

Organic & Biomolecular Chemistry

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

Isostructural Re(I)/^{99m}Tc(I) Tricarbonyl Complexes Functionalized with Benzothiazole Derivatives for Cancer Theranostics

Patrique Nunes,¹ Goreti Ribeiro Morais,¹ Elisa Palma,¹ Francisco Silva,¹ Maria Cristina Oliveira,¹
Vera F. C. Ferreira,¹ Filipa Mendes,¹ Lurdes Gano,¹ Hugo Vicente Miranda,² Tiago F. Outeiro,^{2,3,4}
Isabel Santos,¹ António Paulo^{1*}

¹*Centro de Ciências e Tecnologias e Nucleares, IST, Universidade de Lisboa, Estrada Nacional 10,
2695-066 Bobadela LRS, Portugal*

²*Cell and Molecular Neuroscience Unit, Instituto de Medicina Molecular, Av. Prof. Egas Moniz,
1649-028 Lisboa, Portugal;*

³*Instituto de Fisiologia, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal*

⁴*Department of NeuroDegeneration and Restorative Research, University Medical Center Göttingen,
Waldweg 33, 37073 Göttingen, Germany*

*Corresponding authors: apaulo@ctn.ist.utl.pt

Keywords: Benzothiazoles, Rhenium; Technetium-99m; Tricarbonyl; Antitumoral Activity

Organic and Biomolecular Chemistry: submitted as a full paper

Abstract

Merging classical organic anticancer drugs with metal-based compounds in one single molecule offers the possibility of exploring new approaches for cancer theranostics, i.e., the combination of diagnostic and therapeutic modalities. For this purpose, we have synthesized and biologically evaluated a series of Re(I)/^{99m}Tc(I) tricarbonyl complexes (**Re1-Re4** and **Tc1-Tc4**, respectively) stabilized by a cysteamine-based (N,S,O) chelator and containing 2-(4'-aminophenyl)benzothiazole pharmacophores. With the exception of **Re1**, all the Re complexes have shown a moderate cytotoxicity in MCF7 and PC3 cancer cells (IC₅₀ values in the 15.9-32.1 μM range after 72 h of incubation). The cytotoxic activity of the Re complexes is well correlated with cellular uptake that was quantified using the isostructural ^{99m}Tc congeners. There is an augmented cytotoxic effect for **Re3** and **Re4** (versus **Re1** and **Re2**), and the highest cellular uptake for **Tc3** and **Tc4**, which display a long ether-containing linker to couple the pharmacophore to the (N,S,O)-chelator framework. Moreover, fluorescence microscopy clearly confirmed the cytosolic accumulation of the most cytotoxic compound (**Re3**). Biodistribution studies of **Tc1-Tc4** in mice confirmed that these moderately lipophilic complexes (log D_{o/w} = 1.95-2.32) have a favorable bioavailability. **Tc3** and **Tc4** presented a faster excretion, as they undergo metabolic transformations, in contrast to complexes **Tc1** and **Tc2**. In summary, our results show that benzothiazole-containing Re(I)/^{99m}Tc(I) tricarbonyl complexes stabilized by cysteamine-based (N,S,O)-chelators have potential to be further applied in the design of new tools for cancer theranostics.

Introduction

Many benzothiazole derivatives have shown relevant biological features for drug development, both for diagnostic and therapeutic purposes. This class of compounds has proved to be particularly useful for the design of radioactive probes for nuclear imaging and as cytotoxic drugs for antitumor therapies.¹⁻³

For nuclear imaging, 2-arylbenzothiazole derivatives had a crucial role in the development of radioprobes for the *in vivo* detection of β -amyloid ($A\beta$) aggregates by positron emission tomography (PET) and single-photon emission computed tomography (SPECT).³ The lead compound in these studies has been the so-called "Pittsburgh compound B" (^{11}C -PIB) (Fig. 1). Studies in Alzheimer's disease (AD) patients validated the usefulness of ^{11}C -PIB as a PET imaging probe for $A\beta$ detection. However, ^{11}C -PIB is not approved for clinical use.^{4,5} Nevertheless, structurally related ^{18}F -labeled congeners, such as ^{18}F -Flutemetamol (Fig. 1), have been recently approved by the US Food and Drug Administration (FDA) and European Medicine Agency (EMA) for the visual detection of $A\beta$ burden in patients suspected of AD.⁶ Several metal-based compounds bearing arylbenzothiazole moieties were also evaluated for *in vivo* imaging of $A\beta$ plaques. This included the study of $^{99\text{m}}\text{Tc}$ complexes for SPECT imaging and Gd complexes as contrast agents for magnetic resonance imaging (MRI).⁷⁻⁹ The results for metallic complexes were less encouraging if compared with purely organic molecules, reflecting their poor ability to penetrate the blood-brain barrier and to reach the β -amyloid plaques.

The 2-arylbenzothiazole scaffold is also promising for the development of anticancer drugs. It was shown that such a simple scaffold might afford possible drug candidates upon the introduction of minor functional group changes in its core. Many benzothiazole derivatives have demonstrated activity against a wide range of human tumor cell lines, including ovarian, breast, prostate lung, renal, and colon cancer.¹⁰⁻¹³ From the large number of tested molecules, the most explored as an

antitumour agent is Phortress (Fig. 1), a 2-(4'-aminophenyl)benzothiazole derivative that underwent a Phase 1 clinical trial.¹⁰

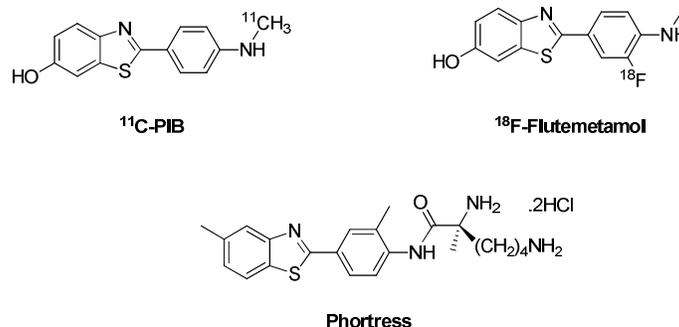


Figure 1. Chemical structures of clinically relevant benzothiazole derivatives.

Merging classical organic anticancer drugs with metal-based compounds in one single molecule offers the possibility of exploring new approaches for cancer theranostics, based on the combination of diagnostic and therapeutic modalities. The metal fragments might confer advantages as theranostic tools, such as the possibility of exploring a larger diversity of chemical structures, which may increase their bioavailability and resistance to metabolic transformation. For this purpose, a large variety of d and f transition metals with nuclear or magnetic properties suitable for medical imaging is available, as for example the aforementioned Tc and Gd.^{14,15}

In the particular case of technetium, non-radioactive Re complexes are often used as reference compounds to assign the chemical identity of the ^{99m}Tc congeners due to the similitude of the chemistry of these two group 7 elements.¹⁴ Hence, when designing metal-based cancer theranostic agents, the Re complexes can be incorporated into the cytotoxic entity that will exert a therapeutic effect while the ^{99m}Tc congeners are part of the imaging entity that will enable the *in vivo* detection of tumor tissues. In addition, there are two beta-emitting radioisotopes of rhenium (¹⁸⁶Re and ¹⁸⁸Re) that present physical properties suitable for radionuclide therapy.¹⁴ Therefore, Tc and Re can be seen as a unique match-pair of d-transition elements that enables the design of radiopharmaceuticals for

diagnostic (^{99m}Tc) and therapeutic applications ($^{186/188}\text{Re}$), offering further possibilities for cancer theranostics by allowing the combination of SPECT imaging with chemo- and/or radiotherapeutic modalities.

Recently, the 2-(4'-aminophenyl)benzothiazole pharmacophore was linked to acyclic or macrocyclic bifunctional chelators (BFCs), which were used to obtain $^{99m}\text{Tc}(\text{I})$ tricarbonyl complexes and Gd (III) complexes. These complexes were evaluated as SPECT- or MRI-imaging compounds for cancer theranostics, respectively.¹⁶⁻¹⁸ These first studies led to encouraging biological results underlying the relevance of benzothiazole-containing metal complexes in this field of research. In this context, we have embarked on the synthesis and biological evaluation of new benzothiazole-containing $^{99m}\text{Tc}(\text{I})$ and Re(I) tricarbonyl complexes stabilized by a (S,N,O)-donor BFC derived from cysteamine. Here, we report on the synthesis and characterization of these new complexes, as well as on their biological evaluation as potential cancer theranostic agents. For the Re complexes, the biological evaluation comprised the assessment of their cytotoxic activity against human breast cancer – MCF7 – and prostate cancer – PC3 – cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the visualization of cellular uptake by fluorescence microscopy. Conversely, the ^{99m}Tc counterparts were used for the quantification of the uptake in the same cell lines, and to ascertain the biodistribution and *in vivo* stability of the complexes in mice.

Results and discussion

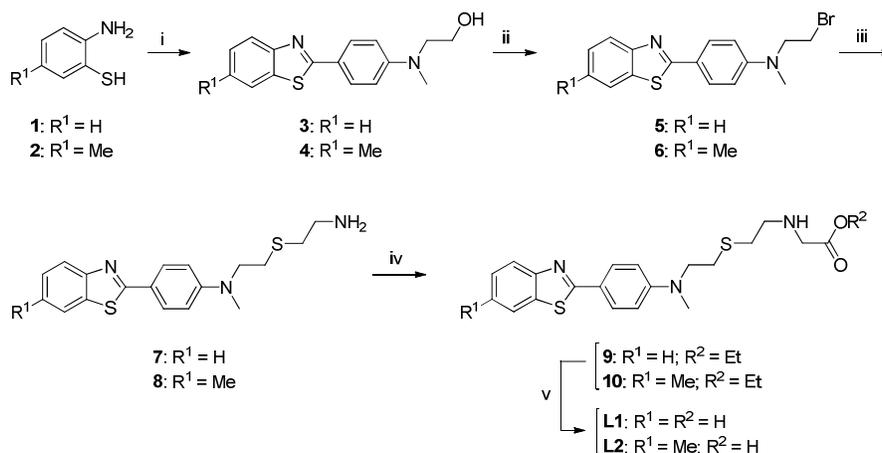
Synthesis and characterization of the ligands

As mentioned above, we have considered (S,N,O)-donor BFCs derived from cysteamine to stabilize the *fac*- $[\text{M}(\text{OH}_2)_3]^+$ (M = Re, ^{99m}Tc) core and to be functionalized with the 2-(4'-aminophenyl)benzothiazole pharmacophore. In the last few years, this core has gained an increasing impact in radiopharmaceutical chemistry following the introduction by Alberto and co-workers of a convenient and fully aqueous-based kit preparation of the organometallic precursors *fac*- $[\text{M}(\text{OH}_2)_3(\text{CO})_3]^+$.^{19,20} This so-called tricarbonyl approach has several intrinsic advantages, including

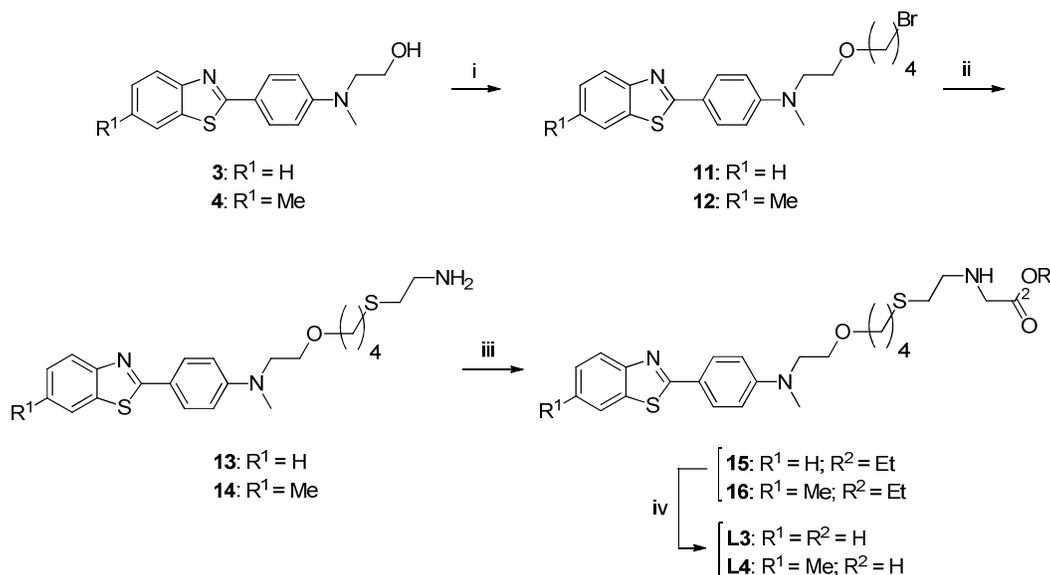
the high stability and kinetic inertness inherent to Tc(I) and Re(I) tricarbonyl complexes when appropriate ligands are used.²¹ Previously, we have shown that the cysteamine-based (S,N,O)-donor ligand 2-(2-(ethylthio)ethylamino)acetic acid affords low-molecular weight and lipophilic ^{99m}Tc(I)/Re(I) complexes with high *in vitro* and *in vivo* stability.^{22,23} We have anticipated that the replacement of the S-terminal ethyl substituent of this chelator by the 2-(4'-aminophenyl)benzothiazole pharmacophore would not compromise the coordination capability of the (S,N,O)-donor set towards *fac*-[M(OH₂)₃]⁺ (M = Re, ^{99m}Tc) and the biological properties of the pharmacophore.

