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Chemical, radiochemical and biological studies of Sm and Ho complexes of H_4dota analogues containing one methylphosphonic/phosphinic acid pendant arm

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This work intends to investigate samarium and holmium complexes with a set of H_4dota-analogues bearing methylphosphonate/methylphosphinic acid pendant arms (H_5do3ap, H_5do3ap^{PrA} and H_4do3ap^{ABn}) as new bifunctional ligands for radiolanthanide targeted therapy. Radiolanthanide complexes of these ligands were prepared and evaluated and thermodynamic solution studies were also performed to get a better insight on the radiochemical results. The radiocomplexes were quantitatively prepared, are hydrophilic and negatively charged. They present a high *in vitro* and *in vivo* stability, a low plasma protein binding and a rapid clearance from the main organs with a high rate of whole body radioactivity excretion. Stability constants with Sm(III) and Ho(III) were determined by potentiometry and the values found are very high for all the ligands. Actually, they are similar to those of H_4dota for the phosphinate analogues and even higher for the phosphonate ligand, being the constants similar to those with other lanthanide(III) ions. The effect of methylphosphorus acid pendant arms functionalization and the use of these ligands as bifunctional chelate agents are discussed.

Keywords: stability constants; radiolanthanides; tetraazamacrocycles; phosphonates; phosphinates; biological studies

Introduction

Macrocyclic ligands can firmly encapsulate various metal ions, namely trivalent lanthanides, forming monomeric and water-soluble complexes. Because of cyclic and preorganized nature of the ligands, the complexes are thermodynamically stable and kinetically inert. This class of compounds has been explored for medical applications, e.g. as contrast agents in magnetic resonance imaging (MRI)[1–6] or as bifunctional chelating agents (BFCA) for the labeling of specific biomolecules for molecular imaging and/or targeted radiotherapy.[7–9] Specifically, H_4dota (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and dota-like derivatives have been widely employed as BFCA due to the formation of stable complexes with metal ions attractive for medical applications.[7–9] Chelators containing phosphonate pendant arms are particularly adequate as bone-seeking agents to be used for MRI contrast agents[10–12] or for bone pain palliation and/or therapy of bone cancer.[13] So far, the most promising radiolanthanide complexes for bone pain palliation or marrow ablation are ^{153}Sm/^{166}Ho/^{177}Lu-H_8dotp (H_8dotp = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylphosphonic acid)), which have been evaluated[14–19] as an alternative to the well-established ^{153}Sm-EDTMP (Quadramet®).[20] However, the relatively slow formation rate, the low selectivity for a specific metal ion and the high osmolarity (due to their high charge) might be a disadvantage of the ^{153}Sm/^{166}Ho-dotp complexes.[10,21] To overcome some of these problems, the interest has been focused on the synthesis of mixed carboxylate/phosphonate or phosphinate dota-like macrocyclic ligands.[12,22–27] Moreover, functionalization of the methylphosphinic acid arm with a propionate (H_5do3ap^{PrA}) or *p*-aminobenzyl reactive groups (H_4do3ap^{ABn}) (Chart 1)[28] enables further conjugation of the chelator to biomolecules while maintaining the strong coordination ability towards lanthanide(III) ions. In addition, it was observed that a presence of a suitable substituent on phosphorus atom in the pendant arm leads to a faster complex formation with metal ions, e.g. with yttrium(III) having radionuclides (^{86,90}Y) useful for imaging and therapy.[25]

Here, we report on the chemical, radiochemical and biological behavior of Sm(III) and Ho(III) complexes of H_5do3ap,

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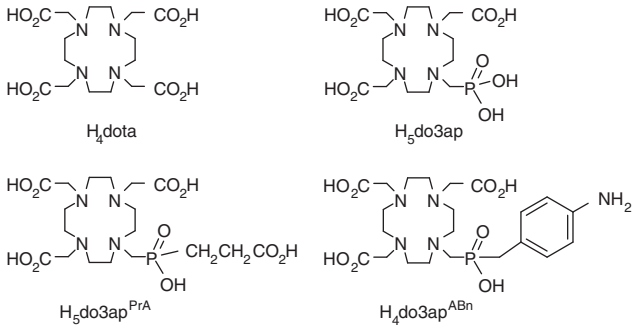

Chart 1. Structures of H_4dota and of the mixed ligands studied in this work.

H_5do3ap^{PrA} and H_4do3ap^{ABn} (Chart 1). The effect of phosphorus acid pendant arm functionalization on the complexation efficiency, on the labeling properties and on the biological profile of ^{153}Sm and ^{166}Ho complexes was evaluated. The results obtained were compared with previous studies performed on the ^{153}Sm/^{166}Ho-H_4dota complexes.[29] To better understand the radiochemical properties and to correlate them with the chemical structure of the ligands, we have also determined the stability constants of the Sm(III) and Ho(III) complexes with the title ligands.

Experimental protocols

Reagents

The ligands were prepared according to the previously described methods.[24–26] Enriched Sm_2O_3 (98.4% ^{152}Sm) was obtained from Campro Scientific and natural Ho_2O_3 (99.9%) from Strem Chemicals. Dibasic calcium phosphate (hydroxyapatite, HA) was purchased from Aldrich Chemical Co., Inc. All materials used for chemical, radiochemical and biological evaluation were reagent grade unless otherwise specified.

