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Original article

# TETA analogue containing one methylenephosphonate pendant arm: Lanthanide complexes and biological evaluation of its $^{153}$Sm and $^{166}$Ho complexes

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ABSTRACT

The thermodynamic stability constants of complexes of 1,4,8,11-tetraazacyclotetradecane-1,4,8-triacetic-11-methylphosphonic acid ($H_5$te3a1p) with $La^{3+}$, $Sm^{3+}$, $Gd^{3+}$, $Ho^{3+}$ and $Lu^{3+}$ metal ions were determined by potentiometric titrations at 298.2 K and with ionic strength 0.10 M in $N(CH_3)_4NO_3$. The complexes are formed relatively fast and the stability constants exhibited are good although lower than those found for the related ligands $H_4$teta and $H_8$tetp. At physiological pH the completely deprotonated complex species predominate, unlike what happens with the other mentioned ligands. The $^{153}$Sm and $^{166}$Ho complexes, $^{153}$Sm/$^{166}$Ho-te3a1p, were synthesised quantitatively at pH 9 and 70 °C, and have shown good *in vitro* stability in human serum and physiological solutions except phosphate buffer (pH 7.4). The *in vivo* behaviour indicated that both complexes have a similar biological pattern, showing a slow tissue clearance, slow rate of total radioactivity excretion and some *in vivo* instability, although with some differences in their extend. These results indicate that the replacement of one acetate pendant arm of $H_4$teta by a methylphosphonate one does not provide promising chelators to stabilize radiolanthanides for *in vivo* application.



## 1. Introduction

Acyclic and macrocyclic polyamines are excellent ligands for binding to metal ions, as they generally form strong complexes with a wide range of metal ions. Among macrocyclic ligands, those based on the cyclen (1,4,7,10-tetraazacyclododecane) and cyclam (1,4,8,11-tetraazacyclotetradecane) frameworks have been most widely studied, particularly the ones appended with arms containing carboxylic and phosphonic acid moieties or its derivatives. Interest in this type of ligands arises mainly from the possible applications of their metal complexes in the medical field, such as in contrast agents for magnetic resonance imaging (MRI) or in radiopharmaceuticals for nuclear medicine imaging and therapy [1–8].

In order to be used in medical applications, metal complexes of acyclic and macrocyclic ligands must be chemically stable under physiological conditions to ensure a desired biodistribution of the chelate, avoid the release of potentially toxic metal ions such as $Gd^{3+}$ used in MRI which accumulates in calcified tissues, and also avoid the transchelation of endogenous metal ions. Thus, such complexes should present high thermodynamic stability as well as strong kinetic inertness to dissociation, and the latter is generally favoured in macrocyclic ligands [4,7,8]. Moreover, when specific targeting is pursued the chelator must be easily functionalized with chemical groups or conjugated to biomolecules with high affinity and selectivity for definite molecular targets [3,4,9]. Radiolanthanides are of great interest in nuclear medicine, and among them $^{153}$Sm and $^{166}$Ho are particularly attractive for therapeutic applications due to their favourable decay properties (strong β emitters with half-lives of respectively 1.95 and 1.1 days) and easy production in a nuclear reactor. However, their complexes often suffer from limited solubility and slow complexation rates, especially those of macrocyclic ligands containing phosphonate pendant arms. As a general rule, a potential radiopharmaceutical must be prepared in quick and mild conditions, and should be widely stable in physiological media and present good chemical properties to ensure an efficient clearance from the body. Therefore, solubility and formation kinetics become also decisive factors when designing such a drug [3,4,10,11].

Aminomethylphosphonates are known to have high affinity to bone and the metal complexes of ligands containing such groups are often retained there [4]. A $^{153}$Sm complex of an acyclic

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polyaminophosphonate is a bone-seeking radiopharmaceutical available for clinical use (Quadramet®), while others have been studied [12]. However, it presents a serious drawback of low kinetic inertness, and attempting to overcome that issue several lanthanide complexes of new macrocyclic ligands bearing phosphonate pendant arms have been studied with diverse success [13–22].

Macrocyclic ligands containing methylphosphonate pendant arms form lanthanide complexes with higher thermodynamic stability constants in comparison to those with acetate arms. Such property, which arises from the increased basicity of macrocyclic amines when appended with methylphosphonate moieties, may or may not translate into better metal ion complexation. To compare directly the performance of different ligands it is necessary to take into account their basicity. That can be done by determining the concentration of free metal ion in solution (pM) for each metal complex system at a given pH, a procedure which requires accurate knowledge of the full set of thermodynamic stability constants. However, the presence of several phosphonate moieties in a ligand causes an increase in the complexity of its speciation in solution and consequently of any possible medical application, as many protonation equilibria appear and water solubility is considerably lowered for the ligand alone as well as for its complexes.

Following our previous work in the field, we decided to study the lanthanide complexes of an analogue of $H_4$teta with a single phosphonate replacing one carboxylate moiety, the ligand 1,4,8,11-tetraazacyclotetradecane-1,4,8-triacetic-11-methylphosphonic acid ($H_5$te3a1p), see Scheme 1. We aimed to find, on the one hand, if the presence of the phosphonate moiety leads to a better complexation of lanthanide ions, and on the other hand, how does such modification affect the production and *in vivo* behaviour of complexes with radiolanthanide ions relevant for nuclear medicine. The introduction of a methylphosphonate pendant arm has also the additional benefit of producing a potential bifunctional chelator, as the phosphonate moiety can be easily replaced by a monophosphinate one in order to allow attachment to a suitable biomolecule. This procedure has already been demonstrated by using *p*-aminophenyl phosphinate or propionyl phosphinate pendant arms where the distant amino or carboxylic functions may be used for bioconjugation while maintaining coordination ability similar to phosphonate pendants [23–25]. In this work we report on the thermodynamic stability of selected lanthanide complexes of the mentioned ligand and on the preparation and biological evaluation of its $^{153}$Sm and $^{166}$Ho complexes *in vitro* and *in vivo*. For a better understanding, the properties found are also compared with those reported for the related ligands $H_4$teta and $H_8$tetp.

