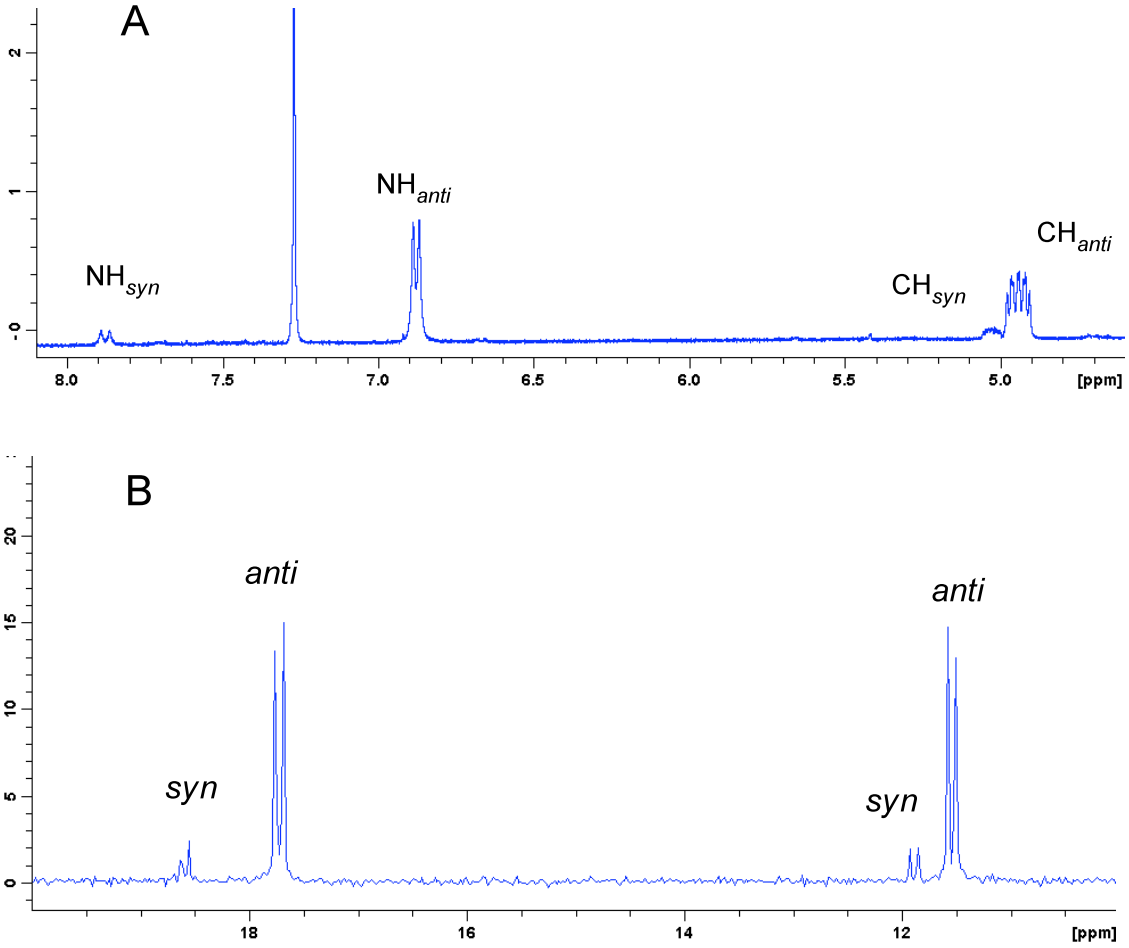


Figure 3. A) ^{1}H-NMR spectra in the diagnostic range (8.1-4.6 ppm) of syn,anti-Re8; B) ^{31}P-NMR spectra of syn,anti-Re8.

ESI mass spectra of the complexes **Re1, Re3, Re8**, show the peaks corresponding to the molecular ion with no additional detectable fragments.

Synthesis and Characterization of ^{188}Re(N)-Compounds

[^{188}Re(N)(cys~)(PNP)]$^{+/0}$ mixed compounds were efficiently prepared in aqueous solution, starting from [Na^{188}ReO$_4$] following the pathway synthesis sketched in Scheme 2.

The labeling procedures were carried out using either a three step (*method A*) or a two-step (*method B*) approach.

The radiochemical yields were optimized considering reaction parameters such as: chemical nature of the nitrido nitrogen (N^{3-}) donor, succession of the reagents, time of incubation and reaction pH.

In view of the fact that in general the rate of ligand substitution to the metal centre is lower for rhenium than it is for technetium, and that the synthesis of the analogues

[^{99m}Tc(N)(cys~)(PNP)]$^{+/0}$ compounds required high temperature, in order to obtain a RCY > 90% [12,17,18,20], the reaction temperature was fixed at 100 °C.