Production of ^{153}Sm and ^{166}Ho

^{153}Sm ($t_{1/2}$ 46.8 h; β 0.67 MeV, 34%; 0.71 MeV, 44%; 0.81 MeV, 21%; γ 0.103 MeV, 28%) and ^{166}Ho ($t_{1/2}$ 26.8 h; β 1.85 MeV, 51%; 1.77 MeV, 48%; γ 80.6 keV, 7.5%; 1.38 MeV, 0.90%) were produced in the ITN Portuguese Research Reactor (1 MW). Irradiation was performed as previously described using nitrate targets prepared from the correspondent isotopically enriched $^{152}Sm_2O_3$ or natural Ho_2O_3 under a thermal neutron flux of 1.3×10^{13} n/cm^2 s and epithermal neutron flux of 2.4×10^{11} n/cm^2 s for 1–3 h.[29,30] 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. The radionuclide purity of the ^{153}Sm and ^{166}Ho solutions was assessed by γ-ray spectrometry using a Ge(Li) detector coupled to an Accuspec B Canberra multichannel analyzer. The γ-ray spectrum processed, following efficiency calibration with a ^{152}Eu source, indicated a high radionuclidic purity of both ^{153}Sm and ^{166}Ho.[29] The targets were reconstituted in water to yield 1% (w/v) ^{153}Sm/^{166}Ho nitrate solution for complex preparation and the activities measured in a dose calibrator (Aloka Curiemeter IGC-3).

Synthesis of ^{153}Sm and ^{166}Ho complexes

Radiolabeling of the macrocyclic ligands was performed by dissolving 5 mg of the ligand in 0.3 mL of water followed by the addition of an adequate amount of ^{153}Sm or ^{166}Ho nitrate solutions to achieve a final volume of 0.5 mL and a ligand concentration of 20, 18 and 17 mM for H_5do3ap, H_5do3ap^{PrA} and H_4do3ap^{ABn}, respectively. The labeling was optimized by changing the pH (6–10) and the temperature. Labeling efficiency, chelation kinetics and stability of the radiolanthanide complexes were accomplished by ascending instant thin layer chromatography using silica gel strips (ITLC-SG) (Polygram, Macherey-Nagel) developed with the mobile phase MeOH:H_2O: conc. aq. NH_3 (2:4:0.2). 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, if present, also remain at the origin and can be assessed by ITLC-SG strips developed with saline. In this system both the radiolanthanide complexes and ^{153}Sm/$^{166}Ho(NO_3)_3$ migrate with $R_f = 1.0$. Radioactive distribution on the ITLC-SG strips was detected using a Berthold LB 505 γ-detector coupled to a radiochromatogram scanner. The radioactivity from samples of protein-binding studies was measured by a γ counter (Berthold LB 2111).

In vitro stability studies

The *in vitro* stability of the complexes was evaluated in saline, 0.1 M phosphate buffer (pH 7.4), 0.1 M Tris-HCl buffer (pH 7.4) and human serum, at 37°C at various time points (up to five days). Typically, 50 μL of each ^{153}Sm- or ^{166}Ho-complex solutions were added to 100 μL of the above media and incubated at 37°C. Daily, an aliquot of each mixture was taken and evaluated by ITLC analysis, as described above and the radiochemical composition of the mixtures was determined.

Adsorption studies

Adsorption of the ^{153}Sm and ^{166}Ho complexes onto HA was determined by incubation with different amounts of HA. Briefly, 50 μL of the complex solution (3–5 MBq/50 μL) was incubated for 1 h at room temperature with 5, 10, 25, 50, 100 and 150 mg of HA in 2 mL of 0.1 M Tris-HCl buffer (pH 7.4). Immediately after incubation, the mixture was centrifuged and the liquid phase separated. The solid phase was washed twice with 2.5 mL of 0.1 M Tris-HCl buffer (pH 7.4). The washing buffer was pooled with the liquid phase. The radioactivity in the liquid and solid phases was measured in a dose calibrator.

Complex charge, lipophilicity and protein binding

The overall charge of the radioactive complex was determined by electrophoresis in 0.1 M Tris-HCl buffer (pH 7.4), as previously described.[30] Lipophilicity was assessed by determination of the partition coefficient (*P*) in *n*-octanol/saline and expressed as log *P*, according to the previously described method.[30] Plasmatic protein binding was determined by a trichloroacetic acid (TCA) precipitation method: 50 μL of complex solution were incubated at 37°C in 500 μL of human blood plasma for 1 h. An aliquot of this solution (100 μL) was added to 1 mL of TCA (5%). The solution was centrifuged and the supernatant separated. The precipitation procedure was repeated twice and the supernatant solution and the precipitated were measured in a γ-counter. The percentage of protein binding is given by the ratio between the activity of the supernatant solution and the activity of the precipitate.