## 2. Experimental

### 2.1. Thermodynamic stability studies

#### 2.1.1. Reagents and equipment

Chemical reagents were obtained from commercial sources in analytical grade. Purified water was obtained from a Millipore Milli-Q demineralization system. The $H_5$te3a1p ligand was obtained as described [26], and a solution of it was prepared in water at $2.00 \times 10^{-3}$ M. Metal ion solutions were prepared at 0.025–0.050 M from nitrate salts in water, kept in excess of $HNO_3$ and standardized by titration with $Na_2H_2$edta [27]. Carbonate-free solutions of the titrant, $N(CH_3)_4OH$, were obtained at *ca.* 0.100 M by treating freshly prepared silver oxide with a solution of $NMe_4I$ under nitrogen. These solutions were standardized by titration with standard $HNO_3$ solutions using Gran's method [28] and discarded every time the carbonate was about 2.0% of the total amount of base. For the backtitrations a 0.100 M standard solution of $HNO_3$ was prepared from a commercial ampoule. $N(CH_3)_4NO_3$ was prepared by neutralisation of a commercial $N(CH_3)_4OH$ solution with $HNO_3$. The setup used for potentiometric titrations has been described before [26]. The ionic strength ($I$) of the experimental solutions was kept constant at $0.10 \pm 0.01$ M with $N(CH_3)_4NO_3$, the temperature was controlled at $298.2 \pm 0.1$ K and atmospheric $CO_2$ was excluded from the cell during titrations by passing purified $N_2$ across the top of the experimental solutions.

#### 2.1.2. Measurements

The $[H^+]$ of the solutions was determined by measurement of the electromotive force of the cell ($E = E'^0 + Q\log[H^+] + E_j$). The term pH is defined as $-\log [H^+]$, and $E'^0$ and $Q$ were determined by titration of a solution with known hydrogen ion concentration at the same ionic strength in the acidic pH range. The liquid junction potential, $E_j$, was found to be negligible under the experimental conditions used. The value of $K_w$ was found to be equal to $10^{-13.80}$ by titration of a solution of known hydrogen ion concentration at the same ionic strength in the alkaline pH range, considering $E'^0$ and $Q$ valid for the entire pH range. Measurements were carried out using 25.00 mL of *ca.* $2.00 \times 10^{-3}$ M ligand solutions diluted to a final volume of 30.00 mL, in the presence of each lanthanide ion for which the metal-to-ligand ratio was 1:1. At the end of each direct titration (from acidic to alkaline pH), a backtitration of the same solution was performed in order to check if equilibrium was attained throughout all the pH range. A minimum of two replicate measurements was taken for each system. Each titration curve contained typically 50–60 points in the 2.5–11.5 pH range.

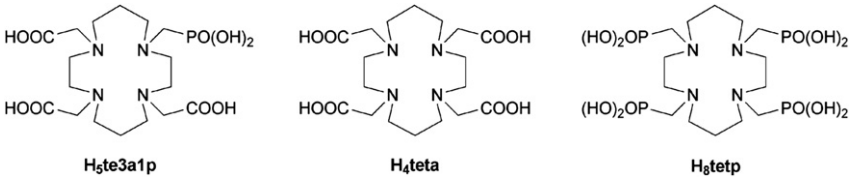
#### 2.1.3. Calculation of equilibrium constants

The calculation of overall equilibrium constants $\beta_{M_mH_hL_l}$ (being $\beta_{M_mH_hL_l} = [M_mH_hL_l]/[M]^m[H]^h[L]^l$ and $\beta_{MLH_{-1}} = \beta_{ML(OH)} \times K_w$) was done by fitting the potentiometric data from titrations with the HYPERQUAD program [29]. Species distribution diagrams were plotted from the calculated constants with the HYSS program [30]. The differences, in log units, between the values of protonated (or hydrolysed) and non-protonated constants provide the stepwise ($\log K$) reaction constants (being $K_{M_mH_hL_l} = [M_mH_hL_l]/[M_mH_{h-1}L_l][H]$). The errors quoted are the standard deviations of the overall stability constants calculated by the fitting program from all the experimental data for each system.

### 2.2. Radiochemical studies

#### 2.2.1. Reagents and equipment

Enriched $Sm_2O_3$ (98.4% $^{152}$Sm) and natural $Ho_2O_3$ (99.9%) were obtained from Campro Scientific and Strem Chemicals, respectively.




**Scheme 1.** Structures of the ligands discussed in this work.

The $^{153}$Sm and $^{166}$Ho activities produced after irradiation were measured by an ionization chamber (Aloka Curiemeter IGC-3). The radionuclidic purity of the $^{153}$Sm and $^{166}$Ho solutions was assessed by γ spectrometry using a Ge(Li) detector coupled to an Accuspec B Canberra multichannel analyzer [15]. The radiolabelling efficiency and stability evaluation of the radiocomplexes were studied by ascending instant thin layer chromatography using silica gel (ITLC-SG) strips (Polygram, Macherey-Nagel). The radioactive distribution on the ITLC-SG strips was detected using a Berthold LB 505 γ detector coupled to a radiochromatogram scanner.

