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Mono- and dicationic Re(I)/^{99m}Tc(I) tricarbonyl complexes for the targeting of energized mitochondria

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ABSTRACT

The enhanced negative mitochondrial membrane potential of tumor cells can increase the cell accumulation of triphenylphosphonium (TPP) derivatives, which prompted us to investigate TPP-containing Re(I)/^{99m}Tc organometallic compounds as probes for in vivo targeting of energized mitochondria. Novel compounds (**Re1**–**Re4**/**Tc1**–**Tc4**) were obtained with bifunctional chelators of the pyrazole-diamine (N,N,N-donors) and pyrazole-aminocarboxylic (N,N,O-donors) type, functionalized with TPP pharmacophores that have been introduced at the central amine of the chelators using different spacers. In this way, dicationic (**Re1**–**Re2**, **Tc1**–**Tc2**) and monocationic (**Re3**–**Re4**, **Tc3**–**Tc4**) complexes with variable lipophilicity were synthesized. The ^{99m}Tc complexes (**Tc1**–**Tc4**) are highly stable under physiological conditions and their chemical identification was done by HPLC comparison with the Re congeners (**Re1**–**Re4**), which were fully characterized by common analytical techniques (electrospray ionization mass spectrometry (ESI-MS), IR, multinuclear NMR). The in vitro biological evaluation of **Tc1**–**Tc4** was performed in a panel of human tumor cell lines (PC-3, MCF-7 and H69), including cell lines overexpressing P-glycoprotein (MCF-7/*MDR1* and H69/Lx4), and in isolated mitochondria. All the tested complexes showed a low to moderate cellular and mitochondrial uptake and did not undergo significant P-glycoprotein (Pgp)-mediated efflux processes. In particular, the dication **Tc2** and the monocation **Tc4** presented the highest cellular and mitochondrial uptake. Their cellular uptake was shown to depend on the mitochondrial ($\Delta\psi_m$) and plasma membrane ($\Delta\psi_p$) potentials. Altogether, the biological properties of these compounds suggest that they might be relevant for the design of radioactive metalloprobes for in vivo targeting of mitochondria.

1. Introduction

Mitochondria are dynamic organelles that play a central role in cellular metabolism. In recent years, there has been an increasing awareness that defects in mitochondrial function contribute to the development and progression of cancer. Several distinct differences between the mitochondria of normal cells and cancer cells are known to occur at the genetic, molecular and biochemical levels, offering the possibility of exploring mitochondria as targets for novel and site-specific anti-cancer agents [1–4]. These differences also render mitochondria suitable markers for the early detection of cancer, based on molecular imaging techniques.

Among the existing molecular imaging modalities (e.g. nuclear imaging, magnetic resonance imaging, computed tomography, ultrasound, bioluminescence and fluorescence imaging), the nuclear techniques single photon emission computed tomography (SPECT) and positron emission tomography (PET) have the advantage of high intrinsic sensitivity and unlimited depth penetration. These favorable features enable the use of SPECT or PET to visualize interactions between physiological targets and adequate ligands, allowing their application in the clinical setting. To fully profit from the intrinsic advantages of SPECT and PET it is necessary to design radioprobes that are able to accumulate in a given target tissue upon specific interaction with a given biomarker of disease, such as an up-regulated membranar receptor or an altered organelle like the energized mitochondria of tumor cells.

The rationale behind the use of radioprobes to target mitochondria of tumor cells relies on the increased negative membrane potential ($\Delta\psi_m$) across the inner membrane of these organelles in cancer cells compared with normal cells [5–8]. Several research groups have used triphenylphosphonium (TPP) derivatives in the design of radioprobes for in vivo targeting of mitochondria [8–17]. The use of TPP derivatives to design this type of probes has been driven by the ability of ^{3}H-triphenylphosphonium and ^{3}H-methyltriphenylphosphonium to accumulate in mitochondria and to measure mitochondrial potentials [10–12,14]. These lipophilic and delocalized cationic molecules penetrate the hydrophobic barriers of the plasma and mitochondrial membranes and accumulate inside the energized mitochondria because of their positive charge [14,18]. Since cancer cells have an increase of

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approximately 60 mV in $\Delta\psi_m$ (i.e., more negative), this difference alone is sufficient to account for a ten-fold accumulation of these compounds in carcinoma cells vs normal cells, in accordance with the Nernst equation [14,18]. The existence of a negative plasma membrane potential ($\Delta\psi_p$) (−30 to −60 mV) further concentrates lipophilic cations in the cytosol, contributing also for their increased accumulation in tumoral mitochondria.

The major part of the work on radiolabeled TPP derivatives has involved PET radioisotopes, such as ^{18}F and ^{64}Cu. Encouraging results were found in terms of selectivity for tumor mitochondria, but unfavorable biodistribution profiles were reported for most of the compounds due to undesirable accumulation in non-target soft tissues [9,13,15–17]. The use of SPECT rather than PET tracers has the potential advantage of wider availability at lower costs. This is particularly true for ^{99m}Tc-based radioprobes, since the ^{99m}Tc radionuclide still is the "work horse" for SPECT imaging in clinical nuclear medicine [19]. Despite these advantages, the efforts made to obtain ^{99m}Tc-labelled TPP derivatives for mitochondria targeting are scarce. To the best of our knowledge, only a mercaptoacetyltriglycine (MAG3) Tc(V) oxocomplex bearing a TPP derivative has been evaluated as a SPECT probe for the targeting of tumoral mitochondria [20]. However, the overall charge of this Tc(V) complex is neutral, which is a potential drawback for the desired purpose. It is worth mentioning that $[^{99m}Tc^{I}(MIBI)_6]^+$ (^{99m}Tc-Sestamibi) and $[^{99m}Tc^{V}(tetrofosmin)_2O_2]^+$, in clinical use as myocardial perfusion imaging agents, have also relevance for tumoral detection due to their ability to accumulate in the mitochondria of tumor cells. Unfortunately, these two complexes are also substrates of multidrug resistance P-glycoprotein (PgP), which may induce a rapid efflux of these cationic and lipophilic radiotracers from tumor cells [19,21,22].

Due to the versatility and unique features of the so-called tricarbonyl approach, the use of the *fac*-$[^{99m}Tc(CO)_3]^+$ core can easily afford complexes of different charge with tunable lipophilicity. We decided to study Tc(I) organometallic complexes bearing TPP derivatives aiming at the design of SPECT probes for in vivo targeting of mitochondria. To achieve this goal, we have explored pyrazole-diamine (N,N,N-donors) and pyrazole-aminocarboxylic (N,N,O-donors) chelators [23–25]. These chelators were functionalized with TPP derivatives and used to synthesize structurally related mono- and dicationic complexes, as depicted in Fig. 1. By using compounds possessing an intrinsic +2 charge *vs* a +1 charge we expected to obtain a further enhancement (i.e. a 100-fold vs 10-fold) of the mitochondrial accumulation in carcinoma cells *vs* normal cells, according to the Nernst equation [14].

Herein, we report on the synthesis, characterization and in vitro evaluation of new ^{99m}Tc(I)/Re(I) tricarbonyl complexes (**Tc1**–**Tc4**/**Re1**–**Re4**), as probes for in vivo targeting of mitochondria. To gain an insight into their relevance for this type of application, the in vitro biological evaluation of the ^{99m}Tc complexes comprised the measurement of cellular uptake in several human tumor cell lines, including Pgp-expressing lines, uptake studies in isolated mitochondria, and the assessment of the influence of $\Delta\psi_m$ and $\Delta\psi_p$ in cellular uptake.

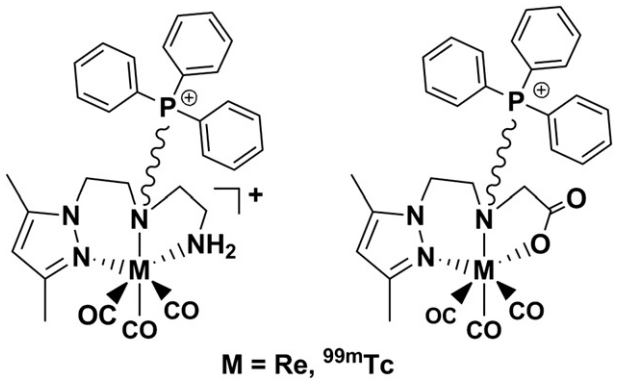


Fig. 1. General molecular structures of the Re and ^{99m}Tc complexes evaluated as probes for the targeting of mitochondria.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis and characterization of the ligands

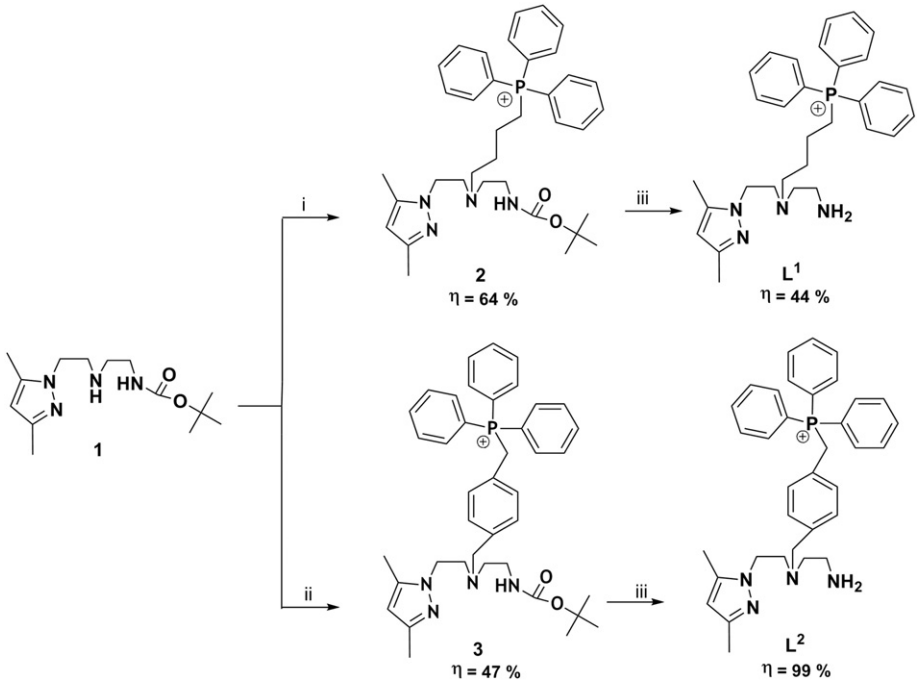
As mentioned above, we have explored bifunctional chelators of the pyrazole-diamine (N,N,N-donors) and pyrazole-aminocarboxylic (N,N,O-donors) type to obtain Re(I)/^{99m}Tc(I) tricarbonyl complexes functionalized with TPP derivatives [23–25]. The use of these chelators has been motivated by the possibility of obtaining structurally related Re(I)/^{99m}Tc(I) tricarbonyl with different lipophilicity and different overall charge, which can be determinant factors of their mitochondria targeting capability. Moreover, these two classes of chelators allowed the functionalization with TPP derivatives using convergent synthetic approaches.

As shown in Schemes 1 and 2, the functionalization of both classes of chelators with the TPP derivatives was done using a common synthetic approach, starting from compounds **1** and **4**, respectively. This approach involved the N-alkylation of the central amine of **1** and **4** with (4-bromobutyl)triphenylphosphonium bromide or (4-bromomethyl)benzyl)triphenylphosphonium bromide [13], carried out in DMF (dimethyl formamide) and in the presence of triethylamine. These N-alkylation reactions afforded compounds **2** and **3** (Scheme 1) and compounds **5** and **6** (Scheme 2), respectively, functionalized with the TPP derivatives at the central amine group. The removal of the tert-butyloxycarbonyl (BOC) protecting group from **2** and **3** with TFA gave the final ligands $\mathbf{L^1}$ and $\mathbf{L^2}$, respectively, which were obtained as trifluoroacetate salts in moderate-to-high yield. In a first attempt, the hydrolysis of the ester function of **5** and **6** was done under basic conditions but the reaction proceeded with the oxidation of the phosphonium group. As indicated in Scheme 2, the use of concentrated HCl avoided the oxidation reaction and led to an efficient hydrolysis of the ester function, yielding the final chelators $\mathbf{L^3H}$ and $\mathbf{L^4H}$, respectively. $\mathbf{L^3H}$ and $\mathbf{L^4H}$ were obtained as zwitterions in moderate yield, after neutralization of the reaction mixture and desalting with a C18 Sep-Pak cartridge that has been eluted successively with distilled water and methanol. The characterization of the new phosphonium-containing ligands ($\mathbf{L^1}$–$\mathbf{L^4H}$) by multinuclear NMR (^{1}H, ^{13}C and ^{31}P), electrospray ionization mass spectrometry (ESI-MS), IR and elemental analysis confirmed the respective formulations.