In vivo biodistribution studies

The *in vivo* behavior of the radioactive complexes was evaluated in groups of 4–5 female CD-1 mice (randomly bred), obtained 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. They were maintained on normal diet *ad libitum* and were sacrificed by cervical dislocation at 30 min and 2 h post injection, according to a previously described method.[29,30] Major organs were dissected, weighed and their radioactivity measured. Results were expressed as percentage of injected dose per gram of organ (% ID/g organ ± 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 (% ID). The *in vivo* stability of the complexes was assessed by urine and blood ITLC analysis, using the above referred experimental conditions for the radiochemical purity evaluation. Animal experiments were carried out in accordance with the EU recommendations on the use of living animal in scientific investigation, and followed the principles of laboratory animal care.

Thermodynamic solution studies

Chemicals and stock solutions for potentiometric titrations

The stock solution of hydrochloric acid (~0.03 mol/dm^3) was prepared from 35% aqueous solution (puriss, Fluka). Commercial NMe_4Cl (99%, Fluka) was recrystallized from boiling *i*-PrOH and the solid salt was dried over P_2O_5 in vacuum to constant weight (this dried form of the salt is extremely hydroscopic). Carbonate-free $(NMe_4)OH$ solution (~0.2 mol/dm^3) was prepared from NMe_4Cl using ion exchanger Dowex 1 in the OH^- form (elution with carbonate-free water, under argon). The hydroxide solution was standardized against potassium hydrogen phthalate and the HCl solution against the 0.2 mol/dm^3 $(NMe_4)OH$ solution. Stock solutions of the metal cations were prepared by dissolving $LnCl_3$ hydrates (99.9%, Strem) in water. The metal content in the solutions was determined by titration with a standard Na_2H_2edta solution. Analytical concentration of a stock solution of the ligand was determined together with refinement of protonation constants using OPIUM software package[31] (see below). Deionized water (Milli-Q, Milipore) was used in all experiments.

Potentiometric titrations

Titrations were carried out in a vessel thermostated at 25.0 ± 0.1°C, at ionic strength I = 0.1 mol/dm^3 (Me_4NCl) and in the presence of excess HCl using a PHM 240 pH-meter, a 2-mL ABU 900 automatic piston burette and a GK 2401B combined electrode (all Radiometer). The initial volume was 5 mL and the ligand concentration was 0.004 mol/dm^3. An inert atmosphere was provided by the constant passage of argon saturated with the vapor of the solvent used in the measurements. Titrations with the lanthanide(III) ions were performed at metal-to-ligand molar ratios of 1:1 (all ligands) and 2:1 (H_5do3ap). The M:L molar ratio 2:1 for the Ln^{3+}–H_5do3ap systems had to be used, as the phosphonate group is able to weakly interact with the second equivalent of a metal ion[32] and, so, the ratio is necessary for a full potentiometric evaluation of the systems. As the complexation was too slow for a conventional titration, the 'out-of-cell' method was used. Each titration consists of 23–25 points in the $-\log[H^+]$ range from 1.8 to 6.0 (at least two parallel titrations for each ratio). Equilibrium was reached after four weeks. The constants determined by this technique are less precise than the standard titrations and are loaded with a high uncertainty due to the less accurate measurements and a small number of experimental points. The constants (with standard deviations) were calculated with program OPIUM.[31] The program minimizes the criterion of the generalized least-squares method using the calibration function.

$$E = E^0 + S \times \log[H^+] + j_1 \times [H^+] + j_2 \times K_w/[H^+]$$

where the additive term E^0 contains the standard potentials of the electrodes used and contributions of inert ions to the liquid junction potential, S corresponds to the Nernstian slope, the value of which should be close to the theoretical value and $j_1 \times [H^+]$ and $j_2 \times K_w/[H^+] = j_2 \times [OH]$ terms are the contributions of the H^+ and OH ions to the liquid-junction potential. It is clear that j_1 and j_2 cause deviation from a linear dependence of E on $-\log[H^+]$ only in strongly acidic and strongly alkaline solutions. The calibration parameters were determined by titration of standard HCl with standard $(Me_4N)OH$ before each titration to give a pair calibration/titration, which was used for calculations of the constants. The overall (concentration) stability constants are defined by $\beta_{mhl} = [M_mH_hL_l]/([M]^m \times [H]^h \times [L]^l)$. The water ion product pK_w (13.81) and stability constants of the Ln^{3+}–OH systems included in the calculation were taken from literature.[33,34] Dissociation constants of the complexes are given as $pK_a = \beta_{m(h+1)l} - \beta_{mhl}$.