*2.2.2. Production of $^{153}$Sm and $^{166}$Ho*

$^{153}$Sm ($T_{1/2} = 46.8$ h; $\beta = 0.67$ MeV, 34%; 0.71 MeV, 44%; 0.81 MeV, 21%; $\gamma = 0.103$ MeV, 28%) and $^{166}$Ho ($T_{1/2} = 26.8$ h; $\beta = 1.85$ MeV, 51%; 1.77 MeV, 48%; $\gamma = 0.081$ MeV, 7.5%; 1.38 MeV, 0.9%) were produced by irradiating isotopically enriched $^{152}$Sm $(NO_3)_3$ or natural $Ho(NO_3)_3$ in the ITN Portuguese Research Reactor (RPI). The samples to be irradiated were prepared by dissolving $^{152}Sm_2O_3$ and $Ho_2O_3$ in $HNO_3$, followed by evaporation to dryness. Irradiation was typically performed at 1 MW, thermal neutron flux of $\sim 1 \times 10^{13}$ neutron cm$^{-2}$ s$^{-1}$ and epithermal neutron flux of $\sim 2 \times 10^{11}$ neutron cm$^{-2}$ s$^{-1}$. The specific activity of the radionuclides, after 3 h irradiation and at EOB, was 110–150 MBq/mg for $^{153}$Sm and 220–260 MBq/mg for $^{166}$Ho. Following irradiation, the radionuclide salts were reconstituted with bidistilled water to produce stock solutions for complex synthesis. An aliquot of each $^{153}$Sm and $^{166}$Ho solutions was taken for the assessment of radionuclidic purity by γ spectrometry.

*2.2.3. Synthesis of radiolabelled complexes*

The $^{153}$Sm and $^{166}$Ho complexes were prepared by dissolving the ligand (5 mg) in bidistilled water (0.3 mL) followed by the addition of an adequate amount of $^{153}$Sm or $^{166}$Ho solutions to achieve a 1:2 metal-to-ligand molar ratio. The pH was adjusted with a freshly prepared 1.0 M NaOH solution and the volume was completed to 0.5 mL with bidistilled water. The final ligand concentrations were 20 mM. Labelling efficiency, reaction kinetics and stability of the radiolanthanide complexes were determined by ITLC-SG strips using MeOH:$H_2O$:conc. aq. $NH_3$ (2:4:0.2) mixture as the eluent. In this system, the $^{153}$Sm/$^{166}$Ho complexes migrate with $R_f = 1.0$, while $^{153}Sm(NO_3)_3$ and $^{166}Ho(NO_3)_3$ remain at the origin. The colloidal radioactive forms also remain at the origin, and their identification was assessed by ITLC-SG strips developed with saline as the eluent. In this system, the radiolanthanide complexes and $^{153}Sm(NO_3)_3$ and $^{166}Ho(NO_3)_3$ migrate with $R_f = 1.0$.

*2.2.4. In vitro studies*

The *in vitro* stability of the radiolanthanide complexes was evaluated in different physiological media at 37 °C and at various time points (up to 72 h). Typically, 50 μL of each $^{153}$Sm or $^{166}$Ho complex was added to 100 μL of different solutions, namely: saline, 0.1 M phosphate buffer (pH 7.4), 0.1 M glycine–HCl (pH 4.0) and human serum. Daily, an aliquot of each mixture was removed and evaluated by ITLC analysis, as described above, to assess the presence of other radiochemical species.

The overall complex charge was determined by electrophoresis on paper strips (Whatman n°1) at 300 V in 0.1 M Tris–HCl buffer (pH 7.4) for 1 h. The radioactive distribution on the strips was analysed using a γ detector coupled to a radiochromatogram scanner.

*2.2.5. Biodistribution studies*

The *in vivo* behaviour of the radioactive complexes was evaluated in groups of 4–5 healthy female CD-1 mice (randomly bred, from Charles River Laboratories, Spain) weighing approximately 20–22 g. Animals were intravenously (i.v.) injected through tail vein with 100 μL (10–15 MBq/100 μL) of the radiolanthanide complex, were maintained on normal diet *ad libitum* and were sacrificed by cervical dislocation at 30 min, 2 h and 24 h post-injection, according to a previously described method [15]. Results were expressed as percentage of injected dose per gram of organ (% I.D./g ± SD). Whole body excretion of the radioactivity was assumed to be the difference between the measured radioactivity in the injected and sacrificed animal and was expressed as percentage of injected dose (% I.D.).

The *in vivo* stability of the complexes was assessed by urine and blood ITLC analysis, using the same experimental conditions as for radiochemical purity evaluation.

Animal experiments were carried out in accordance with the guidelines on the use of living animals in scientific investigation, and followed the principles of laboratory animal care.

## 3. Results and discussion

### 3.1. Thermodynamic stability studies

The thermodynamic stability constants of complexes of $H_5$te3a1p with lanthanide ions ($La^{3+}$, $Sm^{3+}$, $Gd^{3+}$, $Ho^{3+}$ and $Lu^{3+}$) were determined by potentiometric titrations in water solution at 298.2 K and 0.10 M in $N(CH_3)_4NO_3$. Equilibria were attained relatively fast in all the systems studied, typically in 10 min and at the latest within 90 min. Therefore, all titrations could be carried out by conventional in-cell experiments. The backtitrations were in good agreement with the direct ones for all systems, thus confirming that equilibrium was reached during direct titrations. The values of the overall protonation constants of $H_5$te3a1p and those obtained for the stability constants of its lanthanide complexes are presented in Table S1 of the Supplementary Data together with literature values for the protonation and stability constants of related compounds $H_4$teta and $H_8$tetp. The corresponding stepwise stability constants derived for all ligands are collected in Table 1 [17,26,31–33].