Four new BFCs, **L1-L4**, were synthesized by linking 2-arylbenzothiazole pharmacophores to the 3-((2-mercaptoethyl)amino)propanoic acid coordinating unit by using two different aliphatic spacers, displaying or not an ether function (Schemes 1 and 2). The synthesis of **L1-L4** has been achieved in a convergent way, using compounds **3** and **4** as common intermediates. As depicted in Scheme 1, the 2-aryl benzothiazole rings of **3** and **4** were formed through cyclocondensation of the *o*-aminothiophenols **1** and **2**, by reaction with the adequate *N*-substituted benzaldehyde under basic conditions. For **L1** and **L2**, which do not contain any ether function in their structure, the synthesis involved the brominated derivatives **5** and **6** as intermediates. Compounds **5** and **6** were obtained in moderate yield ($\eta = 56-64\%$) from the benzothiazoles **3** and **4** using the Appel reaction (Scheme 1). The synthesis of **L3** and **L4**, having an ether function in the linker between the pharmacophore and the S,N,O-coordinating unit, was also achieved using brominated intermediates (compounds **11** and **12**); **11** and **12** were obtained in fair yield ($\eta = 40-45\%$) by the *O*-alkylation of **3** and **4** with excess of 1,4-dibromobutane (Scheme 2). For all the ligands, the incorporation of the benzothiazole moieties into the (S,N,O)-donor coordination unit was accomplished in a sequential manner, in which the brominated derivatives **5**, **6**, **11**, and **12** acted as electrophiles in *S*-alkylation reactions of 2-aminoethanethiol, followed by the *N*-alkylation of the resulting compounds (**7**, **8**, **13**, and **14**, respectively) with ethyl bromoacetate. Finally, basic hydrolysis of the obtained benzothiazole ester

derivatives (**9**, **10**, **15**, and **16**, respectively) gave the ligands **L1-L4**. All the compounds were characterized by common spectroscopic techniques (^1H and ^{13}C NMR) and by ESI-MS, which confirmed the proposed formulations.



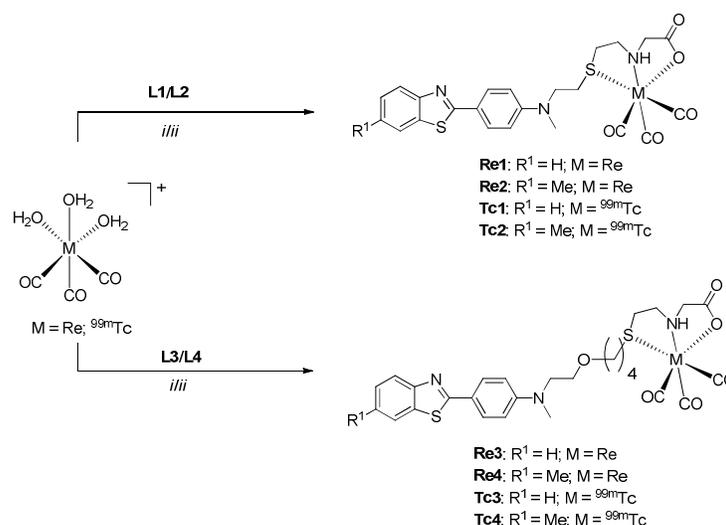
Scheme 1. Synthetic route for the preparation of ligands **L1** and **L2**. Conditions: i) *N*-methyl-*N*-(2-hydroxyethyl)-4-aminobenzaldehyde, pyridine, reflux; ii) CH_2Cl_2 , CBr_4 , PPh_3 , RT; iii) NaOH , EtOH , 2-aminoethanethiol hydrochloride, RT; iv) CH_3CN , KI , K_2CO_3 , ethyl 2-bromoacetate, 82°C ; v) THF, water, NaOH , reflux.



Scheme 2. Synthetic route for the preparation of ligands **L3** and **L4**. Conditions: i) THF, NaH , 1,4-dibromobutane; ii) NaOH , EtOH , 2-aminoethanethiol hydrochloride, RT; iii) CH_3CN , KI , K_2CO_3 , ethyl 2-bromoacetate, reflux; iv) THF, water, NaOH , reflux.

Synthesis and characterization of the organometallic Re and ^{99m}Tc complexes

The synthesis of the Re(I) tricarbonyl complexes **Re1-Re4** was performed by ligand exchange reactions of *fac*-[Re(H₂O)₃(CO)₃]Br with **L**¹–**L**⁴ in refluxing methanol (Scheme 3). After adequate purification, complexes **Re1-Re4** were recovered as yellow microcrystalline solids in moderate to high yield (57-81%). Their characterization was done by common spectroscopic techniques (IR, ¹H and ¹³C NMR), ESI-MS, HPLC and elemental analysis.



Scheme 3. Synthesis of the Re(I) and ^{99m}Tc (I) tricarbonyl complexes. Conditions: i) [Re(H₂O)₃(CO)₃]Br, methanol, reflux, 16 h; ii) [^{99m}Tc (H₂O)₃(CO)₃], H₂O, 100 °C, 30 min.

The positive ESI mass spectra obtained for **Re1-Re4** showed the presence of prominent peaks corresponding to the respective [M+H]⁺ molecular ions; unlike **Re1** and **Re2**, intense peaks that were assigned to [M+Na]⁺ were also observed in the mass spectra of **Re3** and **Re4**. This difference certainly reflects the presence of an ether function in the aliphatic spacers of **L3** and **L4**, which might enhance the interaction with the sodium cation. The IR spectroscopic data obtained for all the complexes corroborated the presence of the *fac*-[Re(CO)₃]⁺ core, detecting two strong bands assignable to ν(C=O) in the 1893-2028 cm⁻¹ range. Moreover, the HPLC analysis of **Re1-Re4** showed that the complexes were of high purity, as only single peaks were detected in the respective chromatograms.

The chemical characterization of **Re1-Re4** by NMR spectroscopy was more demanding. At room temperature, the ^1H NMR spectra of these complexes in $\text{DMF-}d_7$ displayed a set of relatively broad and ill-defined resonances in the area of the methylenic and methyl protons, ranging from 1.71 to 3.96 ppm. The aromatic protons from the 2-(4'-aminophenyl)benzothiazole pharmacophore gave rise to better defined and well resolved signals that appear between 6.90 and 8.10 ppm. The broadness of the resonances from the aliphatic protons suggested that complexes **Re1-Re4** undergo dynamic processes in solution, as confirmed for **Re1** by variable temperature ^1H NMR studies (Fig. 2). The dynamic behavior observed for **Re1-Re4** is most probably due to the occurrence of pyramidal inversion at the coordinated sulfur atom, as we have previously observed for the congener Re(I) tricarbonyl complex with 2-(2-(ethylthio)ethylamino)acetic acid (herein designated as $\text{Re}(\text{CO})_3(\text{NSO})$).²² These findings indicate that **Re1-Re4** most probably contain ligands coordinated in a N,S,O-tridentate fashion, a coordination mode that was confirmed by X-ray diffraction analysis in the case of $\text{Re}(\text{CO})_3(\text{NSO})$.²³

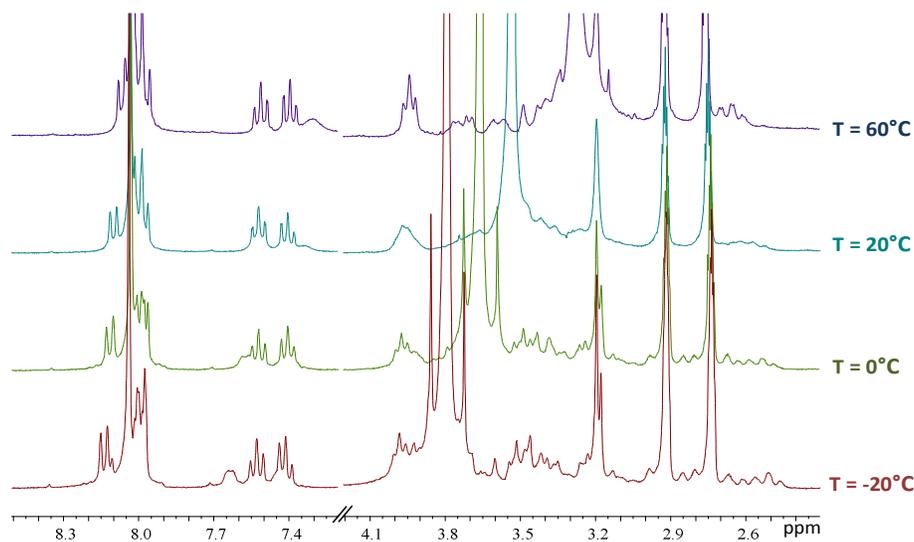


Figure 2. ^1H NMR spectra of complex **Re1** at various temperatures in $\text{DMF-}d_7$.

In aqueous solution and at 100 °C, **L1-L4** readily reacted with $\text{fac-}[^{99\text{m}}\text{Tc}(\text{CO})_3(\text{OH}_2)_3]^+$ affording complexes **Tc1-Tc4**, respectively (Scheme 3). The reactions are almost quantitative (>95%) for final

concentrations of **L1-L4** as low as 10^{-4} M, being complexes **Tc1-Tc4** the unique species formed. Their chemical identity was confirmed by comparison of their HPLC profiles with those of the corresponding rhenium complexes, as exemplified for **Tc1/Re1** in Fig. 3.

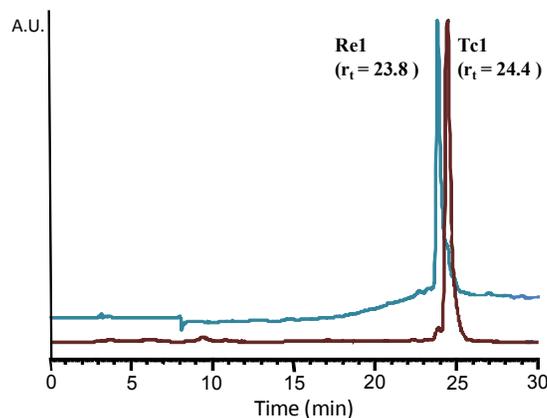


Figure 3. HPLC chromatograms of complexes **Re1** (UV detection) and **Tc1** (radiometric detection).

The characterization of **Tc1-Tc4** comprised also the evaluation of their lipophilicity, which was assessed by the measurement of the respective $\log D_{o/w}$ values (*n*-octanol/0.1 M, phosphate buffered saline (PBS), pH 7.4) using the “shake-flask” method. The $\log D_{o/w}$ values and the HPLC retention times of **Tc1-Tc4** are shown in Table 1. All the complexes are moderately lipophilic with $\log D_{o/w}$ values spanning in a rather narrow range, from 1.98 to 2.32, suggesting that the presence of the ether linkage balances the increase of lipophilicity caused by the larger number of methylenic groups in the linker.

Table 1. HPLC retention times and $\log D_{o/w}$ of complexes **Tc1-Tc4**.

Complex	r_t (min) ^{a,b}	$\log D_{o/w}$
Tc1	24.4 (23.8)	1.98 ± 0.02
Tc2	24.9 (24.1)	2.01 ± 0.05
Tc3	24.0 (23.3)	1.95 ± 0.01
Tc4	26.0 (25.9)	2.32 ± 0.04

a. Using a gradient of aqueous 0.1% CF_3COOH and methanol as the solvent.

b. The values in parentheses are the retention times for the Re congeners.

Biological evaluation of the Re complexes: cytotoxicity assays and microscopy studies

The cytotoxic activity of the ligands and respective Re complexes was tested on human breast cancer – MCF7 – and prostate cancer – PC3 – cell lines. These cancer cells were treated with decreasing concentrations (200 – 0.002 μM) of the different compounds and incubated for 72 h at 37 °C. All the tested compounds were first solubilized in DMSO and diluted in the cell media. The percentage of DMSO never exceeded 1%, a non-toxic concentration. After the treatment, cellular viability was assessed by the MTT assay. The inhibition of growth (%) was calculated by correlation with vehicle-treated cells. The IC_{50} values, expressed in μM concentrations, are average \pm standard deviations of 3 independent experiments with 4 replicates each, and the results are presented in Table 2.