Results and discussion

Radiochemical labeling

The radiolanthanide complexes with H_5do3ap, H_5do3ap^{PrA} and H_4do3ap^{ABn} were prepared by reacting $^{153}Sm/^{166}Ho(NO_3)_3$ with the corresponding ligands (1:2 M/L molar ratio) in water at different pH and temperatures. The labeling efficiency was evaluated by ascending ITLC-SG and the reaction conditions were optimized in order to obtain ^{153}Sm and ^{166}Ho complexes with a high radiochemical purity. As an example, Figure 1 shows the labeling kinetics of H_4do3ap^{ABn} with ^{153}Sm at room temperature as a function of pH (similar results were found for ^{166}Ho). The optimized labeling conditions to achieve the highest chelation efficiencies, expressed as labeling percentage, are summarized in Table 1. The

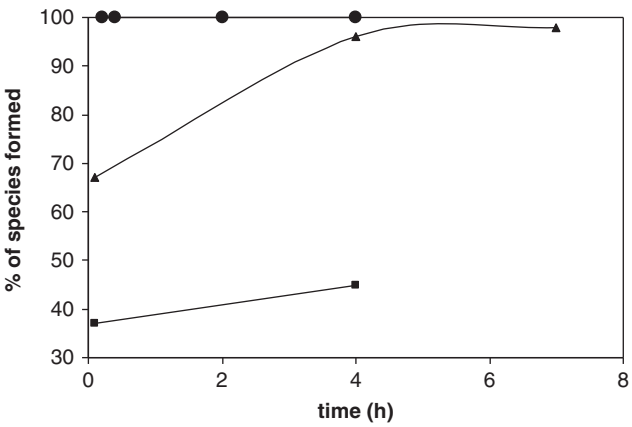

Figure 1. Influence of pH of reaction mixture on the labelling of H_4do3ap^{ABn} with ^{153}Sm at room temperature: ■ pH 3.5; ● pH 6 and 7; ▲pH 9.

maximum complexation yields with ^{153}Sm and ^{166}Ho were achieved at room temperature and pH values in the range 7–9 for H_5do3ap and H_5do3ap^{PrA} and pH 6–7 for H_5do3ap^{ABn}. These labeling conditions are comparable to the ones found for H_4do3ap^{ABn} with the same radiolanthanides (pH 6–7, 5 min, room temp.).[29]

In vitro studies

In vitro *stability, protein binding and lipophilicity*

In vitro studies, such as determination of serum protein binding, lipophilicity and stability of the complexes in physiological media, were performed and the results are listed in Table 2. Results for ^{153}Sm/^{166}Ho-dota were included for a comparison.[29] Lipophilicity was determined by the partition coefficient (*P*) in *n*-octanol/saline. Protein binding was determined by a TCA precipitation method. The overall charge of the complexes was determined by electrophoresis in Tris-HCl buffer (pH 7.4, 0.1 M).

The ^{153}Sm and ^{166}Ho complexes of the investigated ligands have negative overall charge, they are hydrophilic and show a low human serum protein binding. When these results are compared with those obtained for the ^{153}Sm/^{166}Ho-dota complexes under the same experimental conditions, the set of ligands does not show significant differences.[29] However, a complex stability in physiological solutions revealed significant differences when compared to that of complexes of the H_4dota analogues; the most relevant differences were observed for the ^{166}Ho-do3ap^{PrA} complex. In fact, in the presence of human serum, we have observed that another radiochemical species (with a lower R_f) is formed and its abundance increases along the time, while the abundance of the main species decreases (Figure 2(a), trace B).

Hydroxyapatite adsorption studies

It is well recognized that the presence of phosphonate groups leads to *in vivo* bone uptake.[35] The *in vitro* binding onto HA, the main mineral bone component, was studied to anticipate a potential of the complexes to be taken *in vivo* by the bone. Table 3 summarizes the results found after incubation of the ^{153}Sm/^{166}Ho complexes with HA in Tris-HCl buffer (pH 7.4, 0.1 M). The binding values found for complexes of the monophosphorus acid derivatives are very low. Thus, the presence of only one phosphorus acid pendant arm in H_4dota-like ligands does not result in a significant HA binding. It can be concluded that no *in vivo* bone uptake is expected due to the presence of only one phosphonic/phosphinic acid pendant arm in the structure of the ligands.

In vivo biological studies

The biodistribution studies of all ^{153}Sm/^{166}Ho complexes were performed in female CD-1 mice, at 30 min and 2 h after intravenous (i.v.) injection through the tail vein. Results were then compared with data for the corresponding complexes of H_4dota obtained in the same animal model.[29] Tissue distribution data for the most relevant organs, expressed as percentage of injected dose per gram of organ, are graphically presented in Figure 3 and Table 4 for the ^{153}Sm- and ^{166}Ho-complexes, respectively.

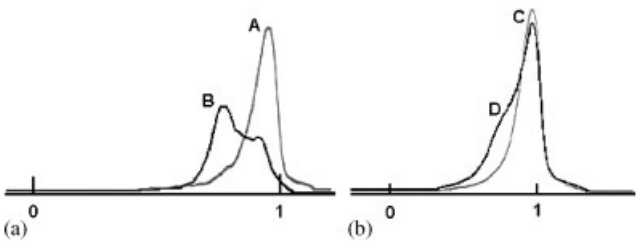

Figure 2. ^{166}Ho-H_5do3ap^{PrA} ITLC-SG chromatographic profile: (a) A: after labelling; B: after 3 days in human serum; (b) C: before injection; D: in urine (30 min post injection).

Table 1. Labeling conditions of ^{153}Sm and ^{166}Ho complexes (L:M ratio 2:1)

Ligand	H_5do3ap	H_5do3ap^{PrA}	H_4do3ap^{ABn}
Efficiency	>98%	>98%	>98%
pH	6–9	7–9	6–7
Time, Temp.	15 min, r.t.	15 min, r.t.	15 min, r.t.

r.t., room temperature.