Only mononuclear complexes could be found in all lanthanide systems, something that is understandable in view of the metal-to-ligand ratio of 1:1 that was used. It is likely that the presence of an excess of lanthanide ion would lead to the formation of dinuclear complexes, but the constants of such species should be very low as has been found for transition metal complexes of $H_4$teta [34]. Additionally, using an excess of lanthanide ions would lead to experimental problems arising from the precipitation of insoluble metal hydroxide species. Furthermore, in the medicinal uses envisaged metal complexes must be prepared with excess of ligand in order to ensure full chelation of the lanthanide ions. It is nonetheless important that such dinuclear complexes were absent in our experimental conditions. Besides the fully deprotonated complexes (ML), all systems showed the presence of several protonated species (MHL to $MH_3L$) as well as a hydroxocomplex species (MLOH).

The values of the stability constants for the fully deprotonated complexes (ML) are of the same order of magnitude than those of $H_4$teta but lower than those of $H_8$tetp. They follow the common trend of increase along the lanthanide series, but with a difference between the values for the lighter lanthanide ions that is higher than for the heavier ones. Consequently, and unlike what was found for $H_4$teta and $H_8$tetp, the lanthanum complex of $H_5$te3a1p has a noticeably lower constant than the other lanthanides.

The stepwise stability constants found for the MHL and $MH_2L$ species (when determined) are very close (in the range of 6.68–6.55 and 6.44–5.68 for the first and the second, respectively) to the first protonation constant of the phosphonate group of the

**Table 1**
Protonation constants ($\log K_{H_iL}$) of $H_5$te3a1p, $H_4$teta and $H_8$tetp, and stability constants ($\log K_{MH_iL}$) of their complexes with lanthanide ions. $T = 298.2$ K and $I = 0.10$ M in $N(CH_3)_4NO_3$.

| Equilibrium quotient [a] | $H_5$te3a1p | $H_4$teta | $H_8$tetp[b] |
|---|---|---|---|
| [HL]/[L][H] | 11.78[c] | 10.59[b] | – |
| [$H_2$L]/[HL][H] | 9.88[c] | 10.08[b] | – |
| [$H_3$L]/[$H_2$L][H] | 6.34[c] | 4.15[b] | 8.85 |
| [$H_4$L]/[$H_3$L][H] | 3.85 [c] | 3.29[b] | 7.68 |
| [$H_5$L]/[$H_4$L][H] | 2.63[c] | 1.84[b] | 6.23 |
| [$H_6$L]/[$H_5$L][H] | 2.14[c] | – | 5.33 |
| [$H_7$L]/[$H_6$L][H] | – | – | 2.28 |
| [LaL]/[La][L] | 12.07 | 12.15[b] | 18.02 |
| [LaHL]/[LaL][H] | – | – | 9.27 |
| [La$H_2$L]/[LaHL][H] | 14.77[d] | – | 8.65 |
| [La$H_3$L]/[La$H_2$L][H] | 4.12 | – | 8.28 |
| [La$H_4$L]/[La$H_3$L][H] | – | – | 6.32 |
| [La$H_5$L]/[La$H_4$L][H] | – | – | 3.37 |
| [LaL]/[LaLOH][H] | 10.9 | 7.58[b] | 10.64 |
| [SmL]/[Sm][L] | 14.38 | 14.15[b] | 19.11 |
| [SmHL]/[SmL][H] | 6.68 | – | 9.63 |
| [Sm$H_2$L]/[SmHL][H] | 6.44 | – | 8.58 |
| [Sm$H_3$L]/[Sm$H_2$L][H] | 4.02 | – | 7.77 |
| [Sm$H_4$L]/[Sm$H_3$L][H] | – | – | 6.15 |
| [Sm$H_5$L]/[Sm$H_4$L][H] | – | – | 3.35 |
| [SmL]/[SmLOH][H] | 10.6 | 7.37[b] | 8.78 |
| [GdL]/[Gd][L] | 14.62 | 13.77[e] | – |
| [GdHL]/[GdL][H] | 6.55 | – | – |
| [Gd$H_2$L]/[GdHL][H] | 6.05 | – | – |
| [Gd$H_3$L]/[Gd$H_2$L][H] | 3.96 | – | – |
| [GdL]/[GdLOH][H] | 10.3 | – | – |
| [HoL]/[Ho][L] | 15.21 | 15.78[b] | 20.03 |
| [HoHL]/[HoL][H] | 6.57 | – | 9.52 |
| [Ho$H_2$L]/[HoHL][H] | 5.75 | – | 9.25 |
| [Ho$H_3$L]/[Ho$H_2$L][H] | 3.98 | – | 7.58 |
| [Ho$H_4$L]/[Ho$H_3$L][H] | – | – | 5.60 |
| [Ho$H_5$L]/[Ho$H_4$L][H] | – | – | 3.10 |
| [HoL]/[HoLOH][H] | 10.5 | 7.03[b] | 10.09 |
| [LuL]/[Lu][L] | 15.49 | 15.31[f] | – |
| [LuHL]/[LuL][H] | 6.68 | – | – |
| [Lu$H_2$L]/[LuHL][H] | 5.68 | – | – |
| [Lu$H_3$L]/[Lu$H_2$L][H] | 3.70 | – | – |
| [LuL]/[LuLOH][H] | 10.4 | – | – |

[a] Charges of equilibrium species are omitted for clarity.
[b] From refs. [17,31].
[c] From ref. [26].
[d] Corresponding to the global equilibrium [La$H_2$L]/[LaL][H]$^2$.
[e] From ref. [32].
[f] From ref. [33] with $I = 0.20$ M in $NaNO_3$.

free ligand $H_5$te3a1p (6.34) [26], which means that the protons in the MHL and $MH_2$L species must be located at amines. The stepwise stability constants for the $MH_3$L species (in the range 4.12−3.70) are significantly lower than the protonation of the phosphonate, therefore pointing to a protonation on an oxygen atom from a phosphonate group involved in the coordination of the lanthanide ion. These assumptions are in agreement with the generally accepted mechanism of complexation of lanthanide ions by tetraaza macrocycles with pendant arms containing oxygen donor atoms, wherein the lanthanide ions are initially captured by the oxygen donor atoms and only bind to the macrocyclic amines upon their deprotonation [35].