Table 2. Cytotoxicity of **L1-L4** and **Re1-Re4** in MCF7 breast and PC3 prostate cancer cells.

Compound	$\text{IC}_{50}^{\text{a}}$ (μM)	
	MCF7	PC3
L1	25.6 \pm 1.4	32.5 \pm 1.1
Re1	90 \pm 1.2	94 \pm 1.3
L2	241 \pm 1.8	89 \pm 1.1
Re2	30.7 \pm 1.0	30.6 \pm 1.0
L3	52.2 \pm 1.1	95 \pm 1.2
Re3	15.9 \pm 1.1	28.8 \pm 1.1
L4	39.3 \pm 1.1	71.3 \pm 1.1
Re4	16.3 \pm 1.2	32.1 \pm 1.2

^a IC_{50} : the concentration that causes a 50% reduction of the cell growth

In general, the complexes **Re2-Re4** exhibited higher cytotoxicity than the respective ligands **L2-L4** in the MCF7 breast and PC3 prostate cell lines. On the contrary, in these cell lines **L1** is more cytotoxic than **Re1**. From all the studied compounds, the complexes **Re3** and **Re4** were shown to be the most potent, with IC_{50} values between 15.9 \pm 1.1 and 32.1 \pm 1.2 μM in both cell lines.

Taking advantage of the fluorescence emission characteristics of the benzothiazole unit of the newly synthesized compounds, the cellular uptake of the most cytotoxic Re complex, **Re3**, was assessed by fluorescence microscopy in MCF7 and PC3 human cancer cells. For comparative purposes, the studies were also performed for the respective ligand **L3**. Compounds **L3** and **Re3** were detected by the emission of blue fluorescence and the nucleus stained with propidium iodide (red emission).

As seen in Figure 4, both complexes **Re3** and **L3** (in a lesser extend) were detected in the cytoplasm of both cell lines. No detectable fluorescence in the blue channel was observed for the DMSO control, confirming that the detected blue emission is due from the compounds **L3** and **Re3**, and not to unspecific signal.

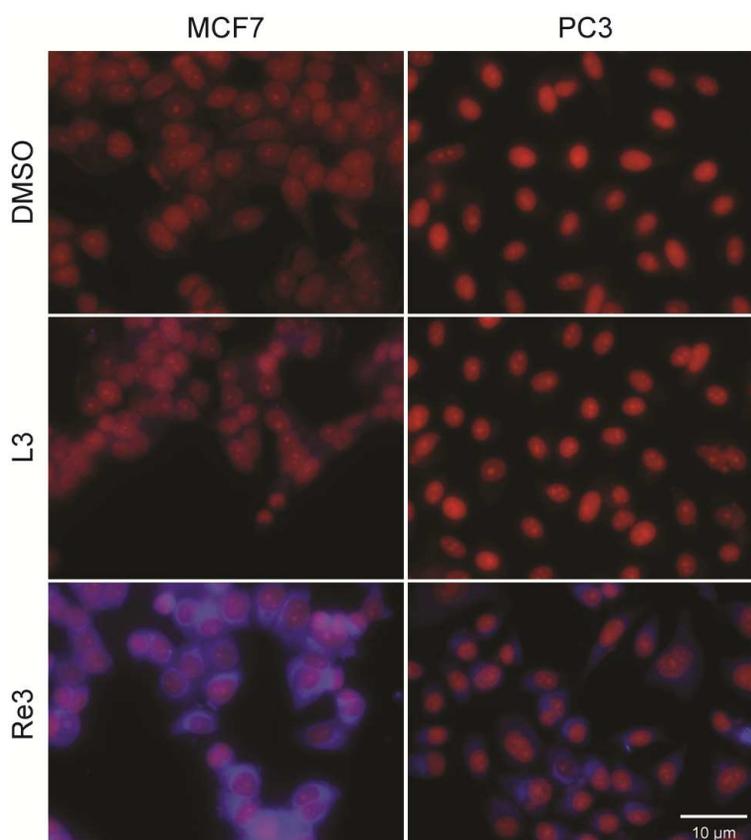


Figure 4. Fluorescence microscopy evaluation of the uptake of **L3** and **Re3** (blue) in human MCF7 and PC3 cells. Nuclei (red) were stained with propidium iodide. Scale bar, 10μm.

The cell entrance/uptake seems to be reduced for **L3** when compared with **Re3**, as less fluorescence was detected from cells incubated with **L3** if compared with those incubated with **Re3**. It is important to mention that in Figure 4 all the images were collected using the same conditions, in particular the acquisition time. A second set of images was collected with longer acquisition time to further evaluate the cellular uptake and localization of **L3** (Figure 5), showing a intracellular cytoplasmic accumulation.

Additionally, the fluorescence spectra obtained for **L3** and **Re3** (at the same concentration), have shown no detectable differences in the maximum wavelength of excitation and emission (Figure 5). Moreover, these experiments have shown that **L3** is a slightly more efficient fluorophore than **Re3**. Hence, the more intense fluorescence detected in the cells incubated with **Re3** are not due to its intrinsic fluorescence properties. Altogether, these data indicate that **Re3** is able to enter into cancer cell lines more easily than the corresponding ligand **L3**. Eventually, this might explain the highest cytotoxicity that has been found for **Re3** in the MCF7 and PC3 cancer cell lines, in comparison with the respective ligand.

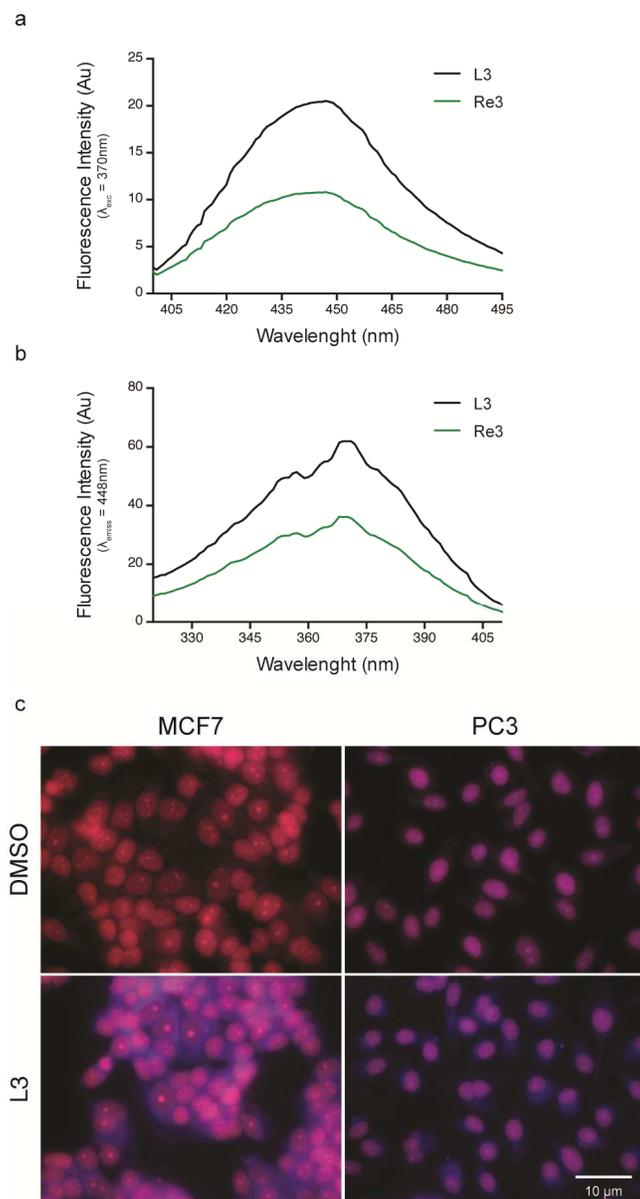


Figure 5. Emission and excitation spectra of **L3** (a) and **Re3** (b). Fluorescence microscopy evaluation of human MCF7 and PC3 cancer cells uptake of L3 (blue). Nuclei (red) were stained with propidium iodide. Note: images were collected with higher acquisition times than those presented in Fig. 4. Scale bar, 10μm.

Biological evaluation of ^{99m}Tc complexes: cellular uptake and biodistribution studies

The microscopy studies performed for **Re3** have confirmed that this complex can reach the cytoplasm of MCF7 breast and PC3 prostate human cancer cells. The isostructural ^{99m}Tc complex, **Tc3**, was used to quantify the cellular uptake in the same cell lines. These studies were extended to

the other radioactive complexes (**Tc1**, **Tc2** and **Tc4**) aiming to correlate their cellular uptake with the cytotoxicity exhibited by the corresponding Re complexes. The breast and prostate cancer cell lines were treated with **Tc1-Tc4** for different times (0 to 18h) and the results show that complexes **Tc1-Tc4** display a high cellular uptake in both cell lines, in a time-dependent manner, with values at 18 h in the range of 14-29% and 23-48% for MCF7 and PC3 cells, respectively (Figure 6). The highest uptakes were observed in the PC3 cells, in particular for the complexes **Tc3** and **Tc4** with values of 38.6% and 31.5% at 5 h and values of 47.1% and 47.9% at 18 h, respectively. These results demonstrate that the complexes **Tc3** and **Tc4** display the highest cellular uptake in the MCF7 and PC3 cells, accumulating in both cell lines over time. This trend seems to be in agreement with the highest cytotoxicity of the Re congeners (**Re3** and **Re4**), particularly in the MCF7 breast cancer cell line.

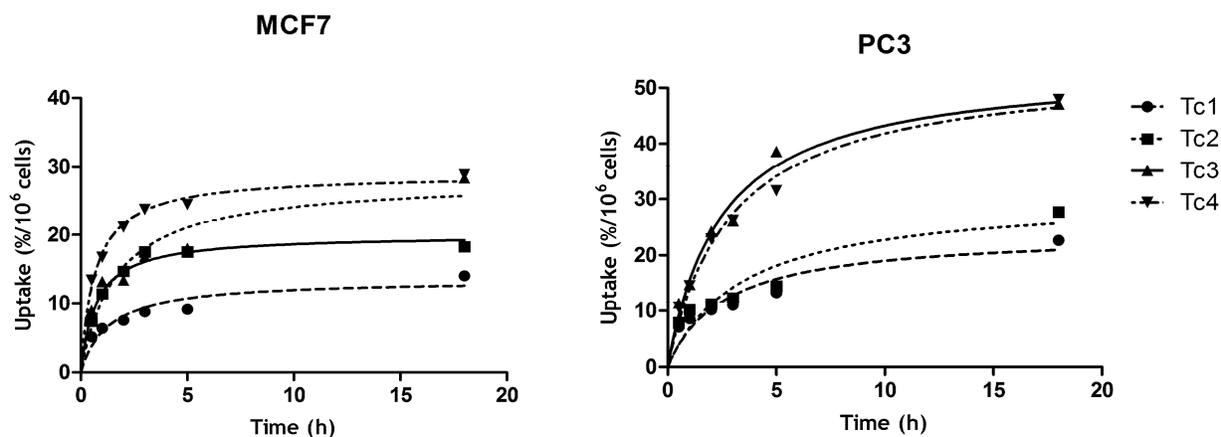


Figure 6. Cellular uptake of **Tc1-Tc4** in human MCF7 breast and PC3 prostate cells, expressed as percentage of total radioactivity per million cells.

As discussed before, all complexes are lipophilic with $\log D_{o/w}$ values within the relatively narrow range of 1.98-2.32. Therefore, the lipophilicity is not expected to be the major cause of the large differences observed between the cellular uptake of **Tc1-Tc4**. This is clearly corroborated by the fact that **Tc1** ($\log P = 1.98 \pm 0.02$) and **Tc3** ($\log P = 1.95 \pm 0.02$), having almost equal $\log P$ values,

present a large difference on the cellular uptake values, which for **Tc3** is as high as 30.9% of the applied activity/million cells in the PC-3 cell line versus 13.3% for **Tc1** at 5 h.