Table 3. Binding of ^{153}Sm/^{166}Ho complexes to hydroxyapatite in Tris-HCl buffer (pH 7.4, 0.1 M)

Ligand	^{153}Sm (%)	^{166}Ho (%)
H_5do3ap	0.7	1.5
H_5do3ap^{PrA}	0.7	2.4
H_4do3ap^{ABn}	0.7	1.7

Table 2. *In vitro* studies

Ligand	^{153}Sm complexes					^{166}Ho complexes				
	% protein binding	log *P*	*In vitro* stability (days)[a]			% protein binding	log *P*	*In vitro* stability (days)[a]		
			HS	S	PB			HS	S	PB
H_5do3ap	3.1	−2.49	2	3	3	2.9	−1.80	1	3	3
H_5do3ap^{PrA}	4.0	−2.46	2	3	2	3.0	−1.88	1	3	2
H_4do3ap^{ABn}	2.3	−2.43	2	3	3	2.7	−2.05	2	3	3
H_4dota[b]	7.0	−2.02	5	5	5	1.4	−1.64	5	5	5

HS, human serum; S, saline; PB, phosphate buffer.
[a]100% of radiocomplex present; selected media.
[b]From Marques *et al.*[29]

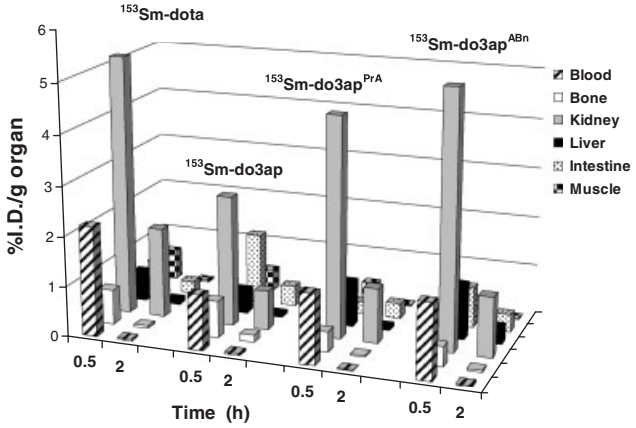

Figure 3. Biodistribution results of ^{153}Sm-complexes of the studied ligands (% ID/g organ) in comparison with the biodistribution of ^{153}Sm-H_4dota in mice ($n = 4–5$).

In general, the *in vivo* studies showed a similar biodistribution profile for all ^{153}Sm/^{166}Ho complexes under study with a rapid clearance from main organs, including the blood stream and soft tissues, which is in agreement with their hydrophilic character and the low plasma protein binding. At 2 h after administration, radioactivity was localized only in organs involved in the excretion routes, especially in the kidneys. In spite of the presence of the methylphosphonate pendant arm in H_5do3ap, the bone uptake of both radiolanthanide complexes is low (0.14 and 0.3% ID/g organ at 2 h, for ^{153}Sm- and ^{166}Ho-do3ap, respectively) and rapidly decreases over time, which is in accordance with the low HA binding observed in the *in vitro* assay. These findings make evident that these complexes are not promising for bone targeting, but the ligand can serve as an alternative metal-binding moiety to the common dota-like ligands in biologically active conjugates.

Table 4. Biodistribution results of ^{166}Ho-complexes (% ID/g organ ± SD) in mice ($n = 4–5$)

Organ	^{166}Ho-H_5do3ap		^{166}Ho-H_5do3ap^{PrA}		^{166}Ho-H_4do3ap^{ABn}	
	30 min	2 h	30 min	2 h	30 min	2 h
Blood	3.0 ± 0.9	0.05 ± 0.02	4.0 ± 0.5	0.03 ± 0.01	1.0 ± 0.2	0.05 ± 0.01
Liver	0.4 ± 0.1	0.19 ± 0.02	0.47 ± 0.05	0.10 ± 0.02	1.5 ± 0.2	1.2 ± 0.1
Intestine	0.4 ± 0.1	0.20 ± 0.03	0.6 ± 0.2	0.15 ± 0.06	0.22 ± 0.06	0.3 ± 0.2
Spleen	0.6 ± 0.3	0.16 ± 0.06	1.3 ± 0.6	0.05 ± 0.02	0.4 ± 0.1	0.6 ± 0.1
Heart	0.7 ± 0.2	0.04 ± 0.01	1.0 ± 0.2	0.02 ± 0.01	0.27 ± 0.01	0.04 ± 0.01
Lung	1.2 ± 0.7	0.08 ± 0.01	2.2 ± 0.3	0.04 ± 0.01	2.4 ± 1.0	0.44 ± 0.06
Kidney	3.0 ± 0.8	2.44 ± 0.02	2.6 ± 0.8	0.9 ± 0.1	3.1 ± 0.7	2.0 ± 0.3
Muscle	0.7 ± 0.3	0.06 ± 0.01	0.6 ± 0.2	0.02 ± 0.01	0.26 ± 0.05	0.03 ± 0.01
Bone	1.4 ± 0.4	0.30 ± 0.02	1.1 ± 0.3	0.04 ± 0.01	0.26 ± 0.04	0.14 ± 0.01
Stomach	0.5 ± 0.1	0.14 ± 0.02	0.9 ± 0.2	0.4 ± 0.1	0.19 ± 0.04	0.13 ± 0.01
Excretion (% ID)	83.6 ± 4.5	96.7 ± 1.0	81.9 ± 3.8	98.2 ± 0.6	78.9 ± 3.9	92.5 ± 0.1