However, unlike all other lanthanide complexes studied here, for $La^{3+}$ it was impossible to include the species LaHL in the chemical model for fitting the titration curves, and therefore only a global constant corresponding to the formation of the $LaH_2$L species could be determined. This feature has been found also for the complexes of $H_4$teta with $La^{3+}$, $Sm^{3+}$ and $Ho^{3+}$ [17,31] and is a consequence of the low abundance of the MHL species in the equilibrium. It happens because of very close values for the stepwise constants corresponding to both protonations, formation of MHL and $MH_2$L species, respectively. In such cases it becomes unfeasible to determine separate constants for those species and only an overall value can be obtained with accuracy. The fact that with the studied ligand only the $La^{3+}$ complex presented such behaviour could be explained by the different sizes of the lanthanide ions together with the flexibility of the cyclam ring. But while the values of the constants for $MH_2$L and $MH_3$L generally decrease along the lanthanide series as expected, the values for the MHL species (when determined) decrease initially and then increase again. This may be an indication that, even in the cases where it was possible to determine constants for the MHL species, their values could be less accurate due to a limited abundance of this species in solution. Probably, in this case just the overall constants for the $MH_2$L species should be considered meaningful.

Additionally, the constant values obtained for the hydroxocomplex species MLOH were determined with lower accuracy due to a slower equilibrium attainment in the high pH region. They are generally higher than those found for similar complexes of $H_4$teta and $H_8$tetp and correspond to the deprotonation of a water molecule in highly alkaline solution, indicating the presence of a water molecule coordinated to the lanthanide ion in such pH range.

The most striking fact about the stability constant values of the lanthanide complexes of $H_5$te3a1p is that they are of the same order of magnitude of the corresponding values of $H_4$teta complexes, and consequently much smaller than those of $H_8$tetp. A direct comparison of the stability constants of complexes of ligands with rather different basicity can, however, lead to erroneous conclusions given that the competition for the ligand between the lanthanide ion and the protons is not taken into account. Therefore, pM values ($-\log[M]$), which are dependant on the protonation constants, have been calculated at pH 7.4 (physiological) and 9.0 on the basis of the full set of stability constants describing each system and are presented in Table 2 [31].

The pM values at pH 7.4 show that $H_5$te3ap is a chelator slightly less efficient than $H_4$teta and clearly less efficient than $H_8$tetp, for all lanthanide ions. At pH 9.0, however, while $H_5$te3a1p is still the less efficient chelator of all three, it can be seen that it becomes closer to the binding ability presented by $H_8$tetp, but finally it is $H_4$teta that presents the highest efficiency except for lanthanum. This behaviour, which could not be foreseen just from analysis of the stability constants, is a consequence of the different protonation properties of the three ligands. It demonstrates that stability constant values must be taken cautiously and comparisons between different ligands must be made with regard to the pH range of intended use.

Overall, when considering the stability constants together with the pM values, it becomes evident that $H_5$te3a1p forms lanthanide complexes that are less stable than those of both $H_4$teta and $H_8$tetp.

**Table 2**
Values of pM [a] for the complexes of $H_5$te3a1p, $H_4$teta and $H_8$tetp with lanthanide ions at pH 7.4 and 9.0, based in the values of the constants from Table S1.

| Ion | pH | $H_5$te3a1p | $H_4$teta | $H_8$tetp |
|---|---|---|---|---|
| $La^{3+}$ | 7.4 | 5.67 | 6.52[b] | 9.69[b] |
| | 9.0 | 8.36 | 10.87 | 11.09 |
| $Sm^{3+}$ | 7.4 | 7.56 | 8.71[b] | 10.65[b] |
| | 9.0 | 10.67 | 14.50 | 12.53 |
| $Gd^{3+}$ | 7.4 | 7.78 | – | – |
| | 9.0 | 10.92 | – | – |
| $Ho^{3+}$ | 7.4 | 8.37 | 10.67[b] | 11.99[b] |
| | 9.0 | 11.50 | 16.69 | 13.53 |
| $Lu^{3+}$ | 7.4 | 8.67 | – | – |
| | 9.0 | 11.79 | – | – |

[a] Values calculated for 100% excess of ligand concentration at $C_L = 2 \times C_M = 2.00 \times 10^{-5}$ M.
[b] From ref. [31].

This is a somewhat unexpected finding given that $H_5$te3a1p is a ligand structurally intermediate between the other two. Based only in the potentiometric determinations performed it is impossible to advance a definite explanation for this fact. We can only hypothesize that the fact could be related to the asymmetric structure of the ligand motivated by the replacement of one acetate pendant arm by a methylphosphonate one.

The species distribution diagrams presented in Figs. 1 and 2 are representative examples (for $La^{3+}$ and $Sm^{3+}$) of the solution behaviour of lanthanide complexes of $H_5$te3a1p, and others may be found in Figs. S1–S3 of the supplementary data. Overall, equimolar solutions of the ligand and each lanthanide ion contain a significant amount of free lanthanide throughout all the acidic pH range, as the complex species start to form only above pH 2. It is only above neutral pH that nearly all the lanthanide ions are coordinated by the ligand, mainly as a fully deprotonated complex species (ML) but also as a hydroxocomplex species at highly alcaline pH. This means that at physiological pH (7.4), the lanthanide complexes are essentially present in the form of the completely deprotonated species ML, unlike what was found for $H_4$teta and $H_8$tetp that at the same pH appear mainly as hydroxocomplex or protonated species, respectively [17].