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

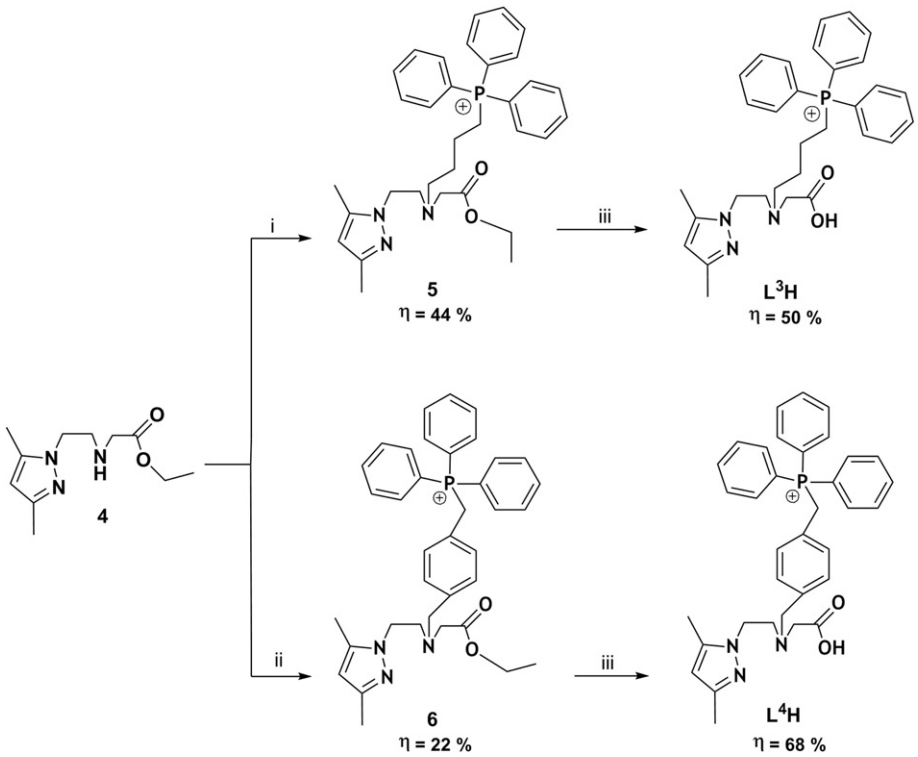
Non-radioactive Re complexes are commonly used as surrogates of the ^{99m}Tc congeners, profiting from the physico-chemical similarities of these group 7 elements. In this way, one can avoid the use of the long-lived β^- emitter ^{99}Tc available in macroscopic amounts for the synthesis and structural analysis of technetium compounds. For $\mathbf{L^1}$–$\mathbf{L^4H}$, the respective Re complexes (**Re1**–**Re4**) were prepared with the goal of using them as surrogates of the ^{99m}Tc congeners, to assign their chemical identity by means of HPLC comparison. **Re1**–**Re4** were obtained in low to moderate yield (24–59%) by reacting *fac*-$[Re(H_2O)_3(CO)_3]Br$ with the corresponding ligands in refluxing methanol or H_2O (Scheme 3). Their purification was done by washing with *n*-hexane and water (**Re1**) or by semi-preparative reverse-phase (RP)-HPLC (**Re2**–**Re4**).

Complexes **Re1**–**Re4** were characterized by elemental analysis, ESI-MS and by common spectroscopic techniques (IR; ^{1}H, ^{31}P and ^{13}C NMR). The positive ESI-MS spectra of **Re1**–**Re4** showed prominent peaks corresponding to the expected molecular-ions, with the correct isotope distribution pattern and without significant fragmentation.

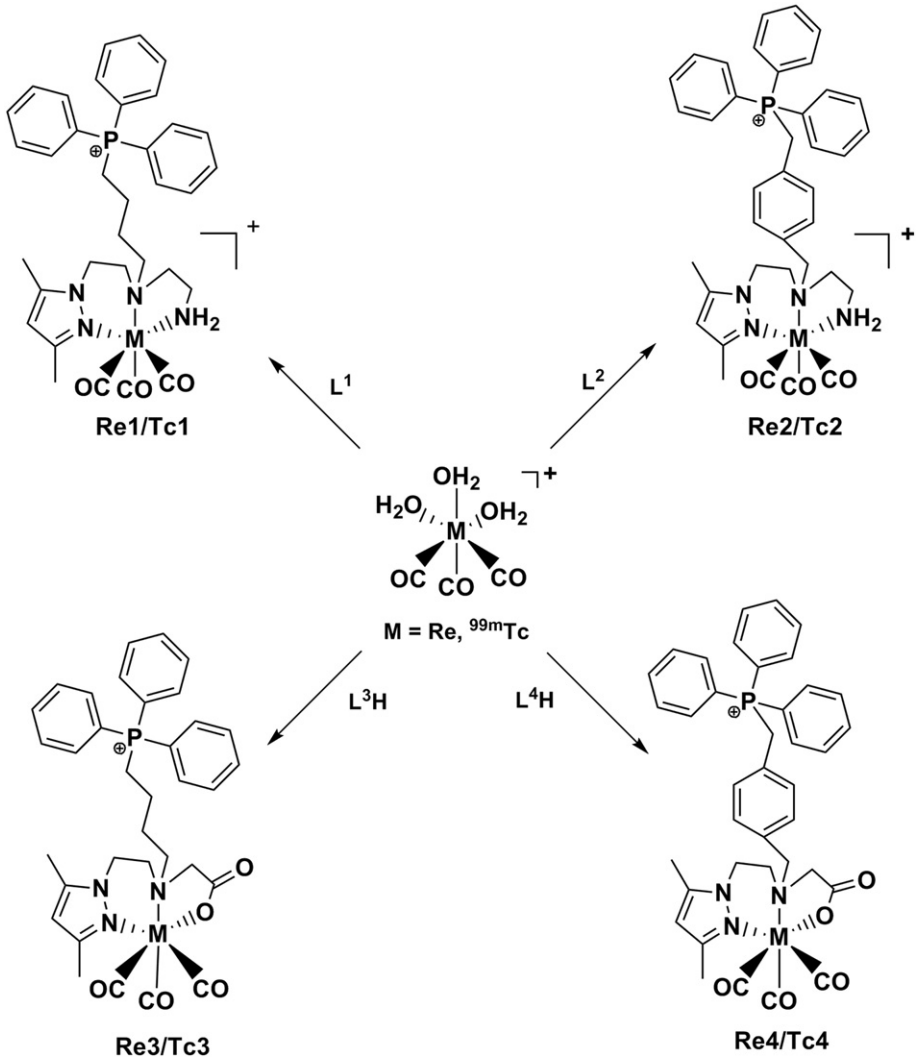


Scheme 1. Synthesis of $\mathbf{L^1}$ and $\mathbf{L^2}$. Reagents and conditions: (i) $Br(CH_2)_4P^+Ph_3$, DMF, NEt_3, r.t., 72 h; (ii) Br-*p*-xylene-P^+Ph_3, DMF, NEt_3, r.t., 72 h; and (iii) TFA, CH_2Cl_2, r.t., 2.5 h.

The IR spectra of **Re1**–**Re4** showed a typical *fac*-$[Re(CO)_3]^+$ pattern with the presence of two strong ν(C≡O) bands centered at 1896 and 2027 cm^{-1}, displaying frequencies comparable to those found for other Re(I) tricarbonyl complexes anchored by the same type of chelators [24,26]. The ^{1}H NMR spectra of **Re1**–**Re4** presented a set of multiplets for the diastereotopic methylenic protons of the framework of the pyrazolyl-diamine or pyrazolyl-aminocarboxylic ligands. The patterns of the ^{1}H NMR spectra were consistent with a facial coordination of the chelators, through the pyrazolyl ring, the central nitrogen atom and the terminal amine (**Re1** and **Re2**) or carboxylate (**Re3** and **Re4**) groups, as previously reported for related complexes [24–26]. The presence of three C≡O resonances, spanning from 193.66 to 196.85 ppm, in the ^{3}C NMR spectra of **Re1**–**Re4** also corroborated the tridentate coordination of the chelators. The presence of a unique



Scheme 2. Synthesis of $\mathbf{L^3H}$ and $\mathbf{L^4H}$. Reagents and conditions: (i) $Br(CH_2)_4P^+Ph_3$, DMF, NEt_3, r.t., 72 h; (ii) Br-*p*-xylene-P^+Ph_3, DMF, NEt_3, r.t., 72 h; (iii) HCl 4 M, reflux, 4 h.



Scheme 3. Synthesis of the Re (**Re1**–**Re4**) and ^{99m}Tc (**Tc1**–**Tc4**) complexes.

singlet in the corresponding $^{31}P\{^{1}H\}$ spectra, between 24.05 and 24.64 ppm, confirmed the presence of the TPP groups, and showed that these groups were not oxidized during the synthesis of the complexes.

The ^{99m}Tc congeners (**Tc1**–**Tc4**) were obtained in aqueous solution, at pH 7.4 (**Tc1** and **Tc2**) or pH 5.5 (**Tc3** and **Tc4**), by reaction of *fac*-$[^{99m}Tc(H_2O)_3(CO)_3]^+$ with the appropriate ligand at 100 °C for 30 min (Scheme 3). Unlike **Tc2**, all the other ^{99m}Tc complexes were obtained in almost quantitative yield (≥95%) using ligand concentrations as low as 10^{-4} M. **Tc2** was synthesized with a moderate yield (70%) due to the formation of an unidentified radiochemical impurity. For this reason, **Tc2** was purified by semi-preparative RP-HPLC, being obtained with a radiochemical purity >99% after purification. Only the purified **Tc2** was used in the in vitro studies described below.

The chemical identity of **Tc1**–**Tc4** was established by comparing their HPLC radiochromatograms with the UV–visible (UV–vis) trace of the fully characterized Re surrogates, as exemplified for **Tc4**/**Re4** in Fig. 2. The lipophilicity of the ^{99m}Tc complexes was assessed by measurement of the respective log $D_{o/w}$ values (n-octanol/0.1 M, phosphate buffered saline (PBS), pH 7.4) using the multiple "shake-flask" method. The log $D_{o/w}$ values and the HPLC retention times of **Tc1**–**Tc4** are shown in Table 1. The dicationic complexes **Tc1** and **Tc2**, anchored by (N,N,N)-donor pyrazolyl-diamine bifunctional chelators (BFCs), are hydrophilic with log $D_{o/w}$ values of -0.93 ± 0.07 and -0.07 ± 0.05, respectively. The monocationic **Tc3** and **Tc4**, anchored by (N,N,O)-donor BFCs, exhibited a lipophilic character with log $D_{o/w}$ values ranging from 0.55 to 0.79. For the complexes with the same donor atom set, replacement of the butylene (**Tc1** and **Tc2**) by a *p*-xylene (**Tc3** and **Tc4**) linker resulted in an increase of lipophilicity.