Method A entail the preliminary formations of the pre-reduced $^{188}Re^{III}$-EDTA intermediate complex by the addition of a fresh perrhenate anion solution to a reaction mixture containing 1 mg of tin chloride and 5 mg of EDTA in an acidic media (pH 1.5). The reduction was completed after 10 min at room temperature. In this first step, low Sn^{2+} concentration (< 1 mg) or pH value > 3 led to the incomplete reduction of the perrhenate.

The *second step* involved the addition of an appropriate source of N^{3-} groups to form, after 45 minutes at room temperature, a mixture of [^{188}Re(N)]-intermediate complexes (*reaction a*). In this *step* different hydrazine-like derivatives, such as NaN_3, SDH and DTCZ were investigated as donors of N^{3-} group. The results evidenced that only DTCZ was able to produce the [^{188}Re(N)]–compounds in high yield. In fact, when the reaction was carried out with SDH or NaN_3, in general efficiently employ with pertechnetate, instead of DTCZ, a low amount (RCY~40%) of the final complex was detected or no reaction occurred and the radioactivity was found to be [$^{188}ReO_4$]$^-$ (Figure 4).

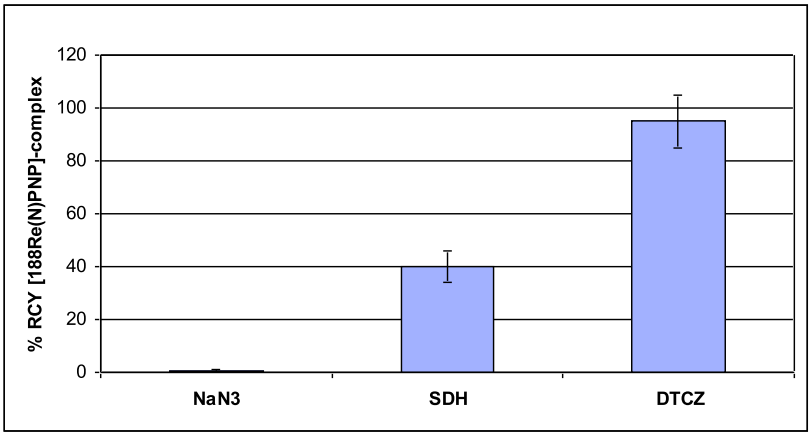


Figure 4. Variation of % RCY as function of the nature of the nitrido nitrogen donor.

In the *third step*, the addition of PNP to the mixture of [^{188}Re(N)]-intermediates (*reaction b, b'*) generated the formation of a single compound, identified as **^{188}Re1** or **^{188}Re2**, depending on the nature of the PNP ligand. TLC and HPLC analysis of **^{188}Re1** or **^{188}Re2** are reported in Table 1 and Figure 2 and compared with the chromatographic data obtained for the analogues [^{99m}Tc(N)(PNP)]-heterocomplexes as well as the corresponding Re-compounds. In these compounds DTCZ is bound to the metal center, of the [M(N)(PNP)] scaffold, as monoanionic chelate through the neutral sulfur atom of the -C=S moiety and the deprotonated terminal amino group.

^{188}Re1 or **^{188}Re2** were found to be kinetically inert as well established by *in vitro* studies carried out incubating the two complexes for 24 h at different pH (in a range 1-10) and temperatures (in a range 37-100 °C) as well as transchelation with a large excess of free cysteine.

Hence, the remarkable stability and kinetic inertness of these compounds prevent their use as precursor in $[^{188}Re(N)(cys\sim)(PNP)]^{+/0}$ synthesis (*reaction c*). In fact, analogously to what previously observed with ^{99m}Tc, all attempts to use this compound in challenge reaction with a large excess of other strong coordinating ligands (cysOEt, NAccys) to produce the corresponding heterocomplexes ($[^{188}Re(N)(cys\sim)(PNP)]^{+/0}$) in appreciable yield were not successful.

However, $[^{188}Re(N)(cys\sim)(PNP)]^{+/0}$ compounds were achieved by adding the two bidentate ligands cys~ and PNP (*reaction d, e, f*) to the buffered reaction vials containing the $[^{188}Re(N)(DTCZ)]_{int}^{2+}$ starting mixture, after 45 min of heating at 100°C.

At this stage the influence of the incubation time and of the pH were evaluated in order to obtain the final mixed compound in high yield.

Figure 5 shows the variation of % RCY of Re complex over the time. The extension of the incubation time from 30 min to 45 min was responsible of a significant variation of the RCY of the final complex, which in general was found to increase of about 50%.