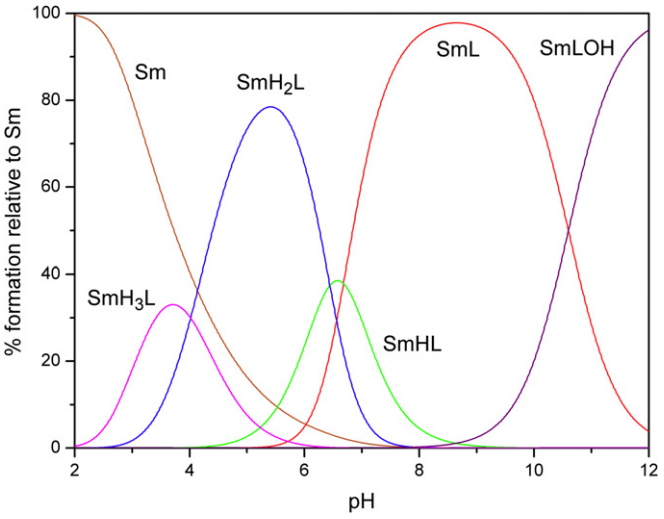



**Fig. 2.** Species distribution diagram for the $Sm^{3+}$ complex of $H_5$te3a1p at $C_M = C_L = 1 \times 10^{-3}$ M.

### 3.2. Radiochemical studies

#### 3.2.1. Radiochemical labelling

$^{153}$Sm and $^{166}$Ho were obtained with high radionuclidic purity, as assessed by γ spectrometry [17]. The reaction conditions for the synthesis of $^{153}$Sm and $^{166}$Ho complexes with $H_5$te3a1p were optimized in order to achieve radiochemical purities higher than 98%. Using the ITLC analysis, the reaction kinetics of $^{153}$Sm- and $^{166}$Ho-$H_5$te3a1p was found to be dependent on chelator concentration, pH, and temperature. Using 1:1 or 1:2 metal-to-ligand molar ratio resulted in poor labelling yields in the pH range 6–9 and at room temperature (RT). Only at pH 9.0 and 70 °C both $^{153}$Sm- and $^{166}$Ho-$H_5$te3a1p complexes were rapidly and quantitatively formed. In Fig. 3 is depicted the labelling kinetics of $H_5$te3a1p with $^{153}$Sm at different temperatures and pH values (similar results were found for $^{166}$Ho labelling).

By ITLC it was found that the radiochemical impurities present in the reactions of $^{153}$Sm/$^{166}$Ho with $H_5$te3a1p at room temperature (RT) and pH 6–9 were not the free radiolanthanides (results not shown). Taking into account the species distribution diagrams, such species may be colloidal radioactive forms other than MLOH hydroxocomplexes, which are only formed at higher pH values (Fig. 2). The complexation rate of $^{153}$Sm/$^{166}$Ho-$H_5$te3a1p was slower than found for the related ligand $H_4$teta but faster than that for $H_8$tetp [17]. Using a 1:2 metal-to-ligand molar ratio, the formation of $^{153}$Sm/$^{166}$Ho-$H_4$teta and $^{153}$Sm/$^{166}$Ho-$H_5$te3a1p was complete in 2.5 h at pH 6/RT and at pH 9.0/70 °C, respectively. The formation rate of $^{153}$Sm/$^{166}$Ho-$H_8$tetp was even slower, and at 70 °C no quantitative labelling could be achieved.

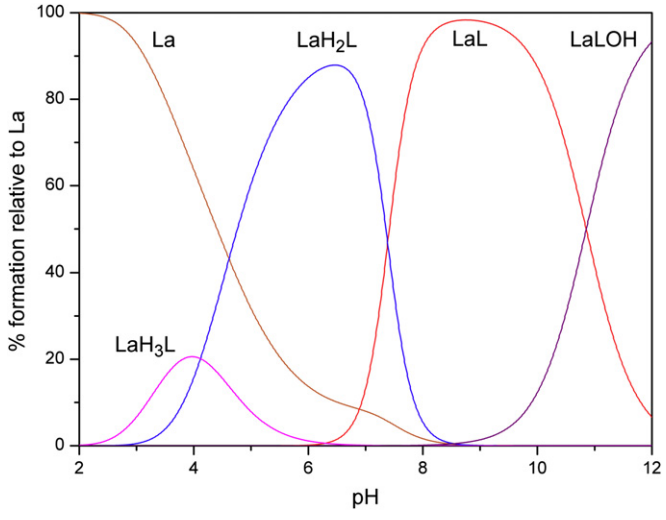



**Fig. 1.** Species distribution diagram for the $La^{3+}$ complex of $H_5$te3a1p at $C_M = C_L = 1 \times 10^{-3}$ M.

#### 3.2.2. In vitro studies

The stability of $^{153}$Sm/$^{166}$Ho-$H_5$te3a1p complexes was evaluated during a period of 72 h at 37 °C in 0.15 M NaCl (saline), 0.1 M phosphate buffer (pH 7.4), 0.1 M glycine–HCl (pH 4.0) and human serum. By ITLC it was found that the $^{153}$Sm/$^{166}$Ho-$H_5$te3a1p complexes were stable up to 72 h in all physiological solutions tested, except in phosphate buffer, pH 7.4. No significant differences were found between the $^{153}$Sm and $^{166}$Ho complexes, therefore results are only shown for $^{153}$Sm-$H_5$te3a1p (Fig. 4). Stability studies were also performed in fresh whole blood at 37 °C. In this medium, the complexes that proved to be stable in saline and in serum also decomposed. The chromatographic analyses have shown about 20%