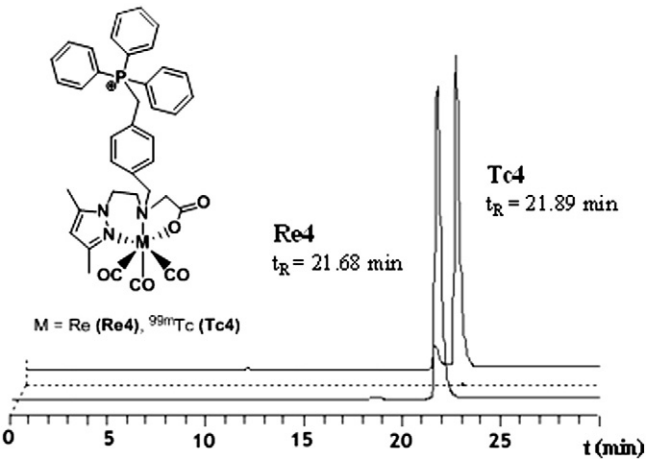


Fig. 2. HPLC chromatograms of co-injected rhenium complex (**Re4**) (UV detection) and ^{99m}Tc complex (**Tc4**) (γ detection).

Table 1
HPLC Retention times and log $D_{7.4}$ of complexes **Tc1**–**Tc4**.

Complex	rt (min)[a,b]	log $D_{7.4}$
Tc1	21.18 (21.08)	−0.93 ± 0.07
Tc2	20.67 (20.49)	−0.07 ± 0.05
Tc3	21.78 (21.60)	0.59 ± 0.05
Tc4	21.89 (21.68)	0.74 ± 0.01

[a] Using a gradient of aqueous 0.1% CF_3COOH and methanol as the solvent.
[b] The values in parentheses are for the Re congeners.

3. Biological evaluation

To assess the ability of **Tc1**–**Tc4** to target mitochondria we have performed their in vitro biological evaluation, which comprised cellular uptake studies in a panel of human tumor cells, including in some cases the corresponding P-glycoprotein (Pgp)-expressing cell lines, and the measurement of their uptake in isolated mitochondria. The effect of $\Delta\psi_p$ and $\Delta\psi_m$ on the cellular uptake has been also evaluated by depolarization of the cellular and/or mitochondrial membrane potentials.

3.1. Cellular uptake

The first screening of the ability of complexes **Tc1**–**Tc4** to accumulate in human tumor cells has been performed using PC-3 prostate cancer cells and MCF-7 breast cancer cells. The cellular uptake of **Tc1**–**Tc4** at 37 °C was time-dependent in both cell lines (Fig. 3A and B) with values in the ranges 3.47–8.30% and 3.12–4.84% for the PC-3 and MCF-7 cells, respectively. The highest uptake was found for complex **Tc2** in PC-3 cell line (8.30% ± 0.63, after 4 h of incubation).

As commented in the introductory part, the cell uptake of lipophilic and cationic radiotracers, such as ^{99m}Tc-Sestamibi can be affected by the expression of Pgp. Hence, we have checked if expression of Pgp would affect the cellular uptake of **Tc1**–**Tc4**. To have a first insight into this

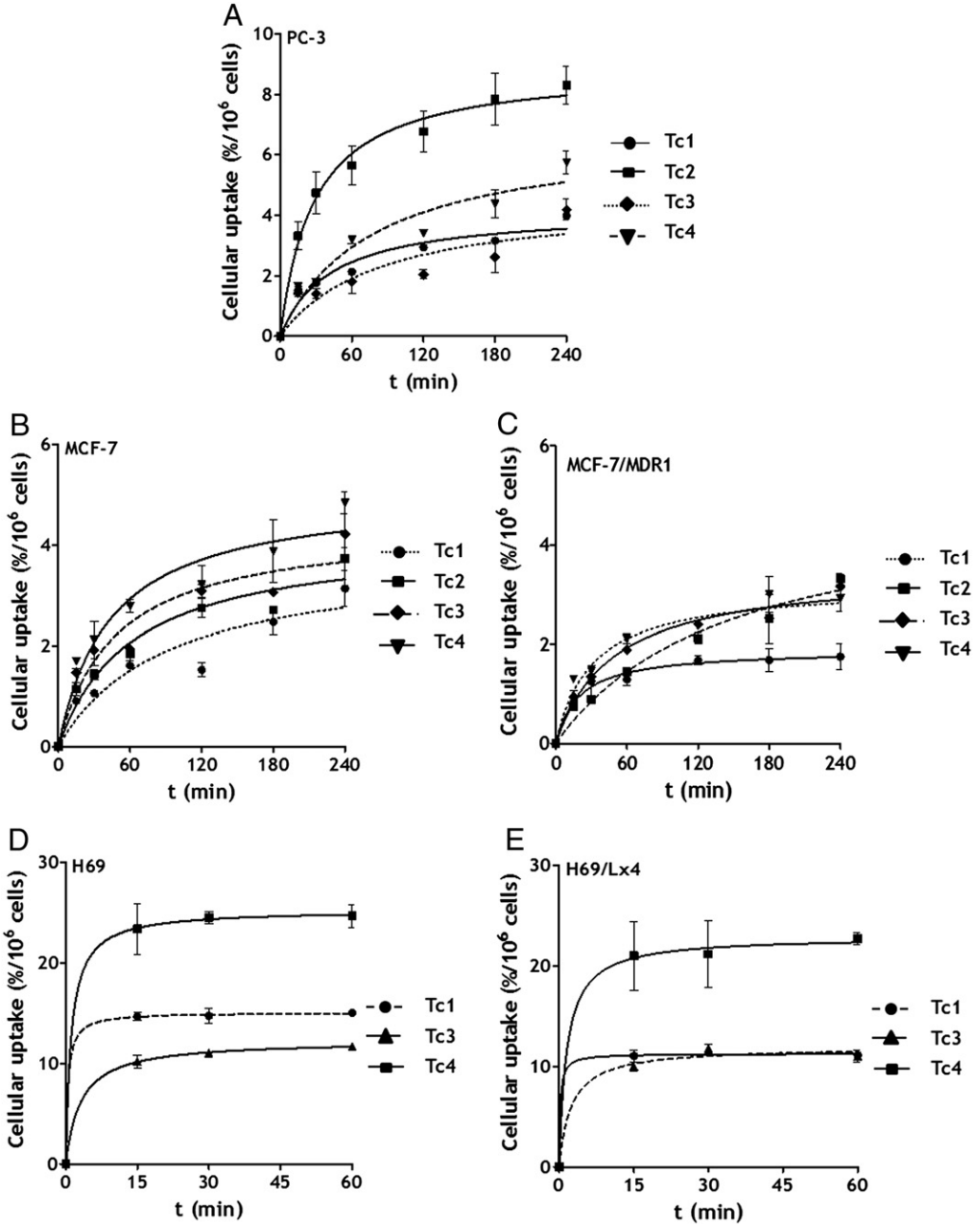


Fig. 3. In vitro cell uptake of complexes **Tc1**–**Tc4** in PC-3 (A), MCF-7 (B), MCF-7/*MDR1* (C), H69 (D) and H69/Lx4 (E) cell lines.

aspect, cellular uptake studies were performed in the Pgp-expressing cell line MCF-7/*MDR1*. Unlike **Tc2**, complexes **Tc1**, **Tc3** and **Tc4** showed a decrease of cellular uptake in the MCF-7/*MDR1* cell line when compared to the one obtained in the parental cell line MCF-7 (Figs. 3A, B and 4A). To further evaluate the effect of Pgp-mediated efflux for complexes **Tc1**, **Tc3** and **Tc4** additional studies were performed in the small cell lung carcinoma cell line H69 and in its derivative drug-resistant cell line H69/Lx4, which presents Pgp overexpression. As can be seen in Figs. 3D, E and 4B, the cellular uptake of **Tc1** is lower in the drug resistant line (H69/Lx4) with values of 14.68% ± 0.37 and 11.04% ± 0.47 for H69 and H69/Lx4, respectively, after 1 h of incubation. The other two tested complexes, **Tc3** and **Tc4**, presented very similar uptake values in both cell lines. The expression of Pgp on the different cell lines has been confirmed by western blot analysis (Fig. 5), using a commercially available antibody as described in Experimental section. The western blot analysis showed no expression of Pgp in the MCF-7, PC-3 and H69 cell lines, in contrary to the MCF-7/*MDR1* and H69/Lx4 cell lines that showed a band between 150 and 225 kDa in agreement with the molecular weight of Pgp (170 kDa) [27].

In summary, the comparison of cellular uptake of the ^{99m}Tc complexes in the pairs of cell lines MCF-7/MCF-7/*MDR1* and H69/H69/Lx4 indicates that **Tc1** has a greater tendency to suffer Pgp-mediated efflux compared with the other complexes under study. However, even for **Tc1** the efflux is much less pronounced than that reported for ^{99m}Tc-Sestamibi in the same cell lines [19,27,28].

3.2. Uptake studies in isolated mitochondria

To assess the mitochondrial targeting ability of the phosphonium-containing complexes **Tc1**–**Tc4**, uptake studies were performed in isolated mitochondria obtained from PC-3. The isolated mitochondria were incubated with the compounds at different intervals of time, up to 2 h of incubation. After appropriate separation of the organelles from the incubation medium, the radioactivity associated with the mitochondria was measured and the mitochondrial uptake determined. The results obtained are presented in Fig. 6.

After 2 h of incubation, the mitochondrial uptake of **Tc1**–**Tc4** ranged between 1.24 and 3.53%, when expressed in percentage of uptake per million cells. With the exception of **Tc3**, the other complexes had a rather similar uptake, particularly the dicationic compounds **Tc1** and **Tc2** that showed almost the same uptake values. The lowest mitochondrial uptake was found for **Tc3**, while **Tc4** has shown the highest mitochondrial uptake. **Tc3** and **Tc4** are both monocationic and lipophilic, being however **Tc4** more lipophilic due to the presence of a *p*-xylene spacer instead of a butylene one. These findings indicate that for the monocationic complexes, **Tc3** and **Tc4**, the lipophilicity seems to play a more important role in the mitochondrial uptake of the complexes. On the contrary, for the dicationic complexes, **Tc1** and **Tc2**, the influence of lipophilicity seems less important being the presence of the +2 charge the major driving force of their accumulation in the mitochondria.

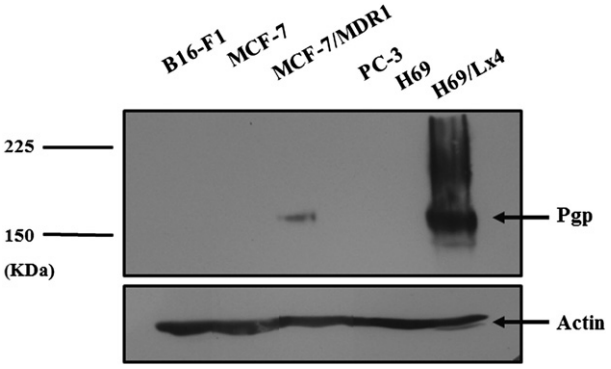


Fig. 5. Western blot analysis of Pgp expression in different cancer cell lines.

Tc4, which is the complex that presented the highest binding to this organelle, was selected for further studies to have an insight into its targeting ability towards tumoral mitochondria versus normal mitochondria. Hence, uptake studies of **Tc4** were performed in parallel using isolated mitochondria that were collected from PC-3 cells and from the human cells of non-tumoral origin HEK. As shown if Fig. 7A, the binding of **Tc4** to tumoral mitochondria is almost 2 times higher than in normal mitochondria. Consistently, **Tc4** reached a cellular uptake plateau that is significantly higher in PC-3 cells than in HEK cells (Fig. 7B), with an approximately two-fold reduction of uptake in the non-tumoral cell line. As mentioned in the introduction, several TPP-containing ^{64}Cu complexes have been evaluated as PET radioprobes for the targeting of tumor mitochondria. Some of these ^{64}Cu complexes have been evaluated in isolated tumor cell mitochondria showing uptake values up to 1.5%, [13] which are lower than those that we have obtained for **Tc4**. These findings suggest that **Tc4** has an enhanced ability to be accumulated by the mitochondria of tumor cells.