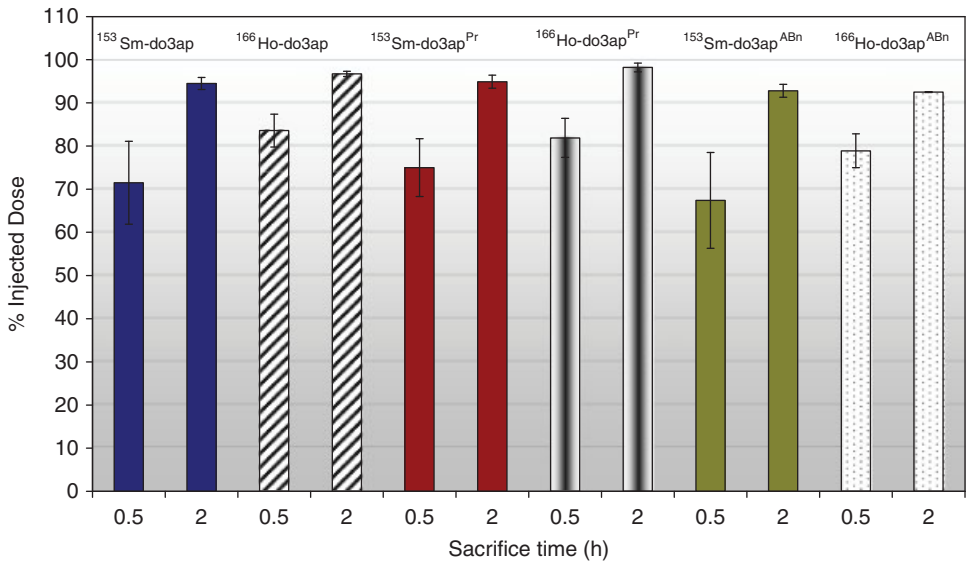

Figure 4. Total excretion (% ID) of the ^{153}Sm/^{166}Ho complexes in mice at 30 min and 2 h after i.v. injection.

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Rapid whole animal body radioactivity clearance was found for all complexes, suggesting the urinary tract as the most important excretory pathway; the results are presented in Figure 4. The main difference observed is related with the rate of total radioactivity clearance. Indeed, at 30 min after administration, the total excretion of all the ^{166}Ho complexes is slightly faster than that of the analogous ^{153}Sm complexes. However, most of the injected activity (>92%) was excreted during 2 h after the administration of the radiolanthanide complexes studied. No significant difference was found between ^{153}Sm and ^{166}Ho complexes with the same macrocyclic ligand. The biodistribution profiles in this study are analogous to those found in a different animal model (rats) with ^{111}In/^{90}Y complexes of the bifunctional phosphinate ligands.[36]

Chromatographic analysis (ITLC-SG) of urine and blood samples taken at the sacrifice times indicated a high *in vivo* stability for the ^{153}Sm/^{166}Ho-do3ap and ^{153}Sm/^{166}Ho-do3ap^{ABn} complexes, since no other radiochemical species than the corresponding intact complexes were detected. The *in vivo* stability and biodistribution profiles of the ^{153}Sm/^{166}Ho complexes with these ligands are comparable to those obtained with the ^{153}Sm/^{166}Ho-dota in the same animal model.[29] In the case of ^{166}Ho-do3ap^{PrA}, a partial degradation of the complex was observed. Figure 2(b) shows the chromatographic profile of an aliquot of urine taken 30 min post injection (trace D) together with the profile of the radiocomplex before the injection (trace C). These findings support the hypothesis of some *in vivo* dissociation of this radiocomplex, leading to a formation of other species. It has been reported that trivalent lanthanides may behave *in vivo* similarly to Fe^{3+}.[37] In blood, the main carrier protein for Fe^{3+} is transferrin, which also forms complexes with trivalent lanthanides. The conditional stability constants for the two binding sites of transferrin with lanthanides(III) are smaller than the ones for Fe^{3+}.[38] However, the serum transferrin is not normally saturated with iron and some binding capacity for the coordination of other hard metal ions is available. *In vivo* degradation was also found for pyridine-containing macrocycles bearing phosphonate pendant arms.[30,39]

The fast tissue clearance with no significant accumulation of radioactivity in any organ and the rapid total clearance suggest that the set of ligands studied can be useful as a BFCA.