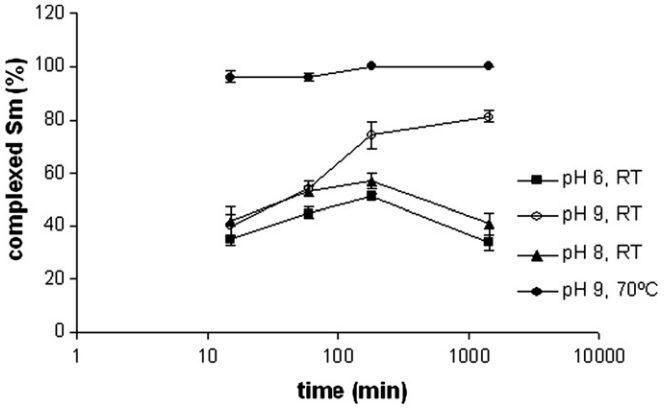



**Fig. 3.** Effect of temperature and pH on the labelling of $H_5$te3a1p with $^{153}$Sm.

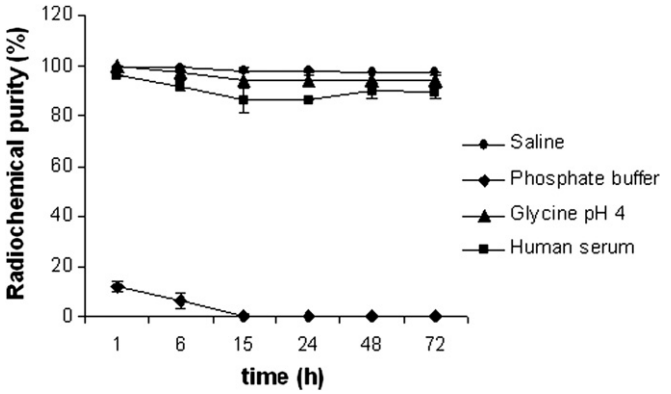



**Fig. 4.** *In vitro* stability of $^{153}$Sm-H$_5$te3a1p in saline, 0.1 M phosphate buffer (pH 7.4), 0.1 M glycine–HCl (pH 4.0) and human serum over time.

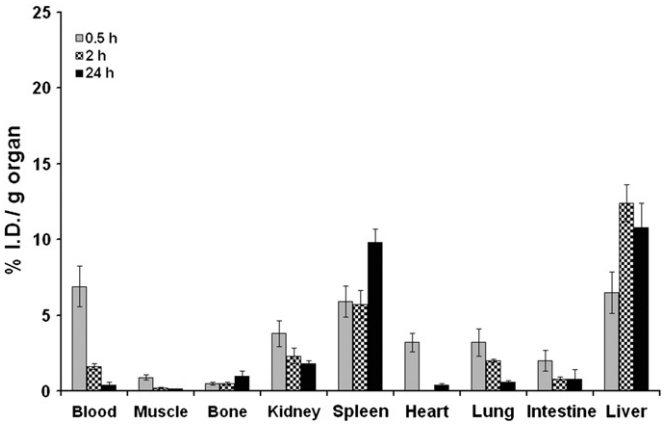



**Fig. 6.** Tissue distribution data (% I.D./g organ) of $^{166}$Ho-te3a1p at 30 min, 2 h and 24 h after i.v. administration in CD-1 mice ($n = 4$–5).

and 30% of radiochemical impurities ($R_f \approx 0$) at 30 min. and 2 h, respectively. Such results indicate that the presence of red blood cells may be responsible for the decomposition of the complexes, due to its own metabolism or competitive displacement by some constituents [15].

The overall charge of the $^{153}$Sm/$^{166}$Ho-H$_5$te3a1p complexes was determined to be negative by electrophoresis in Tris–HCl buffer (pH 7.4). Nevertheless, some instability was found in these systems, as indicated by the presence of some radioactive impurities at the origin.

### 3.2.3. Biodistribution studies

The tissue distribution of the $^{153}$Sm/$^{166}$Ho-te3a1p complexes obtained with high radiochemical purity (>98%) was assessed in CD-1 mice at different time points to investigate both biokinetics and *in vivo* stability. The uptake and clearance from main organs are shown in Figs. 5 and 6, respectively, and the whole animal body radioactivity excretion is graphically represented in Fig. 7.

The biodistribution profile of both complexes presents some differences, the most remarkable being related with the rate of total radioactivity excretion and the washout from most relevant organs. In fact, $^{153}$Sm-te3a1p has shown a significant slower tissue clearance (including blood stream) than $^{166}$Ho-te3a1p and a very low level of total excretion, with less than 30% of the injected activity excreted during 24 h post-injection, while more than 60% of $^{166}$Ho-te3a1p is eliminated at 30 min. However, biodistribution data from both radiolanthanide complexes indicated high liver uptake combined with long hepatic retention of radioactivity, although less pronounced in the case of $^{166}$Ho complex. Actually, most of the activity of any of the complexes does not clear into the intestines. Additionally, high uptake and long residence time in spleen were also detected for the two complexes as well as high accumulation of radioactivity in lungs associated to slow clearance. The elevated fraction of radioactivity taken by the liver, spleen and lungs, associated to the high retention time point out the presence of radiochemical species of colloidal/polymeric nature, suggesting *in vivo* instability of the complexes. Such biological behaviour, in comparison with $^{153}$Sm/$^{166}$Ho-teta, is in agreement with the potentiometric studies which have shown that at physiological pH the stability of the complexes of H$_5$te3a1p is lower than of H$_4$teta (Table 2). Moreover, the stability of the complexes with H$_5$te3a1p also depends on the metal (Table 2, pSm < pHo), a trend also found previously for $^{153}$Sm/$^{166}$Ho-teta in the same animal model [17]. In fact, the $^{153}$Sm-teta complex evidenced *in vivo* instability associated to hepatic retention and lower urinary excretion than the $^{166}$Ho-