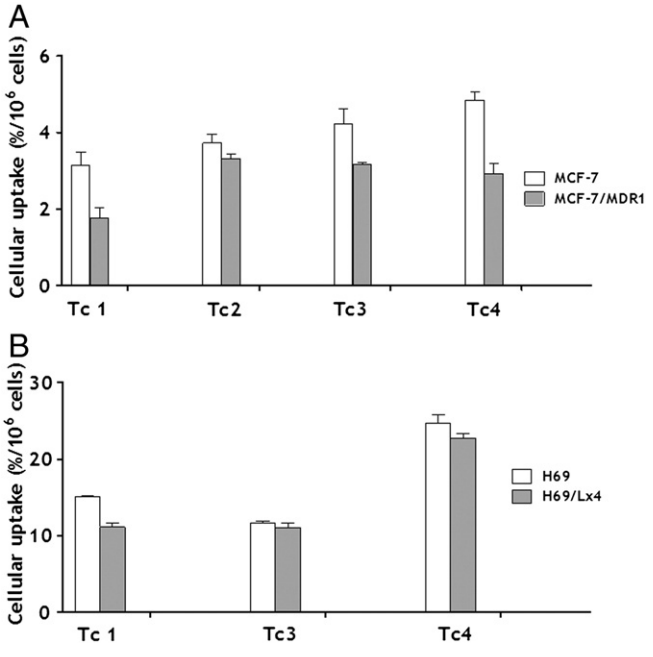


Fig. 4. In vitro cellular uptake of complexes **Tc1**–**Tc4**: in MCF-7 and MCF-7/*MDR1* after 4 h incubation (A) and in H69 and H69/Lx4 after 1 h incubation (B).

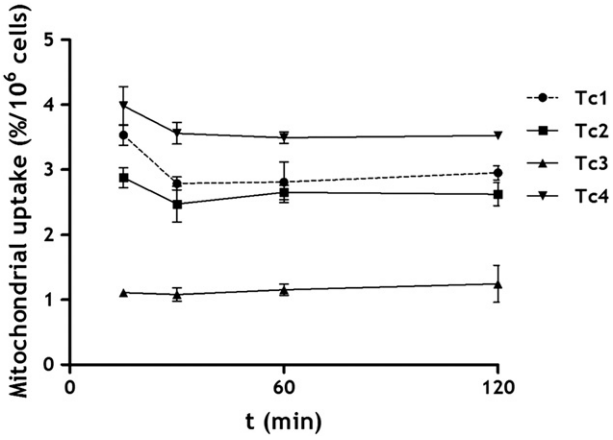


Fig. 6. Uptake of complexes **Tc1**–**Tc4** in isolated mitochondria obtained from PC-3 cells.

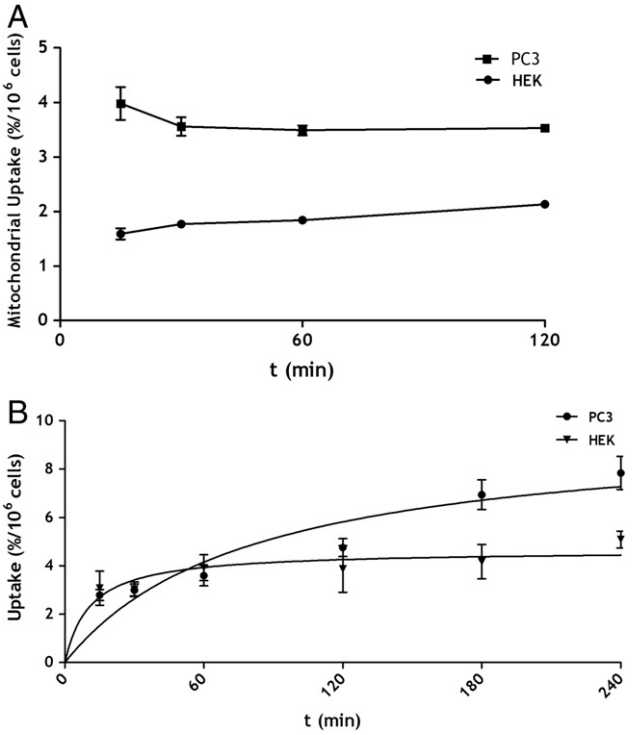


Fig. 7. (A) Comparison of the uptake of **Tc4** in isolated mitochondria obtained from PC-3 and HEK cells; (B) comparison of the in vitro cellular uptake of **Tc4** in PC-3 and HEK cell lines.

3.3. Influence of $\Delta\psi_p$ and $\Delta\psi_m$

The influence of $\Delta\psi_p$ and $\Delta\psi_m$ on the cellular uptake was evaluated for **Tc2** and **Tc4** using PC-3 cells, as these complexes presented the highest cellular uptake in this cell line and a considerable uptake in isolated mitochondria. To evaluate this influence, uptake studies were run under experimental conditions that can reduce or even collapse $\Delta\psi_m$ and $\Delta\psi_p$.

Cellular uptake studies were conducted by incubation of PC-3 cells with the complexes in the presence of a buffer solution with increasing concentrations of K^+ (5 mM, 60 mM and 120 mM) for 4 h. For the K^+ concentration of 120 mM, the study was also run in the presence of valinomycin (Fig. 8A). $\Delta\psi_p$ is generated mainly by the movement of K^+ ions from inside to outside the cell through K^+ channels [29,30]. The increase in K^+ concentration in the extracellular medium from 5 mM to 120 mM leads to the establishment of a balance between the concentrations of K^+ inside and outside the cell, and consequently to membrane depolarization [31]. Valinomycin is a K^+ ionophore that increases membrane permeability to K^+. $\Delta\psi_p$ is nearly zero for high extracellular concentrations of K^+ and, under these conditions, the addition of valinomycin can lead to the collapse of $\Delta\psi_m$ due to the uncoupling of electron chain in oxidative phosphorylation [7,32–35].

As shown in Fig. 8A, the increase in K^+ concentration from 5 to 120 mM led to an approximately two-fold reduction of the cellular uptake for both complexes: from 10.94 to 5.64% and from 8.72 to 4.69% for **Tc2** and **Tc4**, respectively. A further reduction of the uptake, between 60 and 75%, was found when the uptake was measured in the presence of valinomycin. Altogether, these data suggested that both $\Delta\psi_p$ and $\Delta\psi_m$ are important factors of the cellular uptake of these complexes. These findings are in agreement with data from previous studies with TPP derivatives radiolabelled with ^{18}F and ^{3}H [33].

To further investigate the contribution of $\Delta\psi_p$ and $\Delta\psi_m$ to the cellular uptake of **Tc2** and **Tc4**, the studies were also performed in the presence of the ionophores nigericin and ouabain (Fig. 8B) and in the presence of CCCP (carbonyl cyanide m-chlorophenyl hydrazone) (Fig. 8C). In these experiments, the cells underwent a prior incubation with **Tc2** and **Tc4** followed by incubation with the ionophores or with the inhibitor. Nigericin mediates electroneutral K^+/H^+ exchange and is known to collapse the pH gradient across mitochondrial inner membranes, thereby producing a secondary hyperpolarization of $\Delta\psi_m$ [36]. Nigericin can also induce the hyperpolarization of the plasma membrane but this can be avoided by the presence of ouabain, which is a specific inhibitor of the plasma membrane Na^+/K^+ ATPase [32,37]. Finally, CCCP is an inhibitor of oxidative phosphorylation, uncoupling the proton gradient across the inner mitochondrial membrane and resulting in the collapse of $\Delta\psi_m$ [38].

The addition of nigericin induced an increase in the cellular uptake of **Tc2** and **Tc4**, between 30 and 40%, which most probably reflects the combined effects of mitochondrial hyperpolarization and the hyperpolarization of the plasma membrane. As shown in Fig. 8B, the further

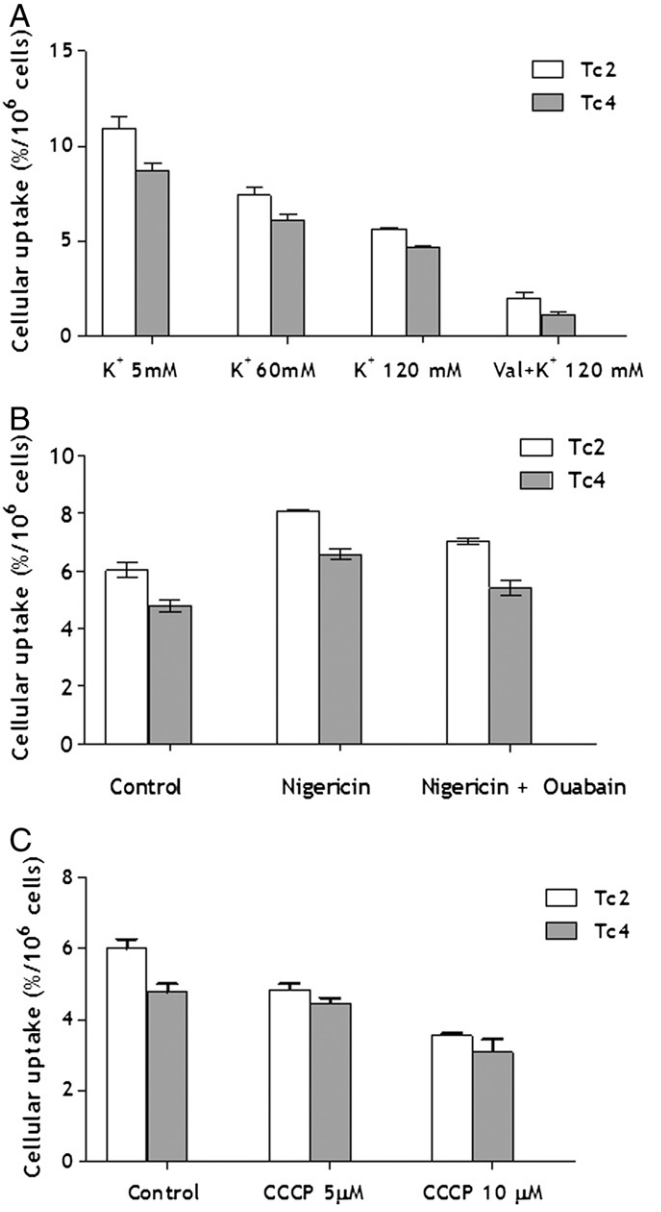


Fig. 8. Effect of alteration in membrane potentials on cellular uptake of complexes **Tc2** and **Tc4** in PC-3 cells. Depolarization of $\Delta\psi_m$ and $\Delta\psi_p$: A using increasing concentrations of K^+ (5, 60, 120 mM) and in the presence of valinomycin (Val, 1 μg/mL); B in the presence of nigericin (5 μg/mL) and nigericin plus ouabain (100 μM); and C in the presence of increasing concentrations of CCCP (5 and 10 μM).

addition of ouabain reduced this increase, but the uptake values were still higher than those obtained in the control experiment (i.e. without addition of nigericin and ouabain) with values of 7.03 *vs* 6.02% (**Tc2**) and 5.39 *vs* 4.78% (**Tc4**), respectively. This trend also indicates that $\Delta\psi_m$ may play a role in the cellular uptake of **Tc2** and **Tc4**. The addition of CCCP at a concentration of 10 μM led to a decrease in the uptake, with values changing from 6.02 to 3.55% (**Tc2**) and from 4.78 to 3.08% (**Tc4**) (Fig. 7C), which is also consistent with the contribution of $\Delta\psi_m$ in the cellular uptake of the complexes.

4. Conclusions

We succeeded in the synthesis of the first examples of TPP-containing organometallic Re(I)/^{99m}Tc(I) complexes. The synthesized complexes presented a dicationic (**Re1**/**Tc1**, **Re2**/**Tc2**) or monocationic character (**Re3**/**Tc3**, **Re4**/**Tc4**). Their lipophilicity was readily tuned by the use of different spacers (butylene *vs p*-xylene) to link the TPP pharmacophore to the central amine of the corresponding tridentate chelators.