The most striking structural differences in complexes **Tc1-Tc4** are related with the linkers used to attach the 2-arylbenzothiazole pharmacophore to the 3-((2-mercaptoethyl)amino)propanoic acid coordinating unit. A shorter ethylenic linker has been used in complexes **Tc1** and **Tc2**, which have shown a much lower cellular uptake than **Tc3** and **Tc4**, containing a longer 2-n-butoxyethyl linker. The use of a longer linker is expected to minimize the interference of the organometallic fragment in molecular interactions involving the pharmacophore and biologically relevant targets. It is worthwhile mentioning that many benzothiazole derivatives, like the parent compound 2-(4'-aminophenyl)-benzothiazole and its derivatives bearing 3' substituents, undergo a selective uptake and biotransformation only in sensitive cancer cell lines, such as MCF-7 breast cancer cells. In these sensitive cell lines, the compounds bind to the aryl hydrocarbon receptor (AhR) with AhR translocation to the nucleus and subsequent induction of the expression and activation of the cytochrome P450 isoform CYP1A1. In contrast, insensitive cells like the prostate carcinoma PC3 cells do not show AhR translocation and CYP1A1 activation upon treatment with the same benzothiazole derivatives.^{12,13,24} We did not fully investigate the mechanisms involved in the cellular uptake of **Tc1-Tc4** and in the cytotoxic activity of the congener **Re1-Re4**, but the higher uptake observed for the ^{99m}Tc complexes in the PC3 cell line in comparison with the MCF7 cell line and the similar cytotoxic activity of the Re complexes in both cell lines, indicates that the interaction with AhR is most likely not responsible for the biological activity exhibited.

Finally, biodistribution studies of complexes **Tc1-Tc4** were performed in CD-1 mice to assess their pharmacokinetic profile and *in vivo* stability. The biodistribution data obtained for **Tc1-Tc4** at 2 min and 1 h post intravenous injection (p.i.), expressed in percent of injected dose per gram of tissue (%ID/g), are presented in Table 3.

Table 3. Biodistribution data (%ID/g) of **Tc1-Tc4** at 2 and 60 min post-injection (p.i.) in CD-1 mice.

Organ	% ID/g \pm SD							
	Tc1		Tc2		Tc3		Tc4	
	2 min	60 min						
Blood	6.40 \pm 1.10	0.58 \pm 0.11	7.00 \pm 0.50	1.00 \pm 0.09	8.00 \pm 2.40	0.70 \pm 0.20	5.20 \pm 0.90	0.84 \pm 0.05
Liver	43.3 \pm 1.90	37.5 \pm 1.60	19.9 \pm 2.60	15.2 \pm 2.40	35.8 \pm 1.60	11.3 \pm 1.20	35.8 \pm 4.40	29.5 \pm 0.60
Intestine	3.10 \pm 0.40	19.5 \pm 1.70	1.40 \pm 0.40	11.5 \pm 3.20	3.90 \pm 0.50	10.3 \pm 1.20	2.40 \pm 0.30	17.2 \pm 0.50
Spleen	3.90 \pm 0.20	0.50 \pm 0.20	3.70 \pm 0.03	0.78 \pm 0.01	2.90 \pm 0.60	0.26 \pm 0.03	5.20 \pm 0.90	0.70 \pm 0.10
Heart	5.40 \pm 0.60	0.90 \pm 0.30	3.80 \pm 0.70	0.90 \pm 0.10	3.70 \pm 0.80	0.42 \pm 0.08	4.40 \pm 0.20	1.10 \pm 0.20
Lung	7.00 \pm 1.00	1.10 \pm 0.20	13.9 \pm 0.70	3.00 \pm 0.80	3.50 \pm 1.00	0.70 \pm 0.20	9.00 \pm 1.00	3.70 \pm 0.60
Kidney	21.6 \pm 2.90	2.70 \pm 0.20	11.8 \pm 4.20	1.90 \pm 0.80	14.9 \pm 2.20	2.00 \pm 0.50	13.2 \pm 0.70	2.10 \pm 0.40
Muscle	1.20 \pm 0.20	0.60 \pm 0.10	0.90 \pm 0.30	0.55 \pm 0.08	0.80 \pm 0.30	0.29 \pm 0.07	1.40 \pm 0.10	0.67 \pm 0.06
Skeletal	1.50 \pm 0.20	0.46 \pm 0.09	1.30 \pm 0.30	0.45 \pm 0.06	1.40 \pm 0.20	0.19 \pm 0.03	1.96 \pm 0.07	0.54 \pm 0.04
Stomach	1.20 \pm 0.40	2.60 \pm 0.70	0.69 \pm 0.10	1.30 \pm 0.30	0.90 \pm 0.60	1.50 \pm 0.50	1.80 \pm 0.50	3.60 \pm 0.70
Brain	0.30 \pm 0.08	0.03 \pm 0.01	0.19 \pm 0.04	0.04 \pm 0.00	0.20 \pm 0.04	0.02 \pm 0.01	0.40 \pm 0.10	0.05 \pm 0.01
Excretion (% IA)		2.70 \pm 0.50		3.60 \pm 2.40		18.4 \pm 1.70		12.4 \pm 3.40

All complexes show a relatively fast blood clearance but a rather low rate of excretion that varied between 2.70 and 18.4 % I.A. At 1 h p.i., most of the activity is retained in the liver and intestine due to the predominantly hepatobiliary excretion of these moderately lipophilic compounds. Despite their neutrality and lipophilicity, **Tc1-Tc4** showed a negligible brain uptake (< 0.40 % ID/g) confirming their inability to cross the blood-brain barrier in mice.

The *in vivo* stability of **Tc1-Tc4** has been assessed by HPLC analysis of the urine and plasma of mice injected with these complexes. At 2 min p.i., most of the radioactivity detected in the plasma corresponds to the intact complexes (Figure 7). However, the complexes with an ether-containing longer linker, **Tc3** and **Tc4**, have a higher tendency to undergo metabolic transformations, as indicated by the HPLC chromatograms obtained for the plasma at 1 h p.i. As exemplified for **Tc4** in Figure 7, a significant part of the circulating activity still corresponds to the intact complex but two metabolites were detected at retention times of 17.2 and 18.7 min, respectively. These metabolites are also the major radiochemical species that are present in the HPLC chromatograms of the urine of mice injected with **Tc4**, at 1h p.i. (data not shown). We did not identify these metabolites but their

formation might be related with transformations involving the ether function from the aliphatic linker used to couple the benzothiazole pharmacophore with the (N,S,O) chelator framework in complexes **Tc3** and **Tc4**. For instance, the ether function can be involved in O-dealkylation processes, followed by oxidation of the resulting alcohol functions, as we have recently showed for $^{99m}\text{Tc(I)}$ tricarbonyl complexes with ether-containing pyrazolyl derivatives.²⁵ The formation of these metabolites *in vivo* can certainly justify that complexes **Tc3** and **Tc4** display a faster excretion rate than **Tc1** and **Tc2**.

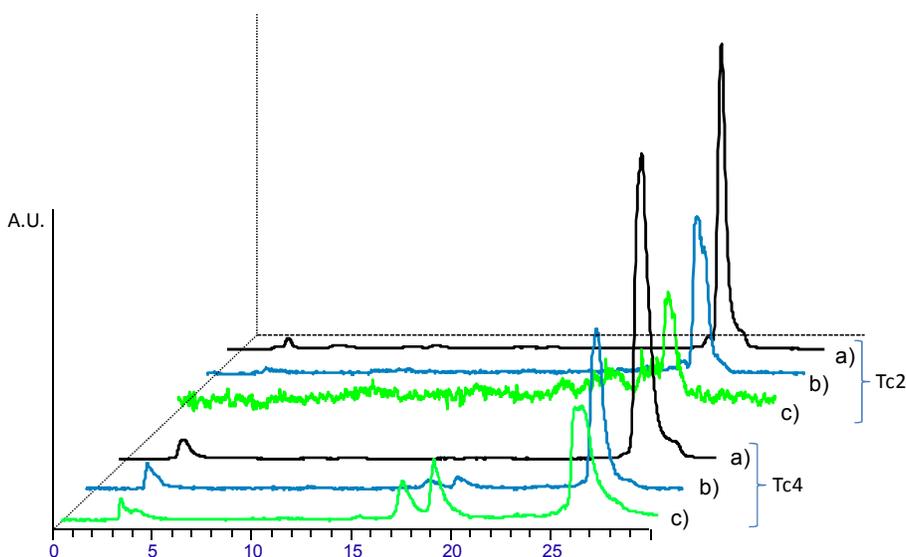


Figure 7. *In vivo* stability studies of complexes **Tc2** and **Tc4**. HPLC chromatograms of : (a) injected preparation; (b) plasma collected at 2 min p.i.; (c) plasma collected at 1 h p.i..

Conclusions

The functionalization of the 3-((2-mercaptoethyl)amino)propanoic acid coordinating unit with the 2-(4'-aminophenyl)benzothiazole pharmacophore, using two different aliphatic spacers containing or not an ether function, led to a new family of benzothiazole-containing chelators: **L1-L4**. These chelators were successfully applied in the synthesis of M(I) (M = Re, ^{99m}Tc) tricarbonyl complexes (**Re1-Re4** and **Tc1-Tc4**), which were biologically evaluated as isostructural tools for cancer theranostics.

The Re complexes have shown a moderate cytotoxic activity against PC3 and MCF7 human tumor cells (IC_{50} values $< 50 \mu\text{M}$ after 72 h of incubation), with the exception of **Re1** that showed relatively high IC_{50} values ($> 50 \mu\text{M}$). In general, the Re complexes are more cytotoxic than the corresponding ligands, which is most probably due to the better ability of the complexes to enter into the cells, as confirmed by fluorescence microscopy studies in the case of **Re3/L3**.

There is a clear influence of the linker on the cytotoxicity of the complexes, being those that display the ether-containing linker, i.e. **Re3** and **Re4** the most active. The cytotoxicity activity of the Re complexes is well correlated with the cellular uptake, which has been quantified using the $^{99\text{m}}\text{Tc}$ congeners, **Tc1-Tc4**. Interestingly, the nature of the linker also strongly influences the *in vivo* behavior of the complexes, as **Tc3** and **Tc4** are more prone to undergo metabolic transformations and display a faster excretion rate in mice.

In summary, the moderate cytotoxicity exhibited by the Re complexes, together with the remarkably high cellular uptake and the reasonably good bioavailability presented by the $^{99\text{m}}\text{Tc}$ congeners, show that the benzothiazole-containing $\text{Re(I)}/^{99\text{m}}\text{Tc(I)}$ tricarbonyl complexes here described hold promise to be further applied in the design of new tools for cancer theranostics. The marked influence of the linker on the biological fate of the complexes and its easy structural modification, as well as the different possibilities available to attach the 2-(4'-aminophenyl)benzothiazole pharmacophore (e.g. N-alkylation *vs* amide-forming reactions) to the cysteamine based (N,S,O)-chelator, are expected to help on the search for better performing compounds and on the elucidation of structure-activity relationships (SAR).

Experimental Section

General information

All chemicals and solvents were of reagent grade and were used without purifications unless stated otherwise. 2-Amino-5-methylbenzenethiol (**2**) was prepared as described in the literature.²⁶ *fac*-[Re(H₂O)₃(CO)₃]Br was synthesized according to literature.²⁷ Na[^{99m}TcO₄] was eluted from a commercial ⁹⁹Mo/^{99m}Tc generator, using 0.9% saline solution. The *fac*-[^{99m}Tc(H₂O)₃(CO)₃] precursor was obtained by labelling of a Isolink®-kit with Na[^{99m}TcO₄], following the procedure described elsewhere.²⁰ The NMR spectra were recorded on a Varian Unity 300 NMR spectrometer at the frequencies of 300 MHz (¹H) and 75 MHz (¹³C). Chemical shifts are reported in parts per million. ¹H and ¹³C chemical shifts were referenced with the residual solvent resonances relative to tetramethylsilane. FTIR spectra were recorded using KBr pellets on a Bruker, Tensor 27 spectrometer. Electrospray ionisation mass spectrometry (ESI-MS) was performed on a QITMS instrument in positive and negative ionization mode. Thin-layer chromatography (TLC) was performed on precoated silica plates 60 F₂₅₄ (Merck). Visualization of the plates was carried out using UV light (254 and 365 nm) and/or iodine chamber. Gravity column chromatography was carried out on silica gel (Merck, 70-230 mesh). HPLC analysis of the Re and ^{99m}Tc complexes was performed on a PerkinElmer LC pump coupled to an LC tuneable UV/Vis detector and to a Berthold LB-507A radiometric detector, using an analytical C18 reversed-phase column (Nucleosil 100-10, 250 x 3 mm) with a flow rate of 1 ml/min. UV detection, 254 nm. Eluents: A - aqueous 0.1% CF₃COOH solution, B - MeOH. The HPLC analysis was done with gradient elution, using the following method: 0-8min., 100% A; 8-8.1min., 100%-75% A; 8.1-14min., 75% A; 14-14.1min., 75%-66% A; 14.1-25min., 66%-0% A; 25-30min., 0% A; 30-30.1min., 0-100% A; 30.1-35min., 100% A.