Thermodynamic solution studies

For a better understanding of the *in vitro/in vivo* studies, the thermodynamic stabilities of the Sm(III) and Ho(III) complexes of H_5do3ap, H_5do3ap^{PrA} and H_4do3ap^{ABn} were determined by potentiometric titrations. As complexation reactions were very slow, out-of-cell method had to be used and time to reach equilibrium was four weeks. Dissociation constants of H_5do3ap,[32] H_5do3ap^{PrA} and H_4do3ap^{ABn} [25] were taken from literature. The values found for the stability constants of the Sm(III) and Ho(III) complexes with the title macrocyclic ligands and the corresponding pM values calculated at physiological pH (pH = 7.4, 1:2 M:L ratio, $c_L = 2c_M = 0.004$ mol/dm^3) are summarized in Table 5. For a comparison, values for the corresponding H_4dota complexes are also included.[40] The studied ligands form complexes with a high thermodynamic stability, and logK_{ML} as well as pM values for the complexes are similar (H_5do3ap^{PrA} and H_4do3ap^{ABn}) or higher (H_5do3ap) than those of the complexes of H_4dota. As it is seen in the distribution diagram, all complexes are fully formed above pH ~ 2.5 (Figure 5). As expected, trends in stability of the complexes inside the lanthanide series are similar for the studied ligands as well as analogous to that for H_4dota.[25,32] The first protonation should take place on the phosphorus acid pendant arm (phosphonate group in H_5do3ap, amino group H_4do3ap^{ABn} or carboxylate group in H_5do3ap^{PrA}) with the lanthanide(III) ion still firmly bound in the macrocycle cavity.[24,41,42] In case of complexes of H_5do3ap, protonated species with the MHL = 131 stoichiometry could be determined similarly to the other lanthanide(III) complexes of the ligand;[32] the species should be out-of-cage complex with

Table 5. Stability and dissociation constants of Sm(III) and Ho(III) complexes with the studied macrocyclic ligands

Metal ion	Species *m h l*	H_4dota[a]	H_5do3ap[b]		H_5do3ap^{PrA} [b]		H_4do3ap^{ABn} [b]	
		log K_{ML}	log β_{MHiL}[c]	log K_{ML} or pK_a (complex)	log βMHiL[c]	log K_{ML} or pK_a (complex)	log β_{MHiL}[c]	log K_{ML} or pK_a (complex)
Sm^{3+}	1 0 1	23.0	28.67 (7)	28.67	25.11 (5)	25.11	24.28 (5)	24.28
	1 1 1	—	33.56 (1)	4.89	30.04 (3)	4.93	29.32 (1)	5.04
	1 2 1	—	—	3.49	31.97 (4)	1.93	30.4 (2)	1.1
	1 3 1	—	37.05 (5)		—	—	—	—
	2 0 1	—	32.64 (7)	3.97	—	—	—	—
pM[d]		16.25	19.23		17.78		16.93	
Ho^{3+}	1 0 1	24.8	28.51 (7)	28.51	25.12 (6)	25.12	24.49 (5)	24.49
	1 1 1	—	33.45 (1)	4.94	30.09 (2)	4.97	29.61 (1)	5.12
	1 2 1	—	—	2.39	31.84 (6)	1.75	31.11 (8)	1.50
	1 3 1	—	35.84 (5)		—	—	—	—
	2 0 1	—	32.62 (8)	4.11	—	—	—	—
pM[d]		17.75	19.07		17.79		17.14	
ΣpK_a[e]		32.71[40]	38.15[32]		34.60[25]		34.08[25]	

[a] $T = 25.0$°C, $I = 1.0$ mol/dm^3 NaCl.[41]
[b] This work, $T = 25.0$°C, $I = 0.10$ mol/dm^3 NMe_4Cl.
[c] Values in parentheses are standard deviations in the last significant figures.
[d] $c_L = 2c_M = 0.004$ mol/dm^3; pH 7.4.
[e] Overall basicity of the ligands: $\Sigma pK_a = pK_a$ (HL) + pK_a (H_2L) + pK_a (H_3L) + pK_a (H_4L) + pK_a (H_5L).