The in vitro evaluation of **Tc1**–**Tc4** has shown that these complexes accumulate in different types of tumor cells, and has also proved that their cell uptake values are not much affected by the expression of Pgp. The mitochondria seem to be a relevant intracellular target of **Tc1**–**Tc4**, as pointed out by the results obtained with isolated mitochondria. For **Tc1** and **Tc3**, presenting a butylenic spacer to link the TPP pharmacophore, there is an enhancement of the mitochondrial uptake for the dicationic complex (**Tc1**) compared with the monocationic counterpart (**Tc3**), as predicted by the Nernst equation. However, such enhancement was not observed for the pair of complexes **Tc2** and **Tc4**, showing that the lipophilicity is also a very important factor of the ability of these complexes to cross the mitochondria membrane.

For the complex with the highest mitochondrial uptake, **Tc4**, the in vitro studies suggested the accumulation in mitochondria of tumor cells by diffusion, in favor of the electrochemical gradient and showing a strong dependency on the mitochondrial and plasma membrane potentials. Moreover, **Tc4** has shown a higher affinity towards tumor mitochondria than towards normal mitochondria, as well as a higher uptake in human tumor cells if compared with normal cells from human origin. Altogether, these results indicate that the new TPP-containing ^{99m}Tc(I) tricarbonyl complexes reported in this manuscript are promising platforms to design radioactive metalloprobes targeted to the mitochondria for in vivo detection of tumor tissues.

5. Experimental section

5.1. Chemistry

Unless otherwise stated, the synthesis of the ligands and complexes was carried out under a nitrogen atmosphere, using standard Schlenk techniques and dry solvents; the work-up procedures were performed under air. The compounds, tert-butyl (2-((2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)ethyl)carbamate (**1**) [39], ethyl 2-((2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)acetate (**4**) [24] and (4-(bromomethyl)benzyl)triphenylphosphonium bromide [13] were prepared according to published methods. The starting material *fac*-[Re(H$_2$O)$_3$(CO)$_3$]Br was synthesized by the literature method [40] Na[^{99m}TcO$_4$] was eluted from a commercial ^{99m}o/^{99m}Tc generator, using 0.9% saline. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Varian Unity 300 MHz spectrometer. ^{1}H, ^{13}C and ^{31}P chemical shifts are given in ppm; ^{1}H and ^{13}C chemical shifts were referenced with the residual solvent resonances relative to SiMe$_4$ and the ^{31}P chemical shifts were referenced with an external 85% H$_3$PO$_4$ solution. IR spectra were recorded as KBr pellets on a Bruker, Tensor 27 spectrometer. ESI-MS was performed at Instituto Tecnológico e Nuclear (ITN) on a quadrupole ion trap mass spectrometry (QITMS) instrument in positive ion mode. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F254 plates, and Instant Thin-layer chromatography (ITLC) on PALL ITLC SG strips. Column chromatography was performed with silica gel 60 (Merck). HPLC analysis of the Re and ^{99m}Tc complexes was performed on a Perkin-Elmer LC pump 200 coupled to a LC 290 tunable UV–vis detector and to a Berthold LB-507A radiometric detector, using an analytical Macherey-Nagel C18 reversed-phase column (Nucleosil 100–10, 250 × 3 mm) with a flow rate of 1 mL min^{-1}. HPLC purifications were performed using a semi-preparative Macherey–Nagel C18 reversed-phase column (Nucleosil 100–7, 250 × 8 mm) with a flow rate of 2.0 mL min^{-1} or a using a preparative Waters μ Bondapak C18 column (150 × 19 mm) at a flow rate of 5.0 mL min^{-1}, UV detection, 254 nm. Gradient elution was used both for analytical and semi-preparative HPLC, with the solvent systems: A — aqueous 0.1% CF_3COOH solution, B — MeOH. The gradient systems used are indicated below for each compound.

5.1.1. (4-((2-((Tert-butoxycarbonyl)amino)ethyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)butyl)triphenylphosphonium bromide, 2

To a solution of tert-butyl (2-((2-(3,5-dimethyl-1H-pyrazol-1-yl) ethyl)amino)ethyl)carbamate (**1**) (668 mg, 1.95 mmol) and (4-bromobutyl)triphenylphosphonium bromide (934 mg, 1.95 mmol) in anhydrous DMF (25 mL) was added triethylamine (density = 0.726 g/mL) (546 μL, 3.92 mmol). The reaction mixture was left to stir at room temperature for 72 h. After removal of the solvent, CH_2Cl_2 and H_2O were added to the residue. The organic phase was separated, dried over $MgSO_4$, filtered and after removal of the solvent the product was further purified by silica gel column chromatography, using CH_2Cl_2/MeOH (95/5) as eluent. The final compound (64%, 850 mg) was recovered from the collected fractions as a yellow oil, after removal of the solvent under reduced pressure.

R_f (SiO_2, CH_2Cl_2/MeOH (90/10)) = 0.42; ^{1}H NMR ($CDCl_3$): δ_H 1.23 (9H, s, BOC), 1.44 (2H, m, CH_2), 1.61 (2H, m, CH_2), 1.96 (3H, s, CH_3-pz), 2.03 (3H, s, CH_3-pz), 2.35 (4H, m, 2 CH_2), 2.64 (2H, m, CH_2), 2.86 (2H, m, CH_2), 3.52 (2H, m, CH_2), 3.79 (2H, m, CH_2), 5.15 (1H, s, $H_{4\text{-pz}}$), 7.55–7.73 (15H, m, PPh_3); ^{13}C NMR ($CDCl_3$): δ_c 11.04 (CH_3-pz), 13.07 (CH_3-pz), 20.00 (d, $J_{P\text{-}C}$ = 13 Hz, CH_2), 22.82 (d, $J_{P\text{-}C}$ = 46 Hz, CH_2), 26.70 (d, $J_{P\text{-}C}$ = 18 Hz, CH_2), 28.07 (CH_3-BOC), 38.14 (CH_2), 44.33 (CH_2), 46.77 (CH_2), 53.25 (CH_2), 54.15 (CH_2), 78.31 ($C_{quaternary}$-BOC), 104.35 (C_4-pz), 117.51 (d, $J_{P\text{-}C}$ = 85 Hz, PPh_3), 130.18 (d, $J_{P\text{-}C}$ = 12 Hz, PPh_3), 133.26 (d, $J_{P\text{-}C}$ = 10 Hz, PPh_3), 135.34 (d, $J_{P\text{-}C}$ = 3.7 Hz, PPh_3), 138.82 ($C_{3/5}$-pz), 146.62 ($C_{3/5}$-pz), 155.80 (C═O); ^{31}P NMR ($CDCl_3$) δ_P 23.88; ESI/MS (+) (m/z): 599.4 M^+, calcd for $C_{36}H_{48}N_4O_2P$ = 599.4 [M^+]; Anal. Calc. for $C_{36}H_{48}N_4O_2PBr$: C, 63.60; H, 7.12; N, 8.25, Found: C, 63.58; H, 6.86; N, 8.35.

5.1.2. (4-(((2-((Tert-butoxycarbonyl)amino)ethyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)methyl)benzyl)triphenylphosphonium bromide, 3

Compound **3** (47%, 858 mg) was prepared and purified as above described for **2**, starting from 709 mg (2.49 mmol) of compound **1** and 1.310 g (2.49 mmol) of (4-(bromomethyl)benzyl)triphenylphosphonium bromide.

R_f (SiO_2, CH_2Cl_2/MeOH (90/10)) = 0.56; ^{1}H NMR ($CDCl_3$): δ_H 1.21 (9H, s, BOC), 1.82 (3H, s, CH_3-pz), 1.97 (3H, s, CH_3-pz), 2.35 (2H, t, CH_2), 2.52 (2H, t, CH_2), 2.91 (2H, t, CH_2), 3.32 (2H, s, CH_2), 3.75 (2H, t, CH_2), 5.02 (2H, d, CH_2), 5.42 (1H, bs, NH), 5.51 (1H, s, H4-pz), 6.67–6.77 (4H, m, Ph), 7.43–7.61 (15H, m, PPh_3); ^{13}C NMR ($CDCl_3$): δ_c 10.48 (CH_3-pz), 12.95 (CH_3-pz), 28.01 (CH_3-BOC), 30.03 (d, $J_{P\text{-}C}$ = 47 Hz, CH_2), 37.94 (CH_2), 46.15 (CH_2), 52.71 (CH_2), 53.18 (CH_2), 57.62 (CH_2), 78.23 ($C_{quaternary}$-BOC), 104.52 (C_4-pz), 117.12 (d, $J_{P\text{-}C}$ = 86 Hz, PPh_3), 125.10 (d, $J_{P\text{-}C}$ = 8.3 Hz, C_{Ph}), 128.60 (d, $J_{P\text{-}C}$ = 2.3 Hz, C_{bz}), 129.73 (d, $J_{P\text{-}C}$ = 12 Hz, PPh_3), 130.73 (d, $J_{P\text{-}C}$ = 5 Hz, C_{Ph}), 133.74 (d, $J_{P\text{-}C}$ = 10 Hz, PPh_3), 134.61 (d, $J_{P\text{-}C}$ = 2.3 Hz, PPh_3), 138.28 ($C_{3/5}$-pz), 139.03 (d, $J_{P\text{-}C}$ = 3.8 Hz, C_{bz}), 146.54 ($C_{3/5}$-pz), 155.76 (C═O); ^{31}P NMR ($CDCl_3$) δ_P 23.67; ESI/MS (+) (m/z): 647.4 M^+, calcd for $C_{40}H_{48}N_4O_2P$ = 647.4 [M^+]; Anal. Calc. for

$C_{40}H_{48}N_4O_2PBr$: C, 66.00; H, 6.65; N, 7.70, Found: C, 65.92; H,[1] 5.78; N, 7.57.

5.1.3 (4-((2-(3,5-Dimethyl-1H-pyrazol-1-yl)ethyl)(2-ethoxy-2-oxoethyl) amino)butyl)triphenylphosphonium bromide, **5**

Compound **5** (44%, 1.149 g) was prepared and purified as above described for **2**, starting from 957 mg (4.25 mmol) of ethyl 2-((2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino)acetate (**4**) and 2.032 g (4.25 mmol) of (4-bromobutyl)triphenylphosphonium bromide.

R_f (SiO_2, CH_2Cl_2/MeOH (90/10)) = 0.47; ^{1}H NMR ($CDCl_3$): δ_H 1.09 (3H, t, CH_3), 1.53 (2H, m, CH_2), 1.66 (2H, m, CH_2), 1.99 (3H, s, CH_3-pz), 2.00 (3H, s, CH_3-pz), 2.50 (2H, t, CH_2), 2.78 (2H, t, CH_2), 3.12 (2H, s, CH_2), 3.58 (2H, m, CH_2), 3.83 (2H, t, CH_2), 3.95 (2H, q, CH_2), 5.49 (1H, s, H4-pz), 7.56–7.75 (15H, m, PPh_3); ^{13}C NMR ($CDCl_3$): δ_c 12.87 (CH_3-pz), 15.16 (CH_3-pz), 15.93 (CH_3), 21.64 (d, $J_{P\text{-}C}$ = 4.5 Hz, CH_2), 23.69 (d, $J_{P\text{-}C}$ = 50 Hz, CH_2), 29.34 (d, $J_{P\text{-}C}$ = 17 Hz, CH_2), 48.82 (CH_2), 55.10 (CH_2), 55.35 (CH_2), 57.22 (CH_2), 62.14 (CH_2), 106.44 (C_4-pz)), 119.93 (d, $J_{P\text{-}C}$ = 86 Hz, PPh_3), 132.22 (d, $J_{P\text{-}C}$ = 15 Hz, PPh_3), 135.38 (d, $J_{P\text{-}C}$ = 9.8 Hz, PPh_3), 136.76 (d, $J_{P\text{-}C}$ = 2.3 Hz, PPh_3), 140.92 ($C_{3/5}$-pz), 148.83 ($C_{3/5}$-pz)), 173.10 (C═O); ^{31}P NMR ($CDCl_3$) δ_P 23.89; ESI/MS (+) (m/z): 542.3 M^+, calcd for $C_{33}H_{41}N_3O_2P$ = 542.3 [M^+]; Anal. Calc. for $C_{33}H_{41}N_3O_2PBr$: C, 63.65; H[1], 6.64; N, 6.75, Found: C, 63.58; H[1], 5.97; N, 6.89.