Synthesis of the ligands

2-[N-methyl-N-(2'-hydroxyethyl)-4'-aminophenyl]-benzothiazole (3) – A solution of *o*-aminothiophenol (557 mg, 4.5 mmol) and *N*-methyl-*N*-(2-hydroxyethyl)-4-aminobenzaldehyde (660 mg, 3.7 mmol) in pyridine (6 mL) was refluxed for 3 hrs. After cooling at room temperature the intense yellow suspension was neutralized with a 3M HCl solution and the solvent was concentrated. The reaction crude was taken in water (50 mL) and extracted with CH₂Cl₂ (3 x 30 ml). The organic phase was dried over Na₂SO₄, filtered and evaporated. The residue was submitted to column chromatographic on silica-gel (CH₂Cl₂:MeOH, 98:2) to give **3** (510 mg, 49%); *R*_f = 0.25 (*n*-hexane/AcOEt 1:1); ¹H NMR (CDCl₃, 300MHz): δ 1.78 (bs, OH, 1H), 3.07 (s, 3H), 3.58 (t, 2H, *J* = 5.7 Hz), 3.86 (t, 2H, *J* = 5.7 Hz), 6.79 (d, 2H, ³*J* = 8.7 Hz), 7.29 (dd, 1H, ³*J* = 8.1 Hz, ³*J* = 8.1 Hz), 7.42 (dd, 1H, ³*J* = 8.1 Hz, ³*J* = 8.1 Hz), 7.82 (d, 2H, ³*J* = 8.1 Hz), 7.94 (d, 2H, ³*J* = 8.7 Hz), 7.98 (d, 2H, ³*J* = 8.1 Hz); ¹³C (CDCl₃, 75 MHz): δ 39.05, 54.67, 60.17, 111.98 (2C), 121.37 (2C), 122.15, 124.38, 126.14, 129.07 (2C); ESI/MS C₁₆H₁₆N₂OS (284) *m/z* 284.9 [M+H]⁺ (100%)

6-Methyl-2-[N-methyl-N-(2'-hydroxyethyl)-4'-aminophenyl]-benzothiazole (4) – Compound **4** was synthesized and purified as described above for compound **3**, starting from 4-methyl-*o*-aminothiophenol (320 mg, 2.30 mmol). Yield: 60% (329 mg, 1.38 mmol); *R*_f = 0.43 (petroleum ether/AcOEt 1:1); ¹H NMR (CDCl₃, 300 MHz): δ 2.45 (s, 3H), 3.06 (s, 3H), 3.52 (t, 2H, *J* = 5.7Hz), 3.85 (t, 2H, *J* = 5.7Hz), 6.78 (d, 2H, ³*J* = 9Hz), 7.23 (d, 1H, ³*J* = 8.1Hz), 7.61 (s, 1H), 7.85 (d, 1H, ³*J* = 8.1 Hz), 7.91 (d, 2H, ³*J* = 9Hz); ¹³C NMR (CDCl₃, 75 MHz): δ 21.5, 39.0, 54.7, 60.1, 111.9 (2C); 121.2, 121.6, 127.7, 128.9, 134.5, 151.5; 168.

2-[N-methyl-N-(2'-fluoroethyl)-4'-aminophenyl]-benzothiazole (5) - To a solution of **3** (0.51 g, 1.8 mmol) in dichloromethane (10 mL) was added carbon tetrabromide (0.65 g, 1.9 mmol) at 0°C followed by triphenylphosphine (0.517 g, 1.8 mmol). The reaction mixture was stirred for 3 h and, thereafter, the solvent concentrated. The residue was submitted to column chromatographic on silica

gel (EtOAc/*n*-hexane 1:6) to afford **5**. Yield: 56% (350 mg, 1.0 mmol) as a yellow oil; $R_f = 0.46$ (AcOEt/*n*-hexane 1: 2); $^1\text{H NMR}$ (CDCl_3 , 300 MHz): δ 3.09 (s, 3H), 3.48 (t, 2H, $J = 7.5$ Hz), 3.80 (t, 2H, $J = 7.5$ Hz), 6.73 (d, 2H, $^3J = 8.7$ Hz), 7.30 (dd, 1H, $^3J = 8.1$ Hz, $^3J = 8.1$ Hz), 7.43 (dd, 1H, $^3J = 8.1$ Hz, $^3J = 8.1$ Hz), 7.83 (d, 1H, $^3J = 8.1$ Hz), 7.96 (d, 2H, $^3J = 8.7$ Hz), 8.01 (d, 1H, $^3J = 8.1$ Hz); ^{13}C (CDCl_3 , 75 MHz): δ 27.89, 38.87, 54.07, 111.56 (2C), 121.38, 122.27, 124.41, 126.12, 129.17 (2C), 150.11; ESI/MS $\text{C}_{16}\text{H}_{15}\text{BrN}_2\text{S}$ (346) m/z 347.0 $[\text{M}+\text{H}]^+$ (97 %), 347.9 $[\text{M}+\text{H}]^+$ (20 %), 348.9 $[\text{M}+\text{H}]^+$ (100 %), 349.9 $[\text{M}+\text{H}]^+$ (20 %).

6-Methyl-2-[*N*-methyl-*N*-(2'-fluoroethyl)-4'-aminophenyl]-benzothiazole (6) – compound **6** was synthesized and purified as described above for **5**, starting from compound **4** (300 mg, 1.00 mmol). Yield: 64% (231 mg, 0.64 mmol); $R_f = 0.39$ (*n*-hexane/AcOEt 1:4); $^1\text{H NMR}$ (CDCl_3 , 300MHz): δ 2.46 (s, 3H), 3.09 (s, 3H), 3.48 (t, 2H, $J = 7.5$ Hz), 3.80 (t, 2H, $J = 7.5$ Hz), 6.73 (d, 2H, $^3J = 8.7$ Hz), 7.24 (d, 1H, $^3J = 8.2$ Hz), 7.62 (s, 1H), 7.87 (d, 1H, $^3J = 8.2$ Hz), 7.95 (d, 2H, $^3J = 8.7$ Hz); ^{13}C NMR (CDCl_3 , 75 MHz): δ 21.1, 27.7, 38.2, 53.4 (CH_2), 111.0 (2C), 120.8, 121.4, 121.7, 127.1, 128.4 (2C), 133.9, 134.2, 149.4, 151.9, 166.9; ESI/MS $\text{C}_{17}\text{H}_{17}\text{BrN}_2\text{S}$ (362) m/z 363.2.0 $[\text{M}+\text{H}]^+$.

2-[*N*-methyl-*N*-(2''-(2''-aminoethylthio)ethyl)-4'-aminophenyl]-benzothiazole (7) – To a solution of 2-aminoethanethiol hydrochloride (296 mg, 2.55 mmol) in anhydrous EtOH (8 mL) was added NaOH (163 g, 4 mmol) under a nitrogen atmosphere. After 1 hr, compound **5** (448 mg, 1.29 mmol) was added and the reaction mixture was stirred for more 20 hrs at room temperature. Then, the solvent was concentrated. The palid brown residue was taken in water (25 mL) and the pH was adjusted to 7 with a 1M HCl solution. The aqueous phase was extracted with CH_2Cl_2 (4 x 25 mL), dried over Na_2SO_4 , filtered and the filtrate was concentrated. The residue was submitted to column chromatographic on silica gel ($\text{CHCl}_3/\text{MeOH}$ 70:30) to afford the desired amine as an oil. Yield: 76% (335 mg, 0.98 mmol); $R_f = 0.64$ ($\text{CHCl}_3/\text{MeOH}$ 3:1); $^1\text{H NMR}$ (CDCl_3 , 300 MHz): δ 2.68 (m,

4H), 2.87 (t, 2H, $J = 6.9$ Hz), 3.04 (s, 3H), 3.59 (t, 2H, $J = 6.9$ Hz), 6.71 (d, 2H, $^3J = 8.7$ Hz), 7.28 (dd, 1H, $^3J = 7.8$ Hz, $^3J = 7.2$ Hz), 7.41 (dd, 1H, $^3J = 7.2$ Hz, $^3J = 7.8$ Hz), 7.82 (dd, 1H, $^3J = 7.8$ Hz), 7.93 (d, 2H, $^3J = 8.7$ Hz), 7.95 (d, 1H, $^3J = 7.8$ Hz); ^{13}C NMR (CDCl_3 , 75 MHz): δ 28.5, 36.4, 38.6, 41.1, 52.4, 111.4 (2C), 121.3, 121.5, 122.2, 124.2, 125.9, 128.9 (2C), 134.4, 150.3, 154.2, 168.5.

6-Methyl-2-[*N*-methyl-*N*-(2'-(2''-aminoethylthio)ethyl)-4'-aminophenyl]-benzothiazole (8) – compound **8** was synthesized and purified as described above for **7**, starting from compound **6** (200 mg, 0.55 mmol). Yield: 35% (68 mg, 0.19 mmol); $R_f = 0.48$ ($\text{CHCl}_3/\text{MeOH}$ 4:1); ^1H NMR (CDCl_3 , 300 MHz): δ 2.42 (s, 3H), 2.65 (t, 2H, $J = 7.5$ Hz), 2.88 (m, 2H), 2.93 (s, 3H), 3.14 (m, 2H), 3.50 (t, 2H, $J = 7.5$ Hz), 6.62 (d, 2H, $^3J = 9$ Hz), 7.19 (d, 1H, $^3J = 8.4$ Hz), 7.55 (s, 1H), 7.82 (m, 3H); ^{13}C NMR (CDCl_3 , 75 MHz): δ 21.4, 21.3, 28.5, 29.5, 38.5, 39.2, 52.1, 111.7 (2C), 121.2, 121.6, 127.6, 128.9 (2C), 134.4, 150.3, 152.1, 167.8, 176.7.

Ethyl 2-[2'-(2''-((4''-(benzo[*d*]thiazol-2-yl)phenyl)(methyl)amino)ethylthio)ethylamino]acetate (9) – To a solution of **7** (100 mg, 0.29 mmol), KI (1.2 mg, 7.3 μmol), and K_2CO_3 (20 mg, 0.15 mmol) in anhydrous CH_3CN (10 mL) was added ethyl 2-bromoacetate (8.2 μL , 0.145 mmol) under nitrogen atmosphere, and the reaction mixture was refluxed overnight. Hence the solvent was concentrated. The mixture was taken in water (30 ml) and extracted with CH_2Cl_2 (3 x 30 ml). The organic phase were dried with Na_2SO_4 , filtered, and the filtrate evaporated. The residue was submitted to column chromatographic on silica gel ($\text{CHCl}_3/\text{MeOH}$ 95:5) to provide **9**. Yield: 58%. (73 mg, 0.17 mmol) as a light yellow oil; $R_f = 0.55$ ($\text{CHCl}_3/\text{MeOH}$ 93:7); ^1H NMR (CDCl_3 , 300 MHz): δ 1.19 (t, 3H, $J = 7.2$ Hz), 2.68 (m, 4H), 2.77 (t, 2H, $J = 6.3$ Hz), 3.00 (s, 3H), 3.36 (s, 2H), 3.55 (t, 2H, $J = 6.2$ Hz), 4.12 (q, 2H, $J = 7.2$ Hz), 6.67 (d, 2H, $^3J = 9.0$ Hz), 7.23 (ddd, 1H, $^4J = 1.2$ Hz, $^3J = 8.1$ Hz, $^3J = 7.5$ Hz), 7.36 (ddd, 1H, $^2J = 1.2$ Hz, $^3J = 8.1$ Hz, $^3J = 8.1$ Hz), 7.77 (d, 1H, $^3J = 8.1$ Hz), 7.88 (d, 2H, $^3J = 9.0$ Hz), 7.90 (d, 1H, $^3J = 7.5$ Hz); ^{13}C NMR (CDCl_3 , 75 MHz): δ 14.0,

28.3, 32.4, 38.4, 47.9, 50.3, 52.2, 60.6, 111.3 (2C), 121.1, 121.3, 122.0, 124.0, 125.7, 128.8 (2C), 134.2, 150.2, 154.1, 168.4, 171.9 (C=O).

Ethyl

2-[2'-(2''-(4'''-(6''''-methylbenzo[d]thiazol-2-yl)phenyl)(methyl)amino)ethylthio)ethylamino] acetate (10) – compound **10** was synthesized and purified as described above for **9**, starting from compound **8** (55 mg, 0.15 mmol). Yield: 55% (37 mg, 0.08 mmol); $R_f = 0.48$ (CHCl₃/MeOH, 97:3); ¹H NMR (CDCl₃, 300 MHz): δ 1.24 (t, 3H, $J = 6.9$ Hz), 2.44 (s, 3H), 2.72 (m, 4H), 2.82 (t, 2H, $J = 5.7$ Hz), 3.03 (s, 3H), 3.40 (s, 2H), 3.59 (t, 2H, $J = 7.2$ Hz), 4.16 (q, 2H, $J = 6.9$ Hz), 6.70 (d, 2H, $^3J = 9.0$ Hz), 7.23 (d, 1H, $^3J = 8.4$ Hz), 7.60 (s, 1H), 7.83 (d, 1H, $^3J = 8.4$ Hz), 7.91 (d, 2H, $^3J = 9.0$ Hz); ¹³C NMR (CDCl₃, 75 MHz): δ 14.2, 21.4, 28.6, 32.6, 38.6, 48.2, 50.3, 52.5, 60.8, 111.5 (2C), 121.1, 121.8, 127.4, 128.8 (2C), 134.2, 134.6, 150.2, 152.4, 167.5, 172.1 (C=O).