The variation of % RCY of the complex evaluated after 45 min of heating at 100 °C at different pH is reported in Figure 6. High radiochemical yields were obtained at pH value in the range 3.2-3.6: at low pH the formation of $[^{188}Re^{V}(N)(DTCZ)(PNP)]^{+}$ mixed compound was observed as main product. Meanwhile, at pH > 3.6 re-oxidation of the intermediate complexes to perrhenate was observed.

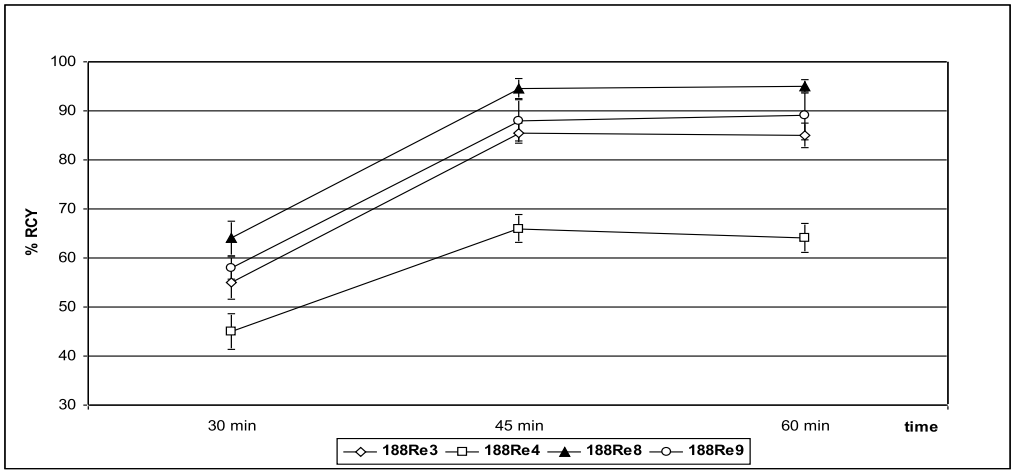


Figure 5. Variation of % RCY over the time of different $[^{188}Re^{V}(N)(cys\sim)(PNP)]^{+/0}$ complexes.

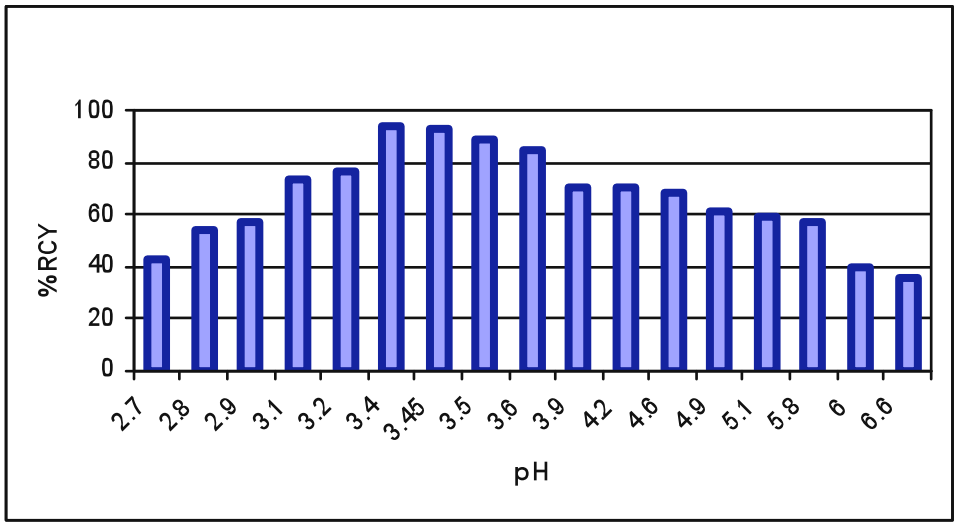


Figure 6. Variation of % RCY of $[^{188}Re^{V}(N)(cys\sim)(PNP3)]^{+/0}$ as function of the pH.

Method B involve the direct formations of the pre-reduced $[^{188}Re^{V}(N)(DTCZ)]_{int}^{2+}$ intermediate complex by the addition of a fresh perrhenate anion solution to a reaction mixture containing tin chloride, EDTA and DTCZ in an acidic media (pH 1.5). Using this method a low amount (0.1 mg) of DTCZ was used. The final asymmetrical compounds were achieved, in high yield after 45 min of heating at 100°C, by adding to the buffered reaction vials the two bidentate ligands cys~ and PNP (*reaction g, h ,i*). In direct comparison with method A the method B affords higher RCY, obviously because of the lower amount of DTCZ used, due to its strong coordination ability to the metal centre.

Chromatographic characterization indicates that the chemical nature of the final mixed complex is independent on the preparation method utilized.

The identity of the new ^{188}ReN-mixed complexes was established by chromatographic methods and by comparison with the well known ^{99m}Tc analogous as well with the corresponding compounds obtained in macroscopic amounts with cold rhenium, characterized by common spectroscopic and analytical methods (Figure 2).