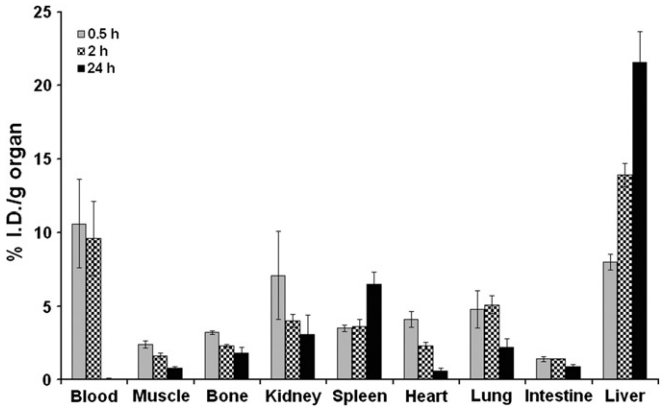



**Fig. 5.** Tissue distribution data (% I.D./g organ) of $^{153}$Sm-te3a1p at 30 min, 2 h and 24 h after i.v. administration in CD-1 mice ($n = 4$–5).

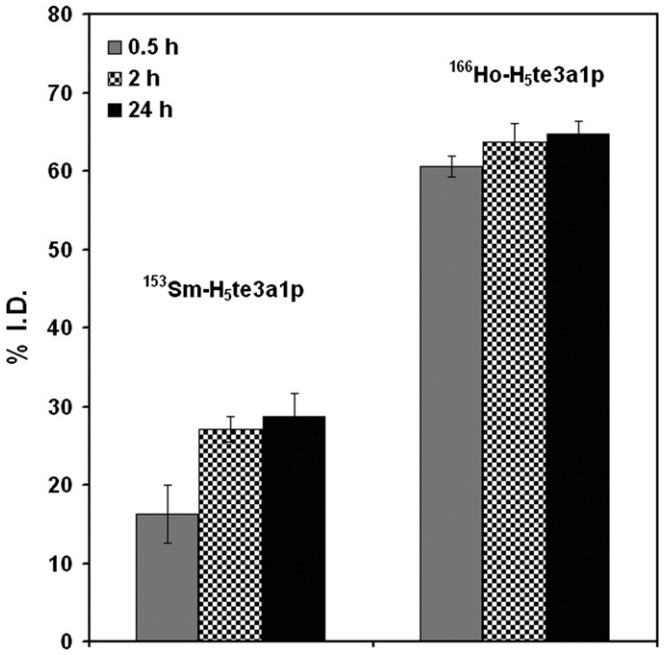



**Fig. 7.** Total radioactivity excretion (% I.D.) of $^{153}$Sm/$^{166}$Ho-te3a1p at 30 min, 2 h and 24 h after i.v. administration in CD-1 mice ($n = 4$–5).

teta, a biological profile previously assigned to free radiolanthanides, as shown by *in vivo* evaluation of $^{153}$Sm/$^{166}$Ho nitrate aqueous solutions [15].

Regarding the bone uptake expected from the introduction of a methylphosphonate pendant arm in the backbone of $H_4$teta, $^{153}$Sm-te3a1p is significantly taken by bone ($3.2 \pm 0.1$% I.D./g, at 30 min. after injection) but the radioactivity decreases over time ($2.3 \pm 0.1$ and $1.8 \pm 0.4$% I.D./g, at 2 and 24 h, respectively). This finding, in addition to the slow blood clearance, led to a very low bone-to-blood ratio. In contrast, the $^{166}$Ho-te3a1p is not significantly taken by the bone. Thus, it is not certain if the bone uptake found for $^{153}$Sm-te3a1p can be assigned to the methylphosphonate arm or to the low stability of the complex at physiological pH, since we and others have already demonstrated that bone is one of the main target organs for released radiolanthanides [15].

To validate the *in vivo* instability of the complexes, blood and urine samples were taken at sacrifice time and were analysed by ITLC. The chromatographic analysis confirmed the instability of $^{153}$Sm/$^{166}$Ho-te3a1p complexes in blood, as radiochemical species other than the injected complexes could be detected both in blood and in urine samples.

## 4. Conclusions

The 1,4,8,11-tetraazacyclotetradecane-1,4,8-triacetic-11-methylphosphonic acid ligand forms thermodynamically stable complexes with the $La^{3+}$, $Sm^{3+}$, $Gd^{3+}$, $Ho^{3+}$ and $Lu^{3+}$ lanthanide ions, although the stability found is somewhat lower than expected. The stability constants show a trend of higher values for the heavier lanthanide ions, as has been frequently found for other macrocyclic ligands. The complexes exist in several protonation degrees throughout the pH range studied, but at physiological pH the completely deprotonated ML species are predominant. High labelling yields could be achieved for both $^{153}$Sm- and $^{166}$Ho-te3a1p complexes, which proved to be stable in human serum and physiological solutions except phosphate buffer (pH 7.4). However, the biological profiles of the complexes showed a slow clearance from main tissues, low level of total radioactivity excretion and *in vivo* instability with both radiolanthanides. Considering also the low bone uptake found for such complexes, we conclude that the replacement of one carboxylic pendant arm in $H_4$teta by a methylphosphonate one does not provide a promising chelator to stabilize radiolanthanides for *in vivo* application.

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## Appendix A. Supplementary information

Supplementary information associated with this article can be found in the online version at doi:10.1016/j.ejmech.2010.09.013.

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