2-[N-methyl-N-(2'-(4'-bromobutoxy)ethyl)-4'-aminophenyl]-benzothiazole (11) – To a suspension of NaH (525 mg, 13.2 mmol) in anhydrous THF (40 mL) was added **3** (2.0 g, 8.05 mmol) under a nitrogen atmosphere. After 30 min, 1,4-dibromobutane (5.6 mL, 47.3 mmol) was added and the solution was left on a vigorous stirring for 3 days at room temperature. Thereafter, the solvent was concentrated and the reaction residue submitted to column chromatographic on silica gel (*n*-hexane/AcOEt 1:1) to afford **11**. Yield: 45 % (1.518 g, 3.62 mmol); $R_f = 0.54$ (CHCl₃/MeOH 99:1); ¹H NMR (CDCl₃, 300 MHz): δ 1.67 (m, 2H), 1.89 (m, 2H), 3.04 (s, 3H), 3.41 (m, 4H), 3.58 (s, 4H), 6.68 (d, 2H, $^3J = 9.0$ Hz), 7.23 (dd, 1H, $^3J = 7.2$ Hz, $^3J = 7.8$ Hz), 7.37 (d, 1H, $^3J = 7.2$ Hz, $^3J = 8.1$ Hz), 7.77 (d, 1H, $^3J = 7.8$ Hz), 7.88 (d, 2H, $^3J = 9.0$ Hz), 7.92 (d, 1H, $^3J = 8.1$ Hz); ¹³C NMR (CDCl₃, 75 MHz): δ 28.2, 29.6, 33.7, 39.2, 52.2, 68.2, 70.4, 111.6 (2C), 121.4, 121.9, 124.5, 126.3, 129.2, 131.9 (2C), 151.4, 164.2.

6-Methyl-(2-(*N*-methyl-*N*-(2'-(4'-bromobutoxy)ethyl)-4'-aminophenyl))-benzothiazole (12) – compound **12** was synthesized and purified as described above for **11**, starting from compound **4** (1.0 g, 3.35 mmol). Yield: 40% (581 mg, 1.34 mmol); $R_f = 0.64$ (*n*-hexane:/AcOEt 1:1); ^1H NMR (CDCl_3 , 300 MHz): δ 1.64 (m, 2H), 1.86 (m, 2H), 2.42 (s, 3H), 3.00 (s, 3H), 3.37 (m, 4H), 3.53 (s, 4H), 6.69 (d, 2H, $^3J = 8.7$ Hz), 7.2 (d, 1H, $^3J = 8.2$ Hz), 7.57 (s, 1H), 7.82 (d, 1H, $^3J = 8.2$ Hz), 7.87 (d, 2H, $^3J = 8.7$ Hz); ^{13}C NMR (CDCl_3 , 75 MHz): δ 21.4, 28.1, 29.5, 33.7, 39.0, 52.0, 70.2, 111.4 (2C), 121.1, 121.3, 121.6, 127.3, 128.7 (2C), 134.1, 134.5, 150.8, 152.3, 167.6; ESI/MS $\text{C}_{21}\text{H}_{25}\text{BrN}_2\text{OS}$ (433) m/z 434.4 $[\text{M} + \text{H}]^+$.

2-[*N*-methyl-*N*-(2'-(4'-(2''-aminoethylthio)butoxy)ethyl)-4'-aminophenyl]-benzothiazole (13) – compound **13** was synthesized and purified as described above for **7**, starting from compound **11** (1.2 g, 2.86 mmol). Yield: 46 % (547 mg, 1.32 mmol); $R_f = 0.42$ (CHCl_3/MeO 4:1); ^1H NMR (CD_3OD , 300 MHz): δ 1.62 (m, 4H), 2.51 (m, 2H), 2.63 (t, 2H, $J = 6.6$ Hz), 2.9 (t, 2H, $J = 6.6$ Hz), 3.08 (s, 3H), 3.46 (m, 2H), 3.64 (s, 4H), 6.73 (d, 2H, $^3J = 9.0$ Hz), 7.28 (dd, 1H, $^3J = 7.5$ Hz, $^3J = 7.8$ Hz), 7.41 (dd, 1H, $^3J = 7.9$ Hz, $^3J = 7.5$ Hz), 7.82 (d, 1H, $^3J = 7.9$ Hz), 7.92 (d, 2H, $^3J = 9.0$ Hz), 7.96 (d, 1H, $^3J = 7.8$ Hz); ^{13}C NMR (CD_3OD , 75 MHz): δ 27.3, 29.8, 32.2, 39.3, 40.5, 52.9, 69.5, 71.7, 112.9 (2C), 122.6, 122.6, 122.7, 125.7, 127.4, 129.9 (2C), 135.3, 153.2, 155.1, 171.1.

6-Methyl-2-[*N*-methyl-*N*-(2'-(4'-(2''-aminoethylthio)butoxy)ethyl)-4'-aminophenyl]-benzothiazole (14) – compound **14** was synthesized and purified as described above for **7**, starting from compound **12** (900 mg, 2.08 mmol). Yield: 86 % (767 mg, 1.79 mmol); $R_f = 0.58$ ($\text{CHCl}_3/\text{MeOH}$ 3:1); ^1H NMR (CDCl_3 , 300 MHz): δ 1.56 (m, 4H), 2.39 (s, 3H), 2.44 (m, 2H), 2.59 (t, 2H, $J = 6.4$ Hz), 2.86 (t, 2H, $J = 6.4$ Hz), 2.97 (s, 3H), 3.35 (m, 2H), 3.51 (s, 4H), 3.76 (s, 2H), 6.67 (d, 2H, $^3J = 8.8$ Hz), 7.17 (d, 1H, $^3J = 8.4$ Hz), 7.54 (s, 1H), 7.80 (d, 1H, $^3J = 8.4$ Hz), 7.85 (d, 2H, $^3J = 8.8$ Hz); ^{13}C NMR (CDCl_3 , 75 MHz): δ 21.3, 26.1, 28.6, 31.4, 34.0, 38.8, 40.3, 52.0, 68.0,

70.6, 111.4 (2C), 121.0, 121.2, 121.5, 127.3, 128.6 (2C), 134.0; 134.4, 150.8, 152.3, 167.5; ESI/MS $C_{23}H_{31}N_3OS_2$ (429.2) m/z 430.3 $[M + H]^+$.

Ethyl 2-[4'-(benzo[d]thiazol-2-yl)phenyl]-5-oxa-10-thia-2,13-diazapentadecan-15-oate (15) – compound **15** was synthesized and purified as described above for **9**, starting from compound **13** (500 mg, 1.20 mmol). Yield: 66% (397 mg, 0.79 mmol); $R_f = 0.53$ ($CHCl_3/MeOH$ 95:5); 1H NMR ($CDCl_3$, 300 MHz): δ 1.23 (t, 3H, $J = 7.2$ Hz), 1.61 (m, 4H), 2.50 (m, 2H), 2.62 (t, 2H, $J = 6.2$ Hz), 2.77 (t, 2H, $J = 6.2$ Hz), 3.04 (s, 3H), 3.40 (m, 4H), 3.57 (s, 4H), 4.15 (q, 2H, $J = 7.2$ Hz), 6.72 (d, 2H, $^3J = 9.0$ Hz), 7.26 (dd, 1H, $^3J = 7.2$ Hz, $^3J = 7.8$ Hz), 7.40 (dd, 1H, $^3J = 7.2$ Hz, $^3J = 8.1$ Hz), 7.80 (dd, 1H, $^4J = 0.6$ Hz, $^3J = 7.8$ Hz), 7.90 (d, 2H, $^3J = 9.0$ Hz), 7.94 (d, 1H, $^3J = 8.1$ Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.9, 25.9, 28.5, 31.2, 31.4, 38.8, 47.4, 49.8, 51.8, 60.6, 67.8, 70.5, 111.2 (2C), 120.8, 121.1, 121.9, 123.9, 125.7, 128.6 (2C), 134.1, 150.8, 154.0, 168.5, 171.5 (C=O); ESI/MS $C_{26}H_{35}N_3O_3S_2$ (501.2) m/z 502.4 $[M + H]^+$.

Ethyl 2-[4'-((6'-methyl)benzo[d]thiazol-2-yl)phenyl]-5-oxa-10-thia-2,13-diazapentadecan-15-oate (16) – compound **16** was synthesized and purified as described above for **9**, starting from compound **14** (700 mg, 1.63 mmol). Yield: 36% (303 mg, 0.59 mmol); $R_f = 0.41$ ($CHCl_3/MeOH$ 97:3); 1H NMR ($CDCl_3$, 300MHz): δ 1.23 (t, 3H, $J = 7.2$ Hz), 1.60 (m, 4H), 2.42 (s, 3H), 2.49 (m, 2H), 2.61 (t, 2H, $J = 6.3$ Hz), 2.76 (t, 2H, $J = 6.3$ Hz), 3.02 (s, 3H), 3.39 (m, 4H), 3.55 (s, 4H), 4.14 (q, 2H, $J = 7.2$ Hz), 6.70 (d, 2H, $^3J = 9.0$ Hz), 7.20 (d, 1H, $^3J = 8.4$ Hz), 7.58 (s, 1H), 7.82 (d, 1H, $^3J = 8.1$ Hz), 7.88 (d, 2H, $^3J = 9.0$ Hz); ^{13}C NMR ($CDCl_3$, 300MHz): δ 13.2, 20.5, 25.2, 27.8, 30.2, 30.7, 38.1, 46.9, 48.8, 51.2, 60.3, 67.2, 69.8, 110.6 (2C), 120.2, 120.5, 120.8, 126.5, 127.8 (2C), 133.2, 133.7, 150.0, 151.5, 166.7; ESI/MS $C_{27}H_{37}N_3O_3S_2$ (515.2) m/z 516.3 $[M + H]^+$.

2-[(2-((2''-(4''-(benzo[d]thiazol-2-yl)phenyl)(methyl)amino)ethyl)thio)ethyl]amino) acetic acid (L1) – A solution of **9** (148 mg, 0.35 mmol) in THF (10 mL) and water (15 mL) was refluxed with NaOH (136 mg, 3.40 mmol) for 17 h. After cooling down, the reaction mixture was neutralized with 1M HCl, followed by extraction with CHCl₃ (2 x 25 mL). The organic phase was dried over Na₂SO₄, filtered and the filtrate concentrated. Recrystallization of the precipitate in methanol afforded **L1** as a white solid. Yield: 58% (80 mg, 0.20 mmol); *R*_f = 0.29 (CHCl₃/MeOH 3:1); ¹H NMR (DMSO, 300 MHz): δ 2.80 (m, 4H), 2.98 (t, 2H, *J* = 8.1 Hz), 3.03 (s, 3H), 3.21 (s, 2H), 3.62 (t, 2H, *J* = 8.1 Hz), 6.84 (d, 2H, ³*J* = 9.0 Hz), 7.39 (ddd, 1H, ⁴*J* = 1.2 Hz, ³*J* = 7.8 Hz, ³*J* = 7.5 Hz), 7.51 (ddd, 1H, ⁴*J* = 1.2 Hz, ³*J* = 7.5 Hz, ³*J* = 8.1 Hz), 7.89 (d, 2H, ³*J* = 9.0 Hz), 7.92 (d, 1H, ³*J* = 8.1 Hz), 8.04 (d, 1H, ³*J* = 7.8 Hz); ¹³C NMR (DMSO, 75 MHz): δ 25.7, 46.4, 49.6, 51.3, 56.1, 111.8 (2C), 120.3, 122.0, 124.5, 126.4, 128.7 (2C), 133.8, 150.7, 153.9, 167.8; ESI/MS C₂₀H₂₃N₃O₂S₂ (401.55) *m/z* 402.2 [M + H]⁺, 424.2 [M + Na]⁺; Anal. calcd. for C₂₀H₂₃N₃O₂S₂·1.4H₂O: C 56.38, H 6.09, N 9.86; found C 56.54, H 6.15, N 9.61.