HPLC profiles of the complexes containing a cys~ derivative ligand show two distinct peaks **a,b** expected as *syn* and *anti* isomers. Nevertheless, the correct attribution of the peaks was not achieved. For neutral **^{188}Re8** and **^{188}Re9** the a/b isomeric ratio was found to be 70/30. Meanwhile, for cationic complexes **^{188}Re3-5** the *syn/anti* isomeric ratio was found to be 60/40. The slight differences in isomeric ratio in the ^{188}Re synthesis, compared with macroscopic Re synthesis can be attributed to the different temperature employed. The high temperature applied in the n.c.a. ^{188}Re preparations

could be responsible for conversion between the two isomeric species and for the variation in isomeric ratio [17,18, 20].

In vitro studies

Stability studies of some representative ^{188}Re-agents were evaluated as variation of the radiochemical purity over the time, and assessing the formation of perrhenate. Studies were performed in duplicate on the purified complexes.

The stability of **^{188}Re5**, **^{188}Re6,** **^{188}Re7** and **^{188}Re9** complexes was compared following incubation at 37 °C for 24 h with: i) glutathione (10 mM), ii) phosphate buffer (0.1 M, pH 7.4) and iii) rat plasma.

All ^{188}Re-complexes were found to be inert toward transchelation with an excess of free glutathione.

On the contrary, differences were observed for ^{188}Re -compounds after incubation in phosphate buffer and in rat plasma. Thus, while **^{188}Re6** and **^{188}Re9** were found stable in all condition as showed in Figure 7 and no evidence of perrhenate (Rt = 15.50 min) was detected, the formation of a more hydrophilic species (Rt = 16.20 min) was observed in HPLC profile of **^{188}Re5** after phosphate buffer and rat plasma exposure which result from a possible hydrolysis of the ester-function of the cysteine derivative ligand. This occurrence was confirmed hydrolyzing the **^{188}Re5** with NaOH solution and comparing the HPLC profile of the basic mixture with those of the phosphate buffer and rat plasma mixtures.

In rat plasma the decomposition of **^{188}Re5** occurred more rapidly than in buffer solution due to the action of esterase enzymes. In fact, at 2 h of incubation the radiochemical purity of the complex was 50%. In rat plasma **^{188}Re7** showed a behavior similar to **^{188}Re5.** The observed degradation could be attributed to the hydrolysis of the amide-function determined by the serum enzymes.

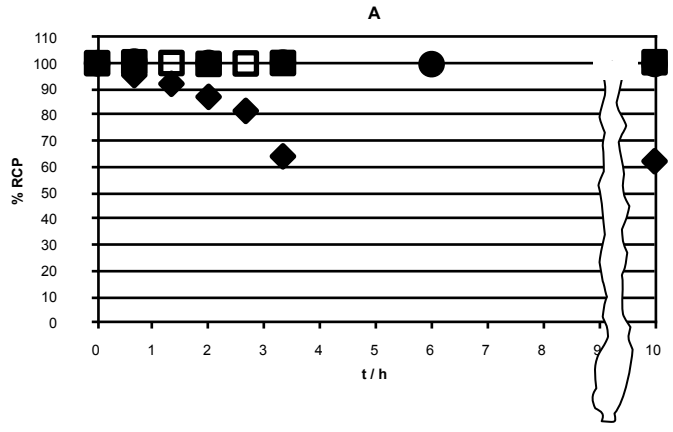


Figure 7. In vitro stability of ^{188}Re5 (-♦-), ^{188}Re6 (-•-) and ^{188}Re9 (-□-) complexes evaluated at 37 °C for 24 h in : A) phosphate buffer (0.1M, pH 7.4); B) rat plasma.

Under these conditions no variation of the isomeric ratios were observed for **^{188}Re5** and **^{188}Re9**.

In vivo studies.

Biodistribution studies were performed in male Wistar rats with **^{188}Re7** and **^{188}Re9** to investigate their organ uptake and excretion pathways. In general, no specific accumulations of the complexes were observed. As Table 2 shows, **^{188}Re7** and **^{188}Re9** present an extremely fast elimination from the blood and from significant organs minimizing the exposure of radiation sensitive organs. The uptake in sensitive organs/ structures such as kidneys, tests, femur (bone marrow) was low and followed by a rapid washout. It is interesting to note that in both cases the renal excretion was extremely rapid, after 60 min *p.i.* the renal activity was found to reduce of 70-80%.

High uptake of **^{188}Re7** and **^{188}Re9** was found in the liver within the 5 min p.i :21.64 ± 2.53; 9.96 ± 1.98 respectively. However, after 60 min the %ID of **^{188}Re7** was 4.51 ± 1.01 and the %ID of **^{188}Re9** was 1.07 ± 0.12. At 360 min an almost quantitative elimination of the compounds was detected.