5.1.4. (4-(((2-(3,5-Dimethyl-1H-pyrazol-1-yl)ethyl)(2-ethoxy-2-oxoethyl) amino)methyl)benzyl)triphenylphosphonium bromide, **6**

Compound **6** (22%, 524 mg) was prepared and purified as above described for **2**, starting from 815 mg (3.62 mmol) of compound **4** and 1.901 g (3.62 mmol) of (4-(bromomethyl)benzyl)triphenylphosphonium bromide.

R_f (SiO_2, CH_2Cl_2/MeOH (90/10)) = 0.46; ^{1}H NMR ($CDCl_3$): δ_H 1.05 (3H, t, CH_3); 1.96 (6H, s, 2 CH_3-pz); 2.82 (2H, t, CH_2); 3.03 (2H, s, CH_2); 3.52 (2H, s, CH_2); 3.77 (2H, t, CH_2); 3.91 (2H, q, CH_2); 5.07 (2H, d, CH_2); 5.55 (1H, s, H4-pz); 6.82 (4H. s, Ph); 7.42–7.62 (15H, m, PPh_3); ^{13}C NMR ($CDCl_3$): δ_c 11.23 (CH_3), 13.69 (CH_3-pz), 14.43 (CH_3-pz), 30.64 (d, $J_{P\text{-}C}$ = 47 Hz, CH_2), 47.57 (CH_2), 53.84 (CH_2), 54.60 (CH_2), 58.24 (CH_2), 60.51 (CH_2), 105.05 (C_4-pz)), 117.75 (d, $J_{P\text{-}C}$ = 86 Hz, PPh_3), 126.10 (d, $J_{P\text{-}C}$ = 8.3 Hz, C_{Ph}), 129.37 (d, $J_{P\text{-}C}$ = 3.0 Hz, C_{Ph}), 130.33 (d, $J_{P\text{-}C}$ = 19 Hz, PPh_3), 131.53 (d, $J_{P\text{-}C}$ = 6.0 Hz, C_{Ph}), 134.38 (d, $J_{P\text{-}C}$ = 9.3 Hz, PPh_3), 135.25 (d, $J_{P\text{-}C}$ = 3.0 Hz, PPh_3), 139.20 ($C_{3/5}$-pz)), 139.43 (d, $J_{P\text{-}C}$ = 3.8 Hz, C_{Ph}), 147.35 ($C_{3/5}$-pz)), 171.26 (C═O); ^{31}P NMR ($CDCl_3$) δ_P 24.21; ESI/MS (+) (m/z): 590.3 M^+, calcd for $C_{37}H_{41}N_3O_2P$ = 590.3 [M^+]; Anal. Calc. for $C_{37}H_{41}N_3O_2PBr$: C, 66.25; H, 6.17; N, 6.27, Found: C, 66.15; H, 5.89; N, 6.59.

5.1.5. (4-((2-Aminoethyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino) butyl)triphenylphosphonium trifluoroacetate, **L^1**

To a solution of **2** (850 mg, 1.25 mmol) in CH_2Cl_2 (5 mL) was added trifluoroacetic acid (density = 1.489 g/mL) (1.62 mL, 21.10 mmol), and the mixture was stirred overnight at room temperature. The solvent was removed under vacuum and the crude product was purified by RP-HPLC on a preparative Waters μ Bondapack. Method: 0–3 min, 75% A; 3–23 min, 75%–50% A; 23–33 min, 50%; 33–43 min 50–0% A; 43–58 min 0% A (t_R = 16.35 min). Compound **L^1** (458 mg, 44%) was recovered from the collected fractions as a transparent oil, after removal of the solvent under vacuum.

^{1}H NMR (CD_3OD): δ_H 1.62 (4H, m, 2 CH_2); 2.11 (3H, s, CH_3-pz); 2.21 (3H, s, CH_3-pz); 2.51 (2H, t, CH_2); 2.80 (2H, m, CH_2); 2.83 (2H, t, CH_2); 2.95 (2H, t, CH_2); 3.37 (2H, m, CH_2); 4.03 (2H, m, CH_2); 5.79 (1H, s, H4-pz); 7.72–7.92 (15H, m, PPh_3); ^{13}C NMR (CD_3OD): δ_c 10.94 (CH_3-pz), 13.21 (CH_3-pz), 21.27 (d, $J_{P\text{-}C}$ = 12 Hz, CH_2), 22.56 (d, $J_{P\text{-}C}$ = 51 Hz, CH_2), 28.70 (d, $J_{P\text{-}C}$ = 17 Hz, CH_2), 38.58 (CH_2), 47.32 (CH_2), 51.94 (CH_2), 54.15 (CH_2), 54.37 (CH_2), 106.15 (C_4-pz)), 115.54 (C_{TFA}), 119.87 (d, $J_{P\text{-}C}$ = 87 Hz, PPh_3), 131.55 (d, $J_{P\text{-}C}$ = 13 Hz, PPh_3), 134.81 (d, $J_{P\text{-}C}$ = 10 Hz, PPh_3), 136.28 (d, $J_{P\text{-}C}$ = 3.4 Hz, PPh_3), 141.27 ($C_{3/5}$-pz)), 148.49 ($C_{3/5}$-pz), 161.50 (C_{TFA}); ^{31}P NMR (CD_3OD) δ_P 24.67; ESI/MS (+) (m/z): 499.4 M^+, calcd for $C_{31}H_{40}N_4P$ = 499.3 [M^+]; Anal. Calc. for $C_{33}H_{40}N_4PF_3O_2$.2 CF_3COOH: C, 52.86; H, 5.04; N, 6.66, Found: C, 52.81; H[1], 5.70; N, 6.93.

5.1.6. (4-(((2-Aminoethyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino) methyl)benzyl)triphenylphosphonium trifluoroacetate, **L^2**

To a solution of **3** (858 mg, 1.18 mmol) in CH_2Cl_2 (5 mL) was added trifluoroacetic acid (density = 1.489 g/mL) (3.00 mL, 39.20 mmol), and the mixture was stirred overnight at room temperature. The solvent was removed under vacuum yielding compound **L^2** (1.157 g, 99%) as a yellow oil, after removal of the solvent under vacuum.

^{1}H NMR (CD_3OD): δ_H 2.25 (3H, s, CH_3-pz); 2.28 (3H, s, CH_3-pz); 3.30 (6H, m, 3 CH_2); 4.18 (2H, t, CH_2); 4.55 (2H, t, CH_2); 4.86 (2H, d, CH_2); 6.16 (1H, s, $H_{4\text{-}pz}$); 7.02–7.34 (4H, m, Ph); 7.57–7.86 (15H, m, PPh_3); ^{13}C NMR (CD_3OD): δ_c 10.91 (CH_3-pz), 11.57 (CH_3-pz), 30.79 (d, $J_{P\text{-}C}$ = 49 Hz, CH_2), 36.57 (CH_2), 45.14 (CH_2), 51.53 (CH_2), 52.80 (CH_2), 57.62 (CH_2), 108.57 (C_4-pz), 118.51 (d, $J_{P\text{-}C}$ = 87 Hz, PPh_3), 118.59 (C_{TFA}), 129.72 (d, $J_{P\text{-}C}$ = 8.3 Hz, C_{bz}), 131.32 (d, $J_{P\text{-}C}$ = 13 Hz, PPh_3), 132.03 (d, $J_{P\text{-}C}$ = 2.3 Hz, C_{bz}), 132.78 (d, $J_{P\text{-}C}$ = 10 Hz, PPh_3), 134.61 (d, $J_{P\text{-}C}$ = 5 Hz, C_{bz}), 135.15 (d, $J_{P\text{-}C}$ = 2.3 Hz, PPh_3), 136.20 (d, $J_{P\text{-}C}$ = 3.8 Hz, C_{bz}), 136.49 ($C_{3/5}$-pz)), 148.01 ($C_{3/5}$-pz)), 161.14 (C_{TFA}); ^{31}P NMR (CD_3OD) δ_P 23.76; ESI/MS (+) (m/z): 647.4 M^+, calcd for $C_{40}H_{48}N_4O_2P$ = 647.4 [M^+]; Anal. Calc. for $C_{42}H_{48}N_4PF_3O_2$.2 CF_3COOH: C, 55.87; H, 5.10; N, 5.67, Found: C, 55.91; H, 5.52; N 5.47.

5.1.7. (2-((2-(3,5-Dimethyl-H-pyrazol-1-yl)ethyl)(4-(triphenylphosphonio)butyl)amino)acetate, **L^3H**

Compound **4** (323 mg, 0.52 mmol) was dissolved in 4 M HCl (12 mL), and the resulting solution was refluxed overnight. After cooling to room temperature, the solution was neutralized to pH 7 and the solvent removed under vacuum. The residue was redissolved in the minimum amount of water and applied on a preconditioned C18 cartridge (Sep-Pak, Waters). The cartridge was eluted with distilled water to remove the salts and then with methanol to recover the title compound. After removal of the solvent from the methanolic fractions, **L^3H** (158 mg, 50%) was recovered as a yellow oil.

IR υ_{max}/cm^{-1}: 1583 (C═O); ^{1}H NMR (CD_3OD): δ_H 1.66 (4H, m, 2 CH_2); 2.10 (3H, s, CH_3-pz); 2.18 (3H, s, CH_3-pz); 2.62 (2H, m, CH_2); 2.85 (2H, t, CH_2); 3.13 (2H, c, CH_2); 3.51 (2H, m, CH_2); 4.02 (2H, t, CH_2); 5.67 (1H, s, H4-pz); 7.74–7.91 (15H, m, PPh_3); ^{13}C NMR (CD_3OD): δ_c 11.19 (CH_3-pz), 13.33 (CH_3-pz), 21.12 (d, $J_{P\text{-}C}$ = 4.5 Hz, CH_2), 22.24 (d, $J_{P\text{-}C}$ = 51 Hz, CH_2), 28.73 (d, $J_{P\text{-}C}$ = 17 Hz, CH_2), 47.60 (CH_2), 54.49 (CH_2), 54.97 (CH_2), 59.93 (CH_2), 105.76 (C_4-pz)), 120.00 (d, $J_{P\text{-}C}$ = 87 Hz, PPh_3), 131.50 (d, $J_{P\text{-}C}$ = 12 Hz, PPh_3), 134.85 (d, $J_{P\text{-}C}$ = 10 Hz, PPh_3), 136.13 (d, $J_{P\text{-}C}$ = 3.0 Hz, PPh_3), 141.28 ($C_{3/5}$-pz)), 148.30 ($C_{3/5}$-pz)), 178.86 (C═O); ^{31}P NMR (CD_3OD) δ_P 25.15; ESI/MS (+) (m/z): 514.3 M^+, calcd for $C_{31}H_{37}N_3O_2P$ = 514.3; Anal. Calc. for $C_{31}H_{36}N_3PO_2$: C, 72.49; H, 7.06; N, 8.18, Found: C, 72.36; H, 6.91; N, 8.05.

5.1.8. 2-((2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)(4-((triphenylphosphonio)methyl)benzyl)amino)acetate, **L^4H**

Compound **L^4H** (68%, 302 mg) was prepared as above described for **L^3H**, starting from 432 mg (0.66 mmol) of **6**.