2-[2''-(2''-(4''-(6''-methylbenzo[d]thiazol-2-yl)phenyl)(methyl)amino)ethylthio)ethyl]amino] acetic acid (L2) – **L2** was synthesized and purified as described above for **L1**, starting from compound **10** (30 mg, 0.067 mmol). Yield: 65% (18 mg, 0.04 mmol); ¹H NMR (CD₃OD, 300MHz): δ 2.46 (s, 3H), 3.09 (s, 2H), 2.85 (m, 4H), 3.42 (s, 3H), 3.69 (m, 4H), 6.84 (d, 2H, ³*J* = 9.0 Hz), 7.28 (d, 1H, ³*J* = 8.4 Hz), 7.70 (s, 1H), 7.76 (d, 1H, ³*J* = 8.4 Hz), 7.87 (d, 2H, ³*J* = 9.3 Hz); ¹³C NMR (CD₃OD, 75 MHz): δ 22.5, 25.3, 28.3, 29.2, 37.8, 67.7, 111.7 (2C), 121.0, 121.2, 127.7, 128.7 (2C), 134.9; ESI/MS C₂₁H₂₅N₃O₂S₂ (415.14) *m/z* 416.3 [M + H]⁺, 438.3 [M + Na]⁺; Anal. calcd. for C₂₁H₂₅N₃O₂S₂·1.6H₂O: C 56.75, H 6.12, N 9.46; found C 55.98, H 6.48, N 9.27.

[4-(benzo[d]thiazol-2-yl)phenyl]-5-oxa-10-thia-2,13-diazapentadecan-15-oic acid (L3) – **L3** was synthesized and purified as described above for **L1**, starting from compound **15** (350 mg, 0.70 mmol). Yield: 52% (172 mg, 0.36 mmol); ¹H NMR (CD₃OD, 300MHz): δ 1.63 (m, 4H), 2.55 (t, 2H,

$J = 7.2$ Hz), 2.75 (t, 2H, $J = 7.2$ Hz), 3.08 (s, 3H), 3.14 (t, 2H, $J = 6.9$ Hz), 3.47 (s or m?, 4H), 3.64 (s, 4H), 6.86 (d, 2H, $^3J = 7.2$ Hz), 7.34 (ddd, 1H, $^3J = 7.8$, 7.5 Hz, $^4J = 1.2$, 0.6 Hz), 7.46 (td, 1H, $^3J = 7.8$; 7.5 Hz, $^4J = 1.2$; 0.6 Hz), 7.89 (m, 4H); ^{13}C NMR (CD_3OD , 75 MHz): δ 27.1, 28.6, 29.8, 32.1, 39.3, 52.9, 66.9, 69.5, 71.7, 112.9 (2C), 121.6, 122.6, 122.7, 125.7, 127.4, 129.9 (2C), 135.3, 153.2, 155.1, 171.1 (C=O); ESI/MS $\text{C}_{24}\text{H}_{31}\text{N}_3\text{O}_3\text{S}_2$ (473.18) m/z 472.2 $[\text{M} - \text{H}]^+$; Anal. calcd. for $\text{C}_{24}\text{H}_{31}\text{N}_3\text{O}_3\text{S}_2 \cdot 2.2\text{H}_2\text{O}$: C 56.16, H 6.95, N 8.19; found C 56.63, H 7.09, N 8.02.

2-[4'-((6'-methyl)benzo[d]thiazol-2-yl)phenyl]-5-oxa-10-thia-2,13-diazapentadecan-15-oic acid (L4) – L4 was synthesized and purified as described above for L1, starting from compound 16 (250 mg, 0.48 mmol). Yield: 65% (154 mg, 0.32 mmol); ^1H NMR (CD_3OD , 300 MHz): δ 1.60 (m, 4H), 2.52 (t, 2H, $J = 7.2$ Hz), 2.74 (t, 2H, $J = 7.2$ Hz), 3.06 (s, 2H), 3.12 (t, 2H, $J = 7.2$ Hz), 3.45 (s, 3H), 3.62 (s, 4H), 6.80 (d, 2H, $^3J = 9.0$ Hz), 7.24 (d, 1H, $^3J = 8.4$ Hz), 7.68 (s, 1H), 7.72 (d, 1H, $^3J = 8.4$ Hz), 7.82 (d, 2H, $^3J = 9.0$ Hz); ^{13}C NMR (CD_3OD , 75 MHz): δ 27.5, 28.9, 30.1, 32.5, 39.7, 53.4, 67.3, 69.9, 72.1, 112 (2C), 123.0, 123.2, 129.8, 129.0 (2C), 135.2.2, 153.4, 155.3, 171.4 (C=O); ESI/MS $\text{C}_{25}\text{H}_{33}\text{N}_3\text{O}_3\text{S}_2$ (487.2) m/z 488.4 $[\text{M} + \text{H}]^+$, 510.4 $[\text{M} + \text{Na}]^+$. Anal. calcd. for $\text{C}_{25}\text{H}_{33}\text{N}_3\text{O}_3\text{S}_2 \cdot 1.5\text{H}_2\text{O}$: C 58.34, H 6.75, N 8.17; found C 57.24, H 6.88, N 8.24.

General procedure for the synthesis of the rhenium complexes (Re1-Re4)

fac- $[\text{Re}(\text{H}_2\text{O})_3(\text{CO})_3]\text{Br}$ was reacted with equimolar amounts of L1-L4 in refluxing methanol for 16 hrs. Thereafter, the solvent was removed under vacuum and the desired complexes were purified by successive washing with water, diethyl ether and n-hexane. The obtained yellow solids were further recrystallized from methanol to afford pure samples of Re1-Re4, as checked by HPLC analysis.

- **Re1** was obtained in 62 % yield (54 mg, 0.08 mmol); ^1H NMR ($\text{DMF-}d_7$, 300 MHz, T = 20 °C): 2.64 (m, 2H), 3.20 (s, 4H), 3.38 (m, 2H), 3.71 (m, 2H), 3.96 (m, 2H), 7.05 (d, 2H, $J = 9.0$ Hz), 7.40 (m, 1H), 7.52 (m, 1H), 7.98 (m, 3H), 8.10 (d, 1H, $J = 7.8$ Hz); ^{13}C NMR ($\text{DMF-}d_7$, 300 MHz, T = 20

°C): δ 39.1, 51.50, 53.32, 54.40, 55.43, 56.40, 113.09, 113.47, 122.93, 123.27, 125.64, 127.40, 129.95, 135.60, 152.30, 155.71, 169.13, 180.16, 193.72, 197.17; IR (KBr) ν : 3410, 2922, 1894, 1699, 1606, 1486, 1455, 1384, 1261, 1191, 1104, 817 cm^{-3} ; ESI/MS (+) $\text{C}_{23}\text{H}_{22}\text{N}_3\text{O}_5\text{S}_2\text{Re}$ (671.0) (m/z): 672.2 $[\text{M} + \text{H}]^+$ (100); Anal. calcd. for $\text{C}_{23}\text{H}_{22}\text{N}_3\text{O}_5\text{S}_2\text{Re}$: C 41.18, H 3.31, N 6.27; found C 41.07, H 3.47, N 6.19.

- **Re2** was obtained in 57% yield (32 mg, 0.05 mmol); ^1H NMR (DMF- d_7 , 300 MHz, T = 20 °C): δ 2.47 (m, 6H), 3.19 (s, 9H), 6.99 (s, 2H), 7.32 (s, 1H), 7.83 (m, 2H), 7.95 (m, 2H); ^{13}C NMR (DMF- d_7 , 300 MHz, T = 20 °C): δ 21.18, 32.33, 38.51, 38.66, 47.99, 50.39, 50.83, 52.65, 54.30, 54.95, 55.66, 55.96, 112.36, 112.72, 121.41, 122.09, 128.35, 129.17, 134.90, 135.24, 151.32, 152.88, 167.69, 181.17, 192.76, 196.56, 198.16; IR (KBr) ν : 3395, 2924, 2028, 1893, 1700, 1604, 1509, 1483, 1437, 1384, 1261, 1193, 1108, 817, 759, 698, 650 cm^{-3} ; ESI/MS (+) $\text{C}_{24}\text{H}_{24}\text{N}_3\text{O}_5\text{S}_2\text{Re}$ (685.07) (m/z) 686.3 $[\text{M} + \text{H}]^+$ (100); Anal. calcd. for $\text{C}_{24}\text{H}_{24}\text{N}_3\text{O}_5\text{S}_2\text{Re}$: C 42.09, H 3.53, N 6.14; found C 42.25, H 3.75, N 6.09.

- **Re3** was obtained in 76 % yield (78 mg, 0.11 mmol); ^1H NMR (DMF- d_7 , 300 MHz, T = 20 °C): δ 1.71 (m, 4H), 3.08 (s, 4H), 3.50 (s, 3H), 3.65 (m, 5H), 6.90 (d, 2H, $J = 8.4$ Hz), 7.39 (m, 1H), 7.50 (m, 1H), 7.95 (m, 3H); ^{13}C NMR (DMF- d_7 , 300 MHz, T = 20 °C): δ 25.01, 28.32, 37.10, 38.54, 51.53, 53.74, 55.76, 68.13, 70.10, 111.90, 120.18, 121.82, 121.97, 124.78, 126.56, 128.79, 134.10, 151.86, 154.14, 168.93, 181.51, 192.31, 196.12, 197.75; IR (KBr) ν : 4008, 2923, 2027, 1896, 1748, 1606, 1559, 1541, 1508, 1487, 1456, 1384, 1350, 1261, 1183, 1101, 816, 691, 651, 567, 516 cm^{-3} ; ESI/MS (+) $\text{C}_{27}\text{H}_{30}\text{N}_3\text{O}_6\text{S}_2\text{Re}$ (743.0) (m/z) 744.4 $[\text{M} + \text{H}]^+$, 766.4 $[\text{M} + \text{Na}]^+$; Anal. calcd. for $\text{C}_{27}\text{H}_{30}\text{N}_3\text{O}_6\text{S}_2\text{Re}$: C 43.65, H 4.07, N 5.66; found C 43.48, H 4.24, N 5.54.

- **Re4** was obtained in 81% yield (86 mg, 0.11 mmol); ^1H NMR (DMF- d_7 , 400 MHz, T = 20 °C): 1.74 (s, 2H), 1.83 (s, 2H), 2.47 (s, 3H), 3.12 (s, 5H), 3.41 (q, 2H, $J = 3.2$ Hz), 3.52 (s, 2H), 3.68 (s, 5H), 6.93 (d, 2H, $J = 7.4$ Hz), 7.33 (s, 1H), 7.85 (m, 2H), 7.94 (d, 2H, $J = 7.4$ Hz); ^{13}C NMR (DMF- d_7 , 400 MHz, T = 20 °C): δ 15.94, 21.68, 26.28, 35.02, 35.23, 37.86, 39.30, 39.59, 52.69, 54.80, 56.08, 66.38, 69.19, 71.13, 112.90, 121.80, 122.65, 128.79, 129.61, 135.52, 152.61, 153.55, 168.26, 171.92, 177.35, 180.44, 194.18, 197.49; IR (KBr) ν : 3490, 2920, 2028, 1895, 1653, 1606, 1558, 1506, 1457, 1384, 1261, 1100, 921, 688 cm^{-3} ; ESI/MS (+) $\text{C}_{28}\text{H}_{32}\text{N}_3\text{O}_6\text{S}_2\text{Re}$ (757.0) (m/z) 758.6 [$\text{M} + \text{H}$] $^+$ (100), 780.5 [$\text{M} + \text{Na}$] $^+$ (80); Anal. calcd. for $\text{C}_{28}\text{H}_{32}\text{N}_3\text{O}_6\text{S}_2\text{Re}$: C 44.43, H 4.26, N 5.55 ; found C 43.97, H 4.6, N 5.43.

General procedure for the synthesis of $^{99\text{m}}\text{Tc}$ complexes

In a nitrogen-purged glass vial, 120 μL of a 1.1×10^{-3} M solution of $\text{L}^1\text{--L}^4$ in propanediol-1,3 was added to 1.2 mL of a solution of the organometallic precursor $\text{fac-}[^{99\text{m}}\text{Tc}(\text{H}_2\text{O})_3(\text{CO})_3]^+$ (1–2 mCi) in saline at pH 7.4. The reaction mixtures were then heated to 100°C for 30 min, cooled to room temperature and analyzed by RP-HPLC, using the method described above. Following this procedure, all $^{99\text{m}}\text{Tc}$ complexes (**Tc1–Tc4**) were synthesized in radiochemical yields >95 % without need of further purification. Their chemical identity was ascertained by HPLC comparison with the Re congeners (see Table 1).

Determination of partition coefficients

The log $D_{\text{o/w}}$ values of complexes **Tc1–Tc2** were determined by the “shake flask” method.²⁸ A mixture of octanol (1 mL) and 0.1 M PBS pH = 7.4 (1mL) was stirred vigorously, followed by the addition of 25 μL of the aqueous solutions of each complex. The mixtures were vortexed and centrifuged (3000 rpm, 10 min, RT) to allow phase separation. Aliquots of 25 μL of the octanol and PBS phases were counted in a gamma counter. The partition coefficient ($P_{\text{o/w}}$) was calculated by

dividing the number of counts of the octanol phase by those from the PBS phase, and the results expressed as $\log D_{o/w}$.