Table 2. Biodistibution in male Wistar rats (n=4) of the complexes **^{188}Re7** and **^{188}Re9**. Results are expressed as %ID.

	%ID ^{188}Re7			**%ID ^{188}Re9**		
Organ	*5 min*	*60 min*	*360 min*	*5 min*	*60 min*	*360 min*
Brain	0.02 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Thyroid	0.05 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.01	0.01 ± 0.00
Harder's gland						
Thymus	0.08 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Heart	0.25 ± 0.02	0.14 ± 0.02	0.08 ± 0.00	0.14 ± 0.02	0.1 ± 0.01	0.06 ± 0.01
Lungs	0.43 ± 0.05	0.16 ± 0.04	0.1 ± 0.02	0.25 ± 0.03	0.1 ± 0.02	0.05 ± 0.04
Spleen	0.04 ± 0.00	0.04 ± 0.02	0.01 ± 0.00	0.04 ± 0.00	0.02 ± 2.00	0.01 ± 0.01
liver	21.64 ± 2.53	4.51 ± 1.01	1.00 ± 0.3	9.96 ± 1.98	1.07 ± 0.12	0.45 ± 0.10
Pancreas	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.08 ± 0.01	0.09 ± 0.07	0.02 ± 0.01
Stomach	2.16 ± 1.2	1.25 ± 1.09	0.43 ± 0.33	1.95 ± 2.12	2.31 ± 2.43	0.11 ± 0.04
Intestine	28.92 ± 4.51	63.77 ± 3.95	74.13 ± 2.17	48.18 ± 6.02	60.21 ± 3.15	64.19 ± 6.49
Adrenal	0.02 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Kidneys	3.88 ± 1.17	0.82 ± 0.25	0.33 ± 0.01	1.45 ± 0.15	0.43 ± 0.04	0.22 ± 0.03
Testes	0.38 ± 0.07	0.11 ± 0.14	0.02 ± 0.01	0.19 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
Femur	0.22 ± 0.11	0.1 ± 0.09	0.04 ± 0.01	0.09 ± 0.02	0.04 ± 0.01	0.03 ± 0.00
Urine	5.00 ± 4.61	3.19 ± 19.22	6.16 ± 7.49	0.98 ± 5.95	22.92 ± 7.52	20.92 ± 7.10

Male NMRI *nu/nu* mice were imaged by the NanoSPECT imaging system at 1 h and at 5 h p.i. Images of the SPECT scans clearly identified the activity in the intestine and

bladder. Representative SPECT images of ^{188}Re-complexes injected animals are showed in Figure 8. The 3D reconstruction uptake of the ^{188}Re yielded similar radioactivity distribution in the bladder and intestine at 5 h (not showed). The NanoSPECT system allows, following image reconstruction, the quantification of activity in defined regions. This has the substantial advantage of enabling assessment of uptake and elimination at multiple time points in the same animal. However, the accuracy of in vivo quantification by this small-animal SPECT imaging system has not yet been extensively validated for ^{188}Re.