IR υ_{max}/cm^{-1}: 1586 (C═O); ^{1}H NMR (CD_3OD): δ_H 2.05 (3H, s, CH_3-pz); 2.18 (3H, s, CH_3-pz); 3.18 (2H, t, CH_2); 3.42 (2H, s, CH_2); 3.98 (2H, s, CH_2); 4.55 (2H, t, CH_2); 4.96 (2H, d, CH_2); 5.77 (1H, s, H4-pz); 6.97 (2H, m, Ph); 7.22 (2H, m, Ph); 7.61–7.91 (15H, m, PPh_3); ^{13}C NMR (CD_3OD): δ_c 10.93 (CH_3-pz), 13.28 (CH_3-pz), 30.33 (d, $J_{P\text{-}C}$ = 47 Hz, CH_2), 45.84 (CH_2), 54.80 (CH_2), 56.58 (CH_2), 59.34 (CH_2), 106.17 (C_4-pz)), 119.11 (d, $J_{P\text{-}C}$ = 86 Hz, PPh_3), 128.72 (d,

[1] Although the H analysis data for this compound are somewhat unsatisfactory, the ESI-MS results and ^{1}H, ^{13}C and ^{31}P NMR data clearly support the proposed formulation.

$J_{P\text{-}C}$ = 8.8 Hz, C_{Ph}), 131.35 (d, $J_{P\text{-}C}$ = 18 Hz, PPh_3), 132.35 (d, $J_{P\text{-}C}$ = 3.3 Hz, C_{Ph}), 132.78 (d, $J_{P\text{-}C}$ = 9.0 Hz, PPh_3), 134.52 (d, $J_{P\text{-}C}$ = 6.0 Hz, C_{Ph}), 135.41 (d, $J_{P\text{-}C}$ = 14 Hz, PPh_3), 136.45 (d, $J_{P\text{-}C}$ = 3.2 Hz, C_{Ph}), 141.49 ($C_{3/5}$-pz)), 148.90 ($C_{3/5}$-pz)), 174.19 (C═O); ^{31}P NMR (CD_3OD) δ_P 23.79; ESI/MS (+) (m/z): 562.3 M^+, calcd for $C_{35}H_{37}N_3O_2P$ = 562.3; Anal. Calc. for $C_{35}H_{36}N_3PO_2$: C, 74.71; H, 6.63; N, 7.47, Found: C, 74.52; H, 6.25; N, 7.37.

5.1.9. General procedure for the synthesis of the Re complexes, **Re1**–**Re4**

fac-[$Re(H_2O)_3(CO)_3$]Br was reacted with equimolar amounts of $\mathbf{L^1}$–$\mathbf{L^4H}$ in refluxing methanol (**Re1**–**Re2**) or H_2O (**Re3**–**Re4**) for 18 h. After this time, the solvent was removed under vacuum and the complexes were purified by RP-HPLC, except **Re1** that was purified by washing with adequate solvents.

5.1.9.1. fac-[Re(CO)$_3$(κ^3-L^1)]Br$_2$, **Re1**. **Re1** (59%, 38 mg) was purified by washing with n-hexane and H_2O.

IR υ_{max}/cm^{-1}: 1897, 2025 (C≡O); ^{1}H NMR (CD_3OD): δ_H 1.77 (2H, m, CH_2), 2.03 (1H, m, CH_2), 2.27 (1H, m, CH_2), 2.37 (3H, s, CH_3-pz), 2.43 (3H, s, CH_3-pz), 2.66 (1H, m, CH_2), 2.74 (1H, m, CH_2), 2.86 (2H, m, CH_2), 3.16 (1H, m, CH_2), 3.44 (1H, m, CH_2), 3.62 (4H, m, CH_2 + CH_2 + CH_2), 4.04 (1H, m, NH_2), 4.22 (1H, m, CH_2), 4.52 (1H, m, CH_2), 5.46 (1H, m, NH_2), 6.19 (1H, s, H4-pz), 7.74–7.93 (15H, m, PPh_3); ^{13}C NMR (CD_3OD): δ_c 11.63 (CH_3-pz), 16.05 (CH_3-pz), 21.17 (CH_2), 22.81 (d, $J_{P\text{-}C}$ = 52 Hz, CH_2), 26.48 (d, $J_{P\text{-}C}$ = 17 Hz, CH_2), 43.66 (CH_2), 48.45 (CH_2), 54.09 (CH_2), 62.67 (CH_2), 67.04 (CH_2), 109.16 (C_4-pz)), 119.65 (d, $J_{P\text{-}C}$ = 81 Hz, PPh_3), 131.55 (d, $J_{P\text{-}C}$ = 12 Hz, PPh_3), 134.87 (d, $J_{P\text{-}C}$ = 10 Hz, PPh_3), 136.29 (d, $J_{P\text{-}C}$ = 3.0 Hz, PPh_3), 145.29 ($C_{3/5}$-pz), 155.00 ($C_{3/5}$-pz), 193.66 (C≡O), 194.73 (C≡O), 195.15 (C≡O); ^{31}P NMR (CD_3OD) δ_P 24.64; ESI/MS (+) (m/z): 385.2 M^{2+}, calcd for $ReC_{34}H_{40}N_4O_3PBr_2$ = 770.3; Anal. Calc. for $ReC_{34}H_{40}N_4O_3PBr_2$: C, 43.93; H, 4.34; N, 6.03, Found: C, 43.79; H, 4.45; N 6.37.

5.1.9.2. fac-[Re(CO)$_3$(κ^3-L^2)](CF$_3$COO)$_2$, **Re2**. **Re2** (45%, 62 mg) was purified by RP-HPLC. Method: 0–3 min, 75% A; 3–28 min, 75%–50% A; 28–33 min, 50%; 33–48 min 50–0% A; 48–58 min 0% A.

IR υ_{max}/cm^{-1}: 1899, 2027 (C≡O); ^{1}H NMR (CD_3OD): δ_H 2.36 (3H. s, CH_3-pz); 2.45 (3H, s, CH_3-pz); 2.52 (3H, m, CH_2 + CH_2 + CH_2); 2.90 (1H, d, CH_2); 3.02 (2H, m, CH_2 + CH_2); 4.23 (1H, m, CH_2); 4.35 (1H, m, CH_2); 4.53 (1H, m, CH_2); 4.61 (1H, m, CH_2); 4.80 (2H, d, CH_2); 5.61 (2H, bs, NH_2); 6.19 (1H, s, $H_{4\text{-}pz}$); 7.12 (2H, d, Ph); 7.41 (2H, d, Ph); 7.67–7.92 (15H, m, PPh_3); ^{13}C NMR (CD_3OD): δ_c 11.54 (CH_3-pz), 16.17 (CH_3-pz), 30.15 (d, $J_{P\text{-}C}$ = 48 Hz, CH_2), 43.28 (CH_2), 47.17 (CH_2), 51.90 (CH_2), 62.13 (CH_2), 69.47 (CH_2), 109.09 (C_4-pz), 118.97 (d, $J_{P\text{-}C}$ = 87 Hz, PPh_3), 118.53 (C_{TFA}), 130.63 (d, $J_{P\text{-}C}$ = 8.3 Hz, C_{Ph}), 131.43 (d, $J_{P\text{-}C}$ = 13 Hz, PPh_3), 132.59 (d, $J_{P\text{-}C}$ = 5.3 Hz, C_{Ph}), 133.35 (d, $J_{P\text{-}C}$ = 10 Hz, PPh_3), 134.58 (d, $J_{P\text{-}C}$ = 3.0 Hz, C_{Ph}), 135.34 (d, $J_{P\text{-}C}$ = 2.3 Hz, PPh_3), 136.57 (d, $J_{P\text{-}C}$ = 3.8 Hz, C_{Ph}), 145.62 ($C_{3/5}$-pz), 155.50 ($C_{3/5}$-pz)), 161.70 (C_{TFA}), 193.72 (C≡O), 194.60 (C≡O), 195.21 (C≡O); ^{31}P NMR (CD_3OD) δ_P 24.05; ESI/MS (+) (m/z): 409.1 M^{2+}, calcd for $ReC_{38}H_{40}N_4O_3P$ = 818.3; Anal. Calc. for $ReC_{42}H_{40}N_4O_7PF_6.CF_3COOH$: C, 45.64; H, 3.57; N 4.84, Found: C, 45.66; H, 3.05; N, 4.77.

5.1.9.3. fac-[Re(CO)$_3$(κ^3-L^3)](CF$_3$COO), **Re3**. **Re3** (45%, 62 mg) was purified by RP-HPLC. Method: 0–3 min, 75% A; 3–3.1 min, 75%–50% A; 3.1–9.0 min, 50%; 9.0–9.1 min 50–35% A; 9.1–30 min 35–0% A; 30–35 min 0% A.

IR υ_{max}/cm^{-1}: 1644 (C═O) 1981, 2027 (C≡O); ^{1}H NMR (CD_3OD): δ_H 1.76 (2H, m, CH_2); 2.01 (2H, m, CH_2); 2.32 (3H, s, CH_3-pz); 2.44 (3H, s, CH_3-pz); 2.56 (1H, m, CH_2); 3.23 (1H, d, CH_2); 3.45 (5H, m, CH_2 + CH_2 + CH_2); 3.64 (1H, d, CH_2); 4.34 (2H, m, CH_2); 6.13 (1H, s, H4-pz); 7.73-7.93 (15H, m, PPh_3); ^{13}C NMR (CD_3OD): δ_c 11.34 (CH_3-pz); 15.72 (CH_3-pz); 21.05 (d, $J_{P\text{-}C}$ = 3.0 Hz, CH_2); 22.75 (d, $J_{P\text{-}C}$ = 52 Hz, CH_2); 26.28 (d, $J_{P\text{-}C}$ = 17 Hz, CH_2); 46.74 (CH_2); 56.84 (CH_2); 63.86 (CH_2); 66.34 (CH_2); 108.89 (C_4-pz)); 115.37 (C_{TFA}); 119.67 (d, $J_{P\text{-}C}$ = 87 Hz, PPh_3); 131.62 (d, $J_{P\text{-}C}$ = 12 Hz, PPh_3); 134.84 (d, $J_{P\text{-}C}$ = 11 Hz, PPh_3); 136.42 (d, $J_{P\text{-}C}$ = 3.0 Hz, PPh_3); 145.07 ($C_{3/5}$-pz)); 155.22 ($C_{3/5}$-pz)); 161.37 (C_{TFA}); 181.54 (C═O); 195.09 (C≡O); 196.38 (C≡O); 196.85 (C≡O); ^{31}P NMR (CD_3OD) δ_P 24.56; ESI/MS (+) (m/z): 784.1 M^+, calcd for $ReC_{34}H_{36}N_3O_5P$ = 784.2; Anal. Calc. for $ReC_{36}H_{36}N_3O_7PF_3.3CF_3COOH$: C, 40.72; H, 3.17; N, 3.39, Found: C, 40.89; H, 2.94; N 3.55.

5.1.9.4. fac-[Re(CO)$_3$(κ^3-L^4)](CF$_3$COO), **Re4**. **Re4** (27%, 30 mg) was purified by RP-HPLC. Method: 0–3 min. 75% A, 3–3.1 min. 75%–50% A, 3.1–9.0 min. 50% A, 9.0–9.1 min 50–35% A, 9.1–30 min 35-0% A, 30–35 min 0% A.