Cell Culture

The human MCF7 breast and PC3 prostate cells (American Type Culture Collection, ATCC) were grown respectively in DMEM and RPMI media, supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin (all from Invitrogen). Cells were cultured at 37 °C in a 5% CO₂ incubator (Heraus, Germany) in a humidified atmosphere, with the medium changed every other day.

Cytotoxicity assays

Cell viability was evaluated by using a colorimetric method based on the tetrazolium salt MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]), which is reduced by living cells to yield purple formazan crystals. Cells were seeded in 96-well plates at a density of 1 - 1.5 x 10⁴ cells per well in 200 µL of culture medium and incubated overnight. After careful removal of the medium, 200 µL of a dilution series of the compounds in fresh medium were added and incubation was performed at 37°C / 5% CO₂ for 72 h. The percentage of DMSO in cell culture medium did not exceed 1%. At the end of the incubation period, the compounds were removed and the cells were incubated with 200 µL of MTT solution (500 µg ml⁻¹). After 3 h, the medium was removed and the purple formazan crystals were dissolved in 200 µL of DMSO by shaking. The cell viability was evaluated by the measurement of the absorbance at 570 nm using a plate spectrophotometer (Power Wave Xs, Bio-Tek). The cell viability was calculated by the ratio between the absorbance of each well and that of the control wells (cells treated with medium containing 1% DMSO). Each experiment was repeated at least three times and each point was determined in at least six replicates. Data were analyzed with GraphPad Prism software.

Fluorescence spectra and fluorescence microscopy analysis

The excitation and emission wavelength scan of **L3** and **Re3** (25 μM in PBS) were recorded in a plate reader (Tecan Infinite 200, Mannendorf, Switzerland). The maximum excitation and emission wavelengths were determined according to the maximum fluorescence intensity level.

MCF7 and PC3 cells, cultured in 35 mm imaging dishes (Ibidi), were treated with vehicle (DMSO) or with the ligands **L3** or the complex **Re3** (100 μM), for 24 h. Cells washed with PBS were fixed and permeabilized in 100% ice-cold methanol (-20°C , 5 min). Cells, washed with PBS 3 times, were incubated with 20 $\mu\text{g}/\text{ml}$ Propidium Iodide (nuclear staining) for 4 min at RT. Cells were washed with PBS 3 times and were kept at 4°C until analysis. Samples were imaged on a widefield fluorescence microscope Zeiss Axiovert 200M (Carl Zeiss MicroImaging) using a 63x Plan-Apochromat using the Red ($\lambda_{\text{ex}} = 540\text{-}552\text{ nm}$, $\lambda_{\text{em}} = 575\text{-}640\text{ nm}$) and Blue ($\lambda_{\text{ex}} = 359\text{-}371\text{ nm}$, $\lambda_{\text{em}} > 397\text{ nm}$) filter sets. Images were acquired using cooled CCD camera (Roper Scientific Coolsnap HQ CCD) using Zeiss Methamorph software. All images were processed using ImageJ – Image Processing and Analysis in Java.²⁹

Cellular uptake

Cellular uptake assays of the $^{99\text{m}}\text{Tc}$ complexes (**Tc1–Tc4**) were performed using PC-3 and MCF-7 cells seeded at a density of 4×10^4 cells in 500 μL medium per well in 24-well plates and allowed to attach overnight. After that period, the medium was removed and cells were treated with fresh medium containing approximately 25 kBq/mL of each $^{99\text{m}}\text{Tc}$ complex and incubated under humidified 5% CO_2 atmosphere, at 37°C for a period of 30 min to 18 h. Cells were washed twice with cold PBS, lysed with 0.1 M NaOH and the cellular extracts counted for radioactivity. Each experiment was performed in duplicate with each point determined in at least four replicates. Cellular uptake data were expressed as an average plus the standard deviation of % of total per million of cells.

Biodistribution studies

All animal experiments were performed in compliance with Portuguese regulations for animal treatment. The animals were housed in a temperature- and humidity-controlled room with a 12h light/12h dark schedule. Biodistribution of complexes **Tc1–Tc4** was evaluated in CD-1 mice (randomly bred, obtained from IFFA, CREDO, Spain) weighing approximately 20–25 g. Animals were intravenously injected in the tail vein with the test complex (1.8–7.8 MBq) diluted in 100 μ L of NaCl 0.9%. Mice were sacrificed by cervical dislocation at 2 min and 1 h after injection. The administered dose and the radioactivity in the sacrificed animals was measured using a dose calibrator (Curiemeter IGC-3, Aloka, Tokyo, Japan). The difference between the radioactivity in the injected and sacrificed animals was assumed to be due to excretion. Tissues of interest were dissected, rinsed to remove excess blood, weighed, and their radioactivity was measured using a γ -counter (LB2111, Berthold, Germany). The uptake in the tissues was calculated and expressed as a percentage of the injected radioactivity dose per gram of tissue.

***In vivo* stability studies**

The *in vivo* stability of **Tc1 - Tc4** was evaluated by urine and blood serum HPLC analysis, using the elution conditions above described for the analysis of these ^{99m}Tc complexes. The urine was collected at sacrifice time and centrifuged for 5 min at 1500 g before RP-HPLC analysis. Blood collected from mice was also centrifuged for 10 min at 1000 g at 4 °C, and the supernatant (serum) was collected. Aliquots of 100 μ L of serum were treated with 200 μ L of cold ethanol for protein precipitation. Samples were centrifuged at 1500 g for more 10 min, at 4 °C. The remaining supernatant was separated and injected through a RP-HPLC column (Nucleosil 100-10, 250 x 3 mm) for analysis.

Acknowledgements

This work was funded by the Fundação para a Ciência e Tecnologia (FCT), Portugal (EXCL/QEQ-MED/0233/2012). The research was carried out within the framework of the European Cooperation COST Action CM1105. FCT is also acknowledged for “Ciência 2008” program (GRM), FCT Investigator Grant (FM) and grants SFRH/BPD/80758/2011 (EP), SFRH/BD/47308/2008 (FS) and SFRH/BPD/64702/2009 (HVM). TFO is supported by the DFG Center for Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB). The authors thank Dr Joaquim Marçalo and Dr Célia Fernandes for Mass Spectroscopy analysis, which were run on a QITMS instrument, acquired with the support of the Programa Nacional de Reequipamento Científico (Contract REDE/1503/REM/2005-ITN) of FCT and is part of RNEM-Rede Nacional de Espectrometria de Massa.

References

1. I. Hutchinson, M-S. Chua, H. L. Browne, V. Trapani, T. D. Bradshaw, A. D. Westwell and M. F. G. Stevens, *J. Med. Chem.*, 2001, 44, 1446.
2. X. Peng, G. Xie, Z. Wang, H. Lin, T. Zhou, P. Xiang, Y. Jiang, S. Yang, Y. Wei, L. Yu and Y. Zhao, *Cell Death and Disease*, 2014, 5, e1143.
3. G. R. Morais, A. Paulo and I. Santos, *Eur. J. Org. Chem.*, 2012, 1279.
4. W. E. Klunk, H. Engler, A. Nordberg, Y. Wang, G. Blomqvist, D. P. Holt, M. Bergstrom, I. Savitcheva, G. F. Huang, S. Estrada, B. Ausen, M. L. Debnath, J. Barletta, J. C. Price, J. Sandell, B. J. Lopresti, A. Wall, P. Koivisto, G. Antoni, C. A. Mathis and B. Langstrom, *Annals of Neurology* 2004, 55, 306.
5. H. Engler, A. Forsberg, O. Almkvist, G. Blomqvist, E. Larsson, I. Savitcheva, A. Wall, A. Ringheim, B. Langstrom, A. Nordberg, *Brain*, 2006, 129, 2856.

6. S. M. Landau, B. A. Thomas, L. Thurffjell, M. Schmidt, R. Margolin, M. Mintun, M. Pontecorvo, S. L. Baker, W. Jagust, *Eur. J. Nucl. Med.*, 2014, **41**, 1398.
7. S. Iikuni, M. Ono, H. Watanabe, K. Matsumura, M. Yoshimura, N. Harada, H. Kimura, M. Nakayama and H. Saji, *Mol. Pharmaceutics*, 2014, **11**, 1132.
8. J. Jia, M. Cui, J. Dai, X. Wang, Yu-S. Ding, H. Jia and B. Liu, *Med. Chem. Commun.*, 2014, **5**, 153.
9. A. F. Martins, J. F. Morfin, A. Kubickova, V. Kubicek, F. Buron, F. Suzenet, M. Salerno, A. N. Lazar, C. Duyckaerts, N. Arlicot, D. Guilloteau, ; C. F. G. C Geraldes, E. Toth, *ACS Med. Chem. Lett.*, 2013, **4**, 436.
10. T. D. Bradshaw and A. D. Westwell, *Curr. Med. Chem.* 2004, **11**, 1241.
11. C. G. Mortimer, G. Wells, J.-P. Crochard, E. L. Stone, T. D. Bradshaw, M. F. G. Stevens and A. D. Westwell, *J. Med. Chem.*, 2006, **49**, 179.
12. A. I. Loaiza-Pérez, V. Trapani, C. Hose, S. S. Singh, J. B. Trepel, M. F. G. Stevens, T. D. Bradshaw and E. A. Sausville, *Mol. Pharmacol.*, 2002, **61**, 13.
13. V. Trapani, V. Patel, C-O. Leong, H. P. Ciolino, G. C. Yeh, C. Hose, J. B. Trepel, M. F. G. Stevens, E. A. Sausville and A. I. Loaiza-Pérez, *Br. J. Cancer*, 2003, **88**, 599-605.
14. E. W. Price and C. Orvig, *Chem. Soc. Rev.*, 2014, **43**, 260.
15. C-T. Yang and K-H. Chuang, *Med. Chem. Commun.*, 2012, **3**, 552-565.
16. S. Tzanopoulou, I. C. Pirmettis, G. Patsis, M. Paravatou-Petsotas, E. Livaniou, M. Papadopoulos and M. Pelecanou, *J. Med. Chem.*, 2006, **49**, 5408.
17. S. Tzanopoulou, M. Sagnou, M. Paravatou-Petsotas, E. Gourni, G. Loudos, S. Xanthopoulos, D. Lafkas, H. Kiaris, A. Varvarigou, I. C. Pirmettis, M. Papadopoulos and Maria Pelecanou, *J. Med. Chem.*, 2010, **53**, 4633.
18. H. K. Kim, M. K. Kang, K. H. Jung, S. H. Kang, Y. H. Kim, J. C. Jung, G. H. Lee, Y. Chang, T. J. Kim, *J. Med. Chem.*, 2013, **56**, 8104.

19. R. Alberto , R. Schibli, A. Egli and A. P. Schubiger, *J. Am. Chem. Soc.*, 1998, **120**, 7987.
20. R. Alberto , K. Ortner , N. Wheatley, R. Schibli and A. P. Schubiger, *J. Am. Chem. Soc.*, **2001**, 123, 3135.
21. G. R. Morais, A. Paulo and I. Santos, *Organometallics*, 2012, **31**, 5693.
22. C. Moura, L. Gano, I. C. Santos, I. Santos and A. Paulo, *Eur J Inorg Chem* 2011, 5405.
23. C. Moura, L. Gano, F. Mendes, P. D. Raposinho, A. M. Abrantes, M. F. Botelho, I. Santos and A. Paulo, *Eur. J. Med. Chem.*, 2012, **50**, 350.
24. I. Fichtner, A. Monks, C. Hose, M. F.G. Stevens and T. D. Bradshaw, *Breast Cancer Res. Treat.* , 2004, **87**, 97.
25. C. Fernandes, L. Maria, L. Gano, I. C. Santos, I. Santos, A. Paulo, *J. Organomet. Chem.*, 2014, **760**, 138.
26. M. Q. Zheng, D. Z. Yin, J. P. Qiao, L. Zhang and Y. X. Wang, *J. Fluorine Chem.*, 2008, **129**, 210.
27. N. Lazarova, S. James, J. Babich and J. Zubieta, *Inorg. Chem. Commun.*, 2004, **7**, 1023.
28. D. E. Troutner, W. A. Volkert, T. J. Hoffman and R. A. Holmes, *Int. J. Appl. Rad. Isot.*, 1984, **35**, 467.
29. M. D. Abramoff, P. J. Magelhaes and S. J. Ram, *Biophotonics Int*, 2004, *11*.

Table of contents entry

A new family of cysteamine-based (N,S,O)-chelators that contain benzothiazole pharmacophores were successfully applied in the synthesis of M(I) (M = Re, ^{99m}Tc) tricarbonyl complexes (**Re1-Re4** and **Tc1-Tc4**), which were biologically evaluated as isostructural tools for cancer theranostics.