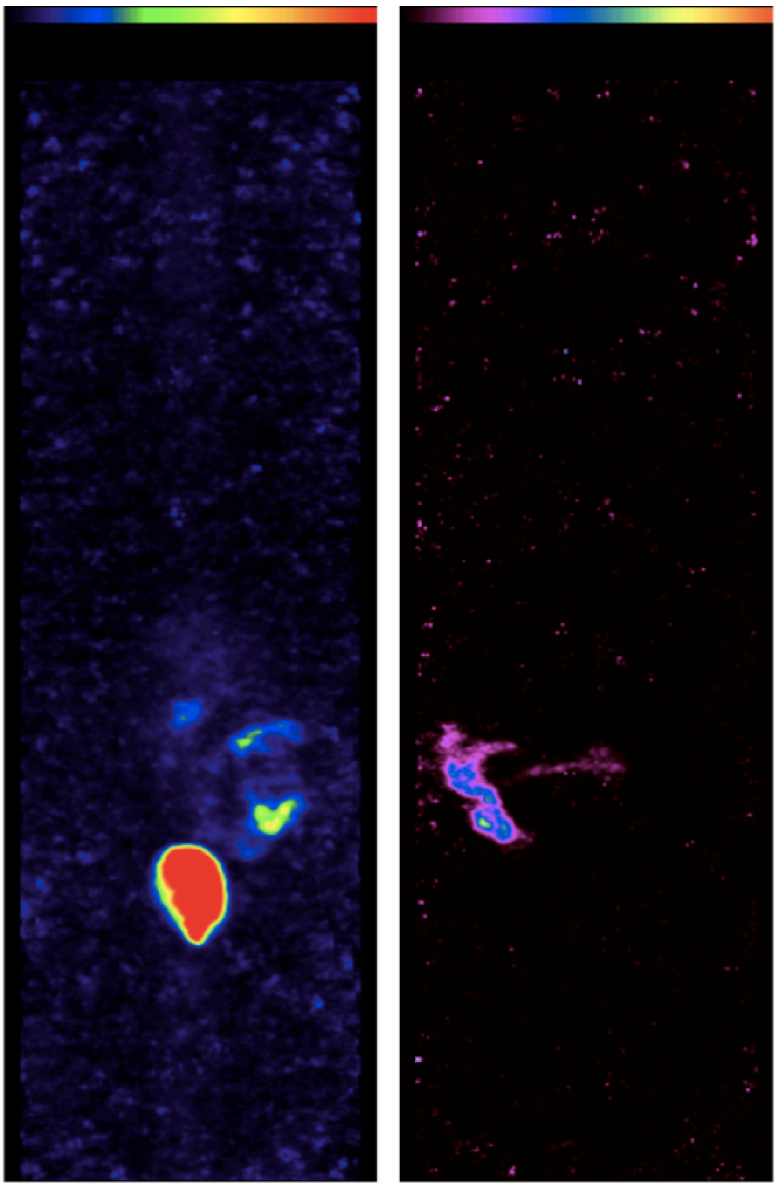


Figure 8. Visualization of eliminated ^{188}Re-activity by SPECT imaging. SPECT images are shown of mice injected with ^{188}Re7 (left) or ^{188}Re8 (right). Images shown are the maximum projections of full body 3D reconstruction at 1 h p.i..

Discussion

The use of radiolabeled compounds for the diagnosis and treatment of degenerative diseases currently constitutes a rapidly expanding field in nuclear medicine [37].

Over the last few years, we described the synthesis of a class of Tc-based asymmetrical nitrido complexes of the type $[Tc(N)(L)(PNP)]^{+/0}$, where L is a bidentate ligand such as a dithiocarbamate or a cysteine derivative, useful in designing either 'essential' and 'receptor-specific' Tc-99m agents [12,13,17,18,20,30].

These heterocomplexes were commonly prepared at tracer level through a two-step procedure which involved the preliminary formation of a mixture of intermediate $[^{99m}Tc{\equiv}N]^{2+}$ species reducing the pertechnetate with tin chloride in presence of SDH as the source of N^{3-} group. This was followed by the simultaneous addition to the reaction vials of the PNP and the bidentate ligand to generate the final mixed compound in high yield. Results from these studies clearly display that it was not possible to apply to ^{188}Re an identical reaction pathway. The differences in redox potential of these two metals and the lower kinetic of substitution on Rhenium center with respect to Technetium impose the use of alternative procedure in order to produce complexes with high radiolabeling efficiency.

Data here reported evidenced that the formation of the $[^{188}Re(N)]^{2+}$ core required more restrictive conditions compared to those required for the formation of the $[^{99m}Tc(N)]^{2+}$ core and that the possibility of obtaining $[^{188}Re(N)]$-compounds in high yield was associated with different factors such as: i) availability of both an efficient reduction of the ^{188}Re-perrhenate anion, and an efficient nitrido nitrogen groups donor; ii) stabilization of the intermediate pre-reduced compound with an appropriate substitution labile ligand that can be replaced by other stronger ligands of interest; iii) correct pH value.

$[^{188}Re(N)(cys{\sim})(PNP)]^{+/0}$ complexes were prepared from perrhenate, in aqueous solution, by a multistep procedure (Scheme 2) based on the preliminary formation of $^{188}Re\ ^{III}EDTA$ precursor which easily undergoes oxidation/ligand exchange reaction to afford the $[^{188}Re{\equiv}N]^{2+}$ core in presence of a low amount of DTCZ (0.1 mg). In this step the nitrogen atom N^{3-} group donor could be also viewed as a suited ligand for the coordination to the metal ion and its stabilization. Indeed the use in excess of DTCZ made possible its participation to the reaction process also as coordinating agent capable of stabilizing the $[^{188}Re{\equiv}N]^{2+}$ core, through the formation of intermediate complexes, for a sufficient time to allow the reaction to be completed.

This behavior was suggested by the fact that when DTCZ was added to the reaction mixture containing the ^{188}Re ^{III}EDTA, a distinct lipophilic intermediate compound (R_T= 17.3, Rf=0.65) was observed (Figure 9). Unfortunately its exact chemical nature was not determined, but, upon addition of the appropriate bidentate ligands, the intermediate was completely converted into the final asymmetrical nitrido complex. Notably, a mixture of three different intermediate complexes were detected when the $[^{188}Re{\equiv}N]^{2+}$ core was produced using the '*oxalate*' method (Figure 9) [28]. Among these, the species indicated as **y** (Rf 0.65) were found corresponding to the intermediate formed by EDTA method; it seems to be the only compound able to react with the chelates to give the final complex. Consequently, using the '*oxalate*' method, the yield of the asymmetrical complex was < 35%.