IR υ_{max}/cm^{-1}: 1647 (C═O) 1904, 2020 (C≡O); ^{1}H NMR (CD_3OD): δ_H 2.25 (1H, m, CH_2), 2.31 (3H, s, CH_3-pz), 2.48 (3H, s, CH_3-pz), 3.04 (1H, d, CH_2), 3.25 (1H, m, CH_2), 3.94 (1H, d, CH_2), 4.41 (2H, m, CH_2), 4.62 (2H, m, CH_2), 4.97 (2H, m, CH_2), 6.13 (1H, s, H4-pz), 7.15 (2H, d, Ph), 7.48 (2H, d, Ph), 7.63–7.92 (15H, m, PPh_3); ^{13}C NMR (CD_3OD): δ_c 10.20 (CH_3-pz), 14.87 (CH_3-pz), 29.02 (d, $J_{P\text{-}C}$ = 48 Hz, CH_2), 45.05 (CH_2), 55,19 (CH_2), 62.73 (CH_2), 67.68 (CH_2), 107.61 (C_4-pz), 117.86 (d, $J_{P\text{-}C}$ = 87 Hz, PPh_3), 119.03 (C_{TFA}), 128.83 (d, $J_{P\text{-}C}$ = 12 Hz, Ph), 130.30 (d, $J_{P\text{-}C}$ = 14 Hz, PPh_3), 131.43 (d, $J_{P\text{-}C}$ = 4.5 Hz, Ph), 131.92 (d, $J_{P\text{-}C}$ = 10 Hz, PPh_3), 133.10 (d, $J_{P\text{-}C}$ = 5.8 Hz, Ph), 134.19 (d, $J_{P\text{-}C}$ = 10 Hz, PPh_3), 135.43 (d, $J_{P\text{-}C}$ = 2.2 Hz, Ph), 144.16 ($C_{3/5}$-pz)), 154.35 ($C_{3/5}$-pz)), 160.70 (C_{TFA}), 180.00 (C═O), 193.90 (C≡O), 194.89 (C≡O), 195.63 (C≡O); ^{31}P NMR (CD_3OD) δ_P 24.14; ESI/MS (+) (m/z): 832.2 M^+, calcd for $ReC_{38}H_{36}N_3O_5P$ = 832.2; Anal. Calc. for $ReC_{40}H_{36}N_3O_7PF_3.2CF_3COOH$: C, 45.06; H, 3.27; N, 3.58, Found: C, 45.16; H 3.56; N, 3.37.

5.2. Synthesis of ^{99m}Tc(I) complexes, **Tc1**–**Tc4**

5.2.1. General method

In a nitrogen-purged glass vial, 40 μL of a 4.4 × 10^{-3} M aqueous solution of $\mathbf{L^1}$–$\mathbf{L^4H}$ was added to 400 μL of a solution of the organometallic precursor *fac*-[$^{99m}Tc(H_2O)_3(CO)_3]^+$ (1–2 mCi) in saline at pH 7.4 (**Tc1**–**Tc2**) or at pH 5.5 (**Tc3**–**Tc4**). The reaction mixture was heated to 100 °C for 30 min, cooled to room temperature and analyzed by RP-HPLC. Method: 0–3 min, 100% A; 3–3.1 min, 100%–75% A; 3.1–9 min, 75% A; 9–9.1 min 75%–66% A; 9,1–20 min, 66%–0% A; 20–25 min, 0% A; 25–25.1 min, 0%–100% A; 25.1–30 min, 100% A.

5.3. Distribution coefficient measurements

The log $D_{o/w}$ values of complexes **Tc1**–**Tc4** were determined by the "shake flask" method under physiological conditions (n-octanol–0.1 M PBS, pH 7.4) [41].

5.4. Cell culture

B16-F1 murine melanotic melanoma, HEK human embryonic kidney (ECACC, UK), MCF-7 human breast cancer (ATCC, Spain) and MCF-7/*MDR1* (kindly provided by Professor David Piwnica-Worms, *Washington University School of Medicine*, St Louis, Missouri, USA) [42], were grown in DMEM (Dulbecco's modified eagle's medium) containing GlutaMax I, while PC-3 human prostate cancer (ATCC, Spain), H69 and H69/Lx4 small cell lung human carcinoma (ECACC, UK) were grown in RPMI 1640 medium. The media were supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin antibiotic solution (all from Invitrogen). The MCF-7/*MDR1* and H69/Lx4 media were supplemented with geneticin 1 mg/mL (Invitrogen) and doxorubicin 0.4 μg/mL (Sigma-Aldrich), respectively. Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C (Heraeus, Germany), with the medium changed every other day. For adherent cell lines (B16-F1, A375, PC-3, MCF-7 and MCF-7/*MDR1*) the cells were adherent in monolayers and, when confluent,

were harvested from the cell culture flasks with trypsin–EDTA (Invitrogen) and seeded in new culture flasks.

For the suspension cell lines, H69 and H69/Lx4, the cells were transferred from culture flasks to plastic tubes, centrifuged for 5 min at 850 g, and the cell pellet resuspended in fresh medium and seeded in new culture flasks.

5.5. Cellular uptake studies

Prior to the cellular uptake assays of the ^{99m}Tc complexes (**Tc1**–**Tc4**), their stability in the cell culture media was evaluated by incubating the complexes with the media at 37 °C for 4 h. Then, an aliquot of the resulting solution was analyzed by ITLC using 1 M HCl/MeOH (5:95) as eluent, and the distribution of radioactivity on the strips was monitored using a Berthold LB 505 detector coupled to a radiochromatogram scanner. No decomposition was observed since single peaks were detected at $R_f = 0.70–0.85$ corresponding to intact **Tc1**–**Tc4**.

Cellular uptake assays of the ^{99m}Tc complexes (**Tc1**–**Tc4**) were performed using PC-3, MCF-7 and MCF-7/*MDR1*cells seeded at a density of 0.4 million/0.5 mL medium per well in 24-well plates and allowed to attach overnight. After that period, the medium was removed and replaced by fresh medium containing approximately 25 kBq/mL of each ^{99m}Tc complex. The cells were incubated again under humidified 5% CO_2 atmosphere, at 37 °C for a period of 15 min to 4 h. After that incubation period the cells were washed twice with cold PBS, lysed with 0.1 M NaOH and the cellular extracts were counted for radioactivity. Each experiment was performed in quadruplicate. Cellular uptake data were expressed as an average plus the standard deviation of % of total per million of cells.

The cellular uptake of **Tc1**, **Tc3** and **Tc4** was also studied on the H69 and H69-LX4 cell lines. For these cells, suspensions were prepared in the respective culture media at a concentration of 2×10^6 cells/mL. The suspensions were then incubated for 1 h in humidified atmosphere with 95% air and 5% CO_2 at 37 °C prior to the evaluation of cellular uptake. The uptake studies were performed after removal of culture medium followed by incubation with the complexes under study, diluted in the respective culture media (0.2 MBq/mL) for 15 min, 30 min and 1 h with constant stirring at 37 ° C (thermostated bath). After the incubation period, samples of 200 μL were collected (in triplicate) to microfuge tubes containing 500 μL of cold PBS. The samples were centrifuged (Eppendorf Centrifuge 5417R) for 3 min at 9400 *g*. The supernatant was discarded and the pellet was washed again in 500 μL of cold PBS. Finally the samples were again centrifuged (10 min, 9400 *g*) and the activity of the cell pellet was measured in a gamma counter.

5.6. Mitochondrial isolation

Mitochondria were isolated from PC-3 and HEK cells in culture using a commercial kit (Mitochondria Isolation Kit for Cultured Cells, Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions. Briefly, after centrifugation (Centrifuge 5804R, Eppendorf) of a cell suspension with $10–15 \times 10^6$ cells for 2 min at 850 g the cell pellet was resuspended in 450 μL of Reagent A supplemented with a cocktail of protease inhibitors (Roche, Basel, Switzerland) and transferred to a microfuge tube. The solution was vortexed at medium speed for 5 s and was incubated on ice for exactly 2 min. After this period, 20 μL of Reagent B was added and the sample was vortexed at maximum speed for 5 s. The sample vial was then incubated on ice for 5 min, vortexing every minute at maximum speed. Then, 450 μL of Reagent C supplemented with a cocktail of protease inhibitors was added and the tube was inverted several times. The sample was centrifuged at 700 *g* (Centrifuge 5417R, Eppendorf) for 10 min at 4 °C and the supernatant was transferred to a new tube and centrifuged again at 12,000 *g* for 5 min. The supernatant was discarded (cytosolic fraction) and pellet corresponded to the mitochondrial fraction. The pellet was washed again with 300 μL of Reagent C and centrifuged to 12,000 *g* for 5 min. The pellet containing the isolated mitochondria was kept on ice before being used in studies of mitochondrial uptake.

5.7. Mitochondrial uptake

The mitochondrial pellet isolated from approximately $10–15 \times 10^6$ cells was resuspended in 2 mL of culture medium. The mitochondrial suspension was incubated with the complexes under study for 15 min, 30 min, 1 h and2 h and 3 h in constant agitation at 37 °C. After the incubation period aliquot samples of 200 μL (in duplicate) were collected to microfuge tubes containing 500 μL of cold PBS. The samples were centrifuged for 3 min at 9400 *g* (Centrifuge 5417R, Eppendorf). After discarding the supernatant, the pellet was washed again with 500 μL of cold PBS, and samples centrifuged (10 min, 9400 g). The activity of the cell pellet was measured in gamma counter. The % of mitochondrial uptake was calculated as described for cellular uptake.

5.8. Alteration of cellular and mitochondrial potential membrane

The assays were performed in PC-3 cells, using buffer solutions with different concentrations of K^+ (5, 60, 120 mM). A normal extracellular concentration (i.e. in the culture medium) of K^+ was considered to be 5 mM [43,44]. For this concentration of K^+ (5 mM) a buffer was used with the following composition (mM): NaCl (145), KCl (5.4), $CaCl_2$ (1.2), $MgSO_4$ (0.8), NaH_2PO_4 (0.8), dextrose (5.6), HEPES (5) and 1% (v/v) fetal bovine serum with a final pH of 7.4. The solutions of 60 and 120 mM K^+ were prepared by replacement of NaCl by K^+-aspartate at the desired concentration, in order to avoid cytosolic and mitochondrial swelling [34]. For these solutions the concentration of Ca^{2+} was further reduced to 0.1 mM and Cl^- was maintained at a concentration of 20 mM. Stock solutions of valinomycin, nigericin, ouabain and CCCP were prepared in DMSO.

The uptake studies were performed after removal of culture medium followed by incubation of cells with the complex under study. The ^{99m}Tc complexes were incubated with buffer solutions of different concentrations of K^+ (5, 60, 120 mM) in a total volume of 500 μL. When indicated, valinomycin (1 μg/mL) was added to the buffer solution with 120 mM K^+. After 4 h of incubation the procedure followed was the same as described above for cellular uptake.

In a different set of assays Valinomycin, nigericin, ouabain and CCCP were added after the incubation of cells with ^{99m}Tc complexes in culture medium for 4 h. After this period, valinomycin (1 μg/mL), nigericin (5 μg/mL), ouabain (100 μM) and CCCP (5 μM and 10 μM) were added and the cell further incubated at 37 °C for 15 min. After this second incubation period, the medium was removed and the procedure was followed as described for cellular uptake.

5.9. Evaluation of Pgp expression

Western blot experiments were performed to evaluate the levels of expression of Pgp in the following cell lines: B16-F1, A375, PC-3, MCF-7 and MCF-7/*MDR1*, H69 and H69/Lx4. Cells were lysed in Cell Lytic-MT Extraction reagent (Sigma) supplemented with complete protease inhibitor cocktail tablets (Roche). After 15 min on ice, lysates were centrifuged at 14,000 *g* for 10 min at 4 °C to pellet the cellular debris and the supernatants collected for further use. The total protein content was determined in aliquots of cell lysates by using the DC Protein Assay Kit (Biorad, Hercules, California, USA), based on the method of Lowry modified. For the standard calibration curve ultrapure bovine serum albumin (Biorad) was used. Aliquots of protein (30 μg) from each sample were analyzed using standard western blot procedures. Briefly, protein extracts were subjected to electrophoresis on a 7% SDS-polyacrylamide gel and transferred

electrophoretically onto nitrocellulose membranes. The blots were blocked with PBS-T (phosphate buffered saline with Tween 20) containing 5% nonfat dry milk for 1 h. Then, the blotting membranes were incubated with primary antibodies against Pgp (1:350, C219, mouse monoclonal, Abcam) and actin (1:8000, mouse monoclonal, Sigma) overnight. Membranes were washed with PBS-T (phosphate buffered saline with Tween 20) and incubated for 1 h with secondary antibody (goat anti-mouse IgG-HRP, Biorad) diluted 1:3000. Finally, membranes were developed using the SuperSignal WetsPico Substrate kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

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