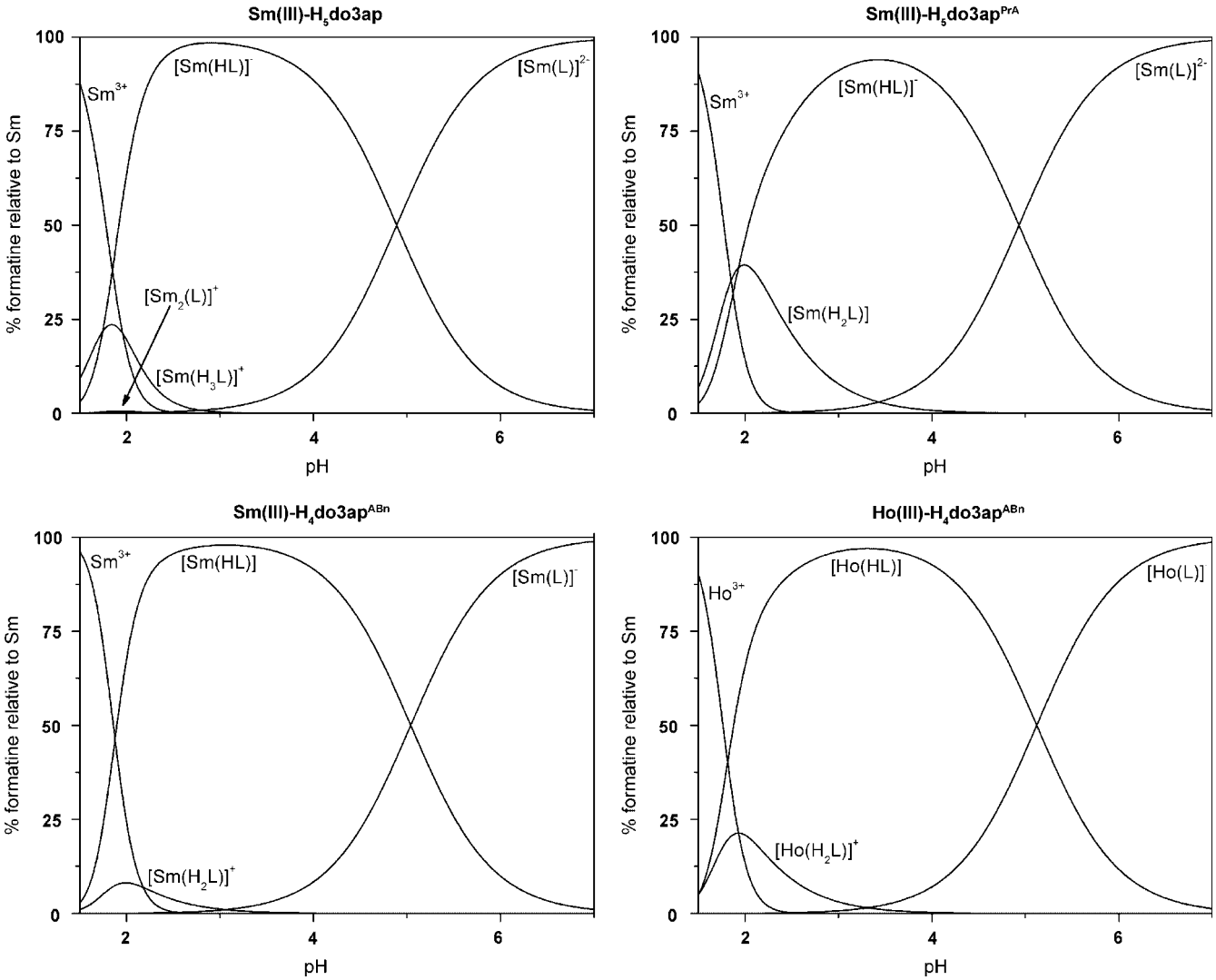

Figure 5. Species distribution diagrams of the Sm(III)-H_5do3ap, Sm(III)-H_5do3ap^{PrA} and Sm(III)/Ho(III)-H_4do3ap^{ABn} systems (L:M = 2:1; $c_L = 2\,c_M = 0.004$ mol dm^{-3}).

double protonated ring nitrogen atoms as an intermediate species present in acidic solutions during the complex formation.[43] A weak interaction ($\log K_{M2L} \sim 4$, Table 5) of the H_5do3ap complexes with the second metal ion (formation of the M_2L species) was found similarly to other lanthanide(III) complexes of the ligand.[32] According to the species distribution diagrams (Figure 5), the fully deprotonated ML species are the final products of complexation reactions under pH optimized for radioisotope labeling and the same negatively charged species should be present *in vivo* under physiological pH. This result also agrees with the overall negative charge of the radiocomplexes as it was proved by electrophoresis. As other lanthanide(III) complexes, the studied ligands have been proved to be highly kinetically inert,[25,32,41] our present solution studies also confirmed that the title ligands are suitable for conjugation and radionuclide labeling of biological vectors.

Concluding remarks

Complexation of ^{153}Sm and ^{166}Ho with the ligands under study is fast and maximum radiolabeling yields were achieved at room temperature, using a 1:2 M:L molar ratio and pH values in the range 7–9 for H_5do3ap and H_5do3ap^{PrA} and pH 6–7 for H_4do3ap^{ABn}. Based on the solution studies, the final radiolabeled species formed under these reaction conditions must be the $[M(L)]^{z-}$ ($z = 1$ or 2) species; therefore, the ^{153}Sm/^{166}Ho-do3ap, ^{153}Sm/^{166}Ho-do3ap^{PrA} and ^{153}Sm/^{166}Ho-do3ap^{ABn} complexes prepared have a negative overall charge. They are hydrophilic, exhibit a low protein binding and a good stability *in vitro*. The complexes show a good biological profile, with a fast clearance from most organs and a high total excretion at 2 h post injection. Moreover, it is also possible to conclude that the functionalization of the phosphorus acid pendant arm by propionic acid or *p*-aminobenzyl side chains does not influence the radiochemical properties, the biological profile and the bone uptake of the macrocyclic complexes. Additionally, the results suggest that the use of the phosphinic acid pendant arms to modify the H_4dota skeleton might be a suitable alternative to other dota-like ligands for the design of new BFCA valuable for radiolanthanide imaging and/or targeted therapy.

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