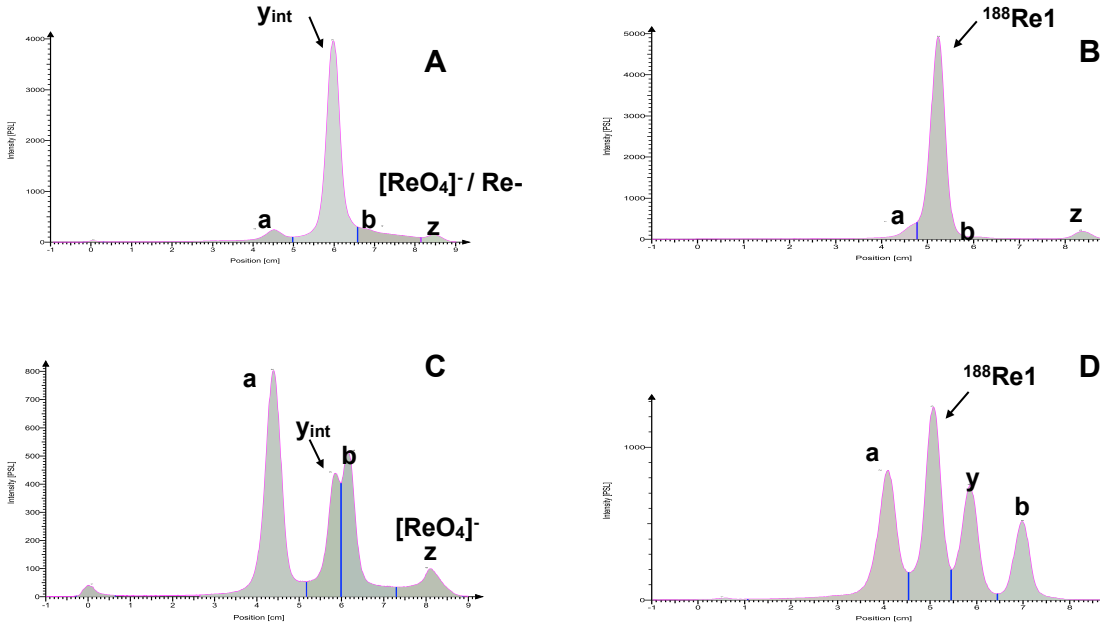


Figure 9: Chromatographic profile of $[^{188}Re{\equiv}N]_{int}^{2+}$ intermediate (A, C) and ^{188}Re1 (C, D) produced using 'EDTA' method or 'oxalate' method respectively.

These stable intermediate compounds were not found when SDH was used instead of DTCZ.

The final mixed compounds were obtained, at 100 °C, by adding to the buffered $[^{188}Re^{V}{\equiv}N]^{2+}$ solution (pH 3.2-3.6) the two bidentate ligands.

It is interesting to note that analogously to what previously observed in ^{99m}Tc-preparation [12] when the two chelates (PNP and cys~) react with the $[^{188}Re{\equiv}N]_{int}^{2+}$ intermediate in a pH range value of 3.2-3.6, only the asymmetrical $[^{188}Re(N)(cys\sim)(PNP)]^{+/0}$ species were produced and no concomitant formation of the symmetrical $[^{188}Re(N)(PNP)_2]$ and $[^{188}Re(N)(cys\sim)_2]$ compounds occurred. This fact further confirms

the high specificity and selectivity of these reactions. However, the need to use DTCZ as N^{3-} group donor, whose excess can compete as ligand in $[^{188}Re(N)(cys\sim)(PNP)]^{+/0}$ formation, make a relative high amount of the cysteine derivate ligand (5 - 2 mg) to be required in order to obtain the final complex in high radiochemical yield.

Data collected from the first and second step reaction provide a clear contribute to elucidate the mechanism of formation of the $[M^{V}\equiv N]^{2+}$ core from the reaction of $[M^{VII}O_4]^-$ with donors of N^{3-}groups, and in presence of a reducing agent. The results showed that the overall process does not proceed through the formation of an intermediate mono-oxo M^{V}-species, which is subsequently converted into a nitrido complex by substitution of the $M^{V}=O$ with the $M\equiv N$ group. Instead, the key step in the reaction is the preliminary reduction of $[M^{VII}O_4]^-$ to afford an intermediate M^{III}- species, stabilized as M^{III}EDTA, which is then re-oxidized to give the final $[M^{V}\equiv N]^{2+}$ core in presence of a suitable source of nitrido nitrogen atoms.

These informations nicely suggest that the formation of M^{III}-complexes would be readily accessible in physiological solution than the M^{V}-complexes. This conclusion may have also a deep impact on the interpretation of the mechanism of formation of mono-oxo M=O complexes.

In vitro studies carried out on both monocationic and neutral ^{188}Re(N)-complexes clearly indicate that these compounds were completely stable toward reoxidation to perrhenate in buffered solution and in plasma, and toward ligand exchange reaction in presence of a large excess of free glutathione. This behavior can be explained considering different issues inherent to the properties of the $[M\equiv N]^{2+}$ core. This moiety, in general, confers to the complexes a particular stability over a wide range of pH values and a strong resistance to red-ox processes compared to the M(O)-complexes.

A further explanation of the high stability of the complexes appears to be correlated to the chemical nature of the atoms set coordinated around to the $[M\equiv N]^{2+}$ group and composed by P, P, S(N;O) as proof of the concept that arrangements of two π-acceptor phosphorus and two π-donor atoms around the $[M\equiv N]^{2+}$ core confer to the final complex a remarkable thermodynamic stability and kinetic inertness.

Ex vivo studies of ^{188}**Re7** and ^{188}**Re9** showed that the complexes were efficiently cleared from the blood and no specific uptake or long-term retention was observed in other significant organ reducing the background activity and minimizing the exposure of radiation sensitive organs. The activity was eliminated both through the hepatobiliary system and urinary tract with a major urinary excretion for ^{188}**Re9.**

However, high level of radioactivity was found in the intestinal lumen, suggesting that the complexes may excrete rapidly in the feces. This latter behavior was already observed in a series of related ^{99m}Tc(N)-complexes [13,15,17,18,20].

In both cases no activity in thyroid and no increase of the %ID in the stomach were found indicating high *in vivo* stability of the complexes.

MicroSPECT/CT is a non-invasive imaging modality that can monitor the behavior of radiotherapeutic agents in the same animal across different time points. Analysis of the images permit, thank to the decay properties of the ^{188}Re, to assess the distribution and the therapeutic efficacy of the corresponding ^{188}Re-agents.

In vivo imaging of ^{188}**Re7** and ^{188}**Re9** showed a rapid distribution and elimination of the complexes. Data on uptake/elimination analyzed by imaging is similar to the results of the biodistribution studies. In particular, there is a positive correlation for the accumulation of both complexes in radiation sensitive organs such as kidneys, tests, femur (bone marrow) estimate with biodistribution studies (%ID) and with microSPECT semiquantification imaging analysis (SUV). All these findings are of fundamental importance in view of the potential application of these agents in preparation of ^{188}Re- based target specific radiopharmaceuticals.

Conclusion

The present study illustrate the synthesis, characterization and the biological evaluation of a series of $[^{188}Re(N)(cys\sim)(PNP)]^{+/0}$ mixed ligand compounds as model for the development of $^{188}Re(N)$-based target specific radiopharmaceuticals.

The complexes were efficiently prepared in aqueous solution from perrhenate using a multistep procedure based on the preliminary formation of the labile $^{188}Re^{III}EDTA$ species, which undergo to oxidation/ligand exchange reaction to give in presence of DTCZ, the $[^{188}Re{\equiv}N]^{2+}$ moiety.

The most important think that makes DTCZ an excellent reagent in formation of the $[^{188}Re{\equiv}N]^{2+}$ core may be attributed to its combined capability to donate N^{3-} groups and, concomitantly, to stabilize the metal center as coordinating agent.

Nevertheless, the excess of DTCZ combined with its good coordinating properties may generate a limitation in ligand exchange reaction when other co-ligands (cys~) are added to obtain the final $[^{188}Re(N)(cys\sim)(PNP)]^{+/0}$ mixed compounds; consequently a relative high amount of the cysteine derivate ligand is required to obtain the final complex in high radiochemical yield.

However, the fact that cysteine derivative ligands strongly bound the $[^{188}Re(N)(PNP)]$ moiety, both in NH_2S and OS coordination mode, thus giving monocationic **^{188}Re3-^{188}Re7** and neutral **^{188}Re8** and **^{188}Re9**, prompt us to use this amino acid as chelating system to incorporate bioactive peptides into the final complex. The compounds were found to be remarkable stable *in vitro* and *in vivo* toward reoxidation to perrhenate and toward ligand exchange reactions.

These findings could be conveniently applied in the development of $[^{188}Re(N)(PNP)]$- radiolabeled peptides useful as target specific radiopharmaceuticals. However, the low labeling efficiency limits but not excludes the possibility to apply $[^{188}Re(N)(PNP)]$-technology to the preparation of target specific radiotherapeutical agents.

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