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Biological evaluation of ^{153}Sm and ^{166}Ho complexes with tetraazamacrocycles containing methylcarboxylate and/or methylphosphonate pendant arms

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Summary. ^{153}Sm and ^{166}Ho complexes with two series of tetraazamacrocyclic ligands containing methylcarboxylate and/or methylphosphonate pendant arms were synthesized and their charge, lipophilicity, protein binding and *in vitro* and *in vivo* behaviour evaluated. The first series has the same backbone, a 14-membered tetraazamacrocycle containing a pyridine unit with different pendant arms, namely methylcarboxylates (ac_3py14) or methylphosphonates (MeP_2py14 and P_3py14). The second series comprises 12- to 14-membered tetraazamacrocycles having methylcarboxylates and/or methylphosphonates as pendant arms (*trans*-DO2A2P, TRITA, TRITP, TETA and TETP). The ^{153}Sm/^{166}Ho complexes with the 14-membered tetraazamacrocycles containing the pyridine unit are neutral, hydrophilic, have a significant plasmatic protein binding, are unstable *in vivo* and present a slow rate of radioactivity excretion and high hepatic retention. ^{153}Sm/^{166}Ho complexes with the 12- to 14-membered tetraazamacrocycles are quantitatively prepared, except those with TETP. These complexes are hydrophilic, have an overall negative charge and present a medium to low plasmatic protein binding.

The ^{153}Sm/^{166}Ho-*trans*-DO2A2P, ^{153}Sm/^{166}Ho-TRITA and ^{166}Ho-TRITP complexes are stable *in vitro* and *in vivo*, presenting a rapid clearance from main organs and a high rate of whole body radioactivity excretion. Biological profile of ^{153}Sm/^{166}Ho-TRITA complexes makes them promising candidates for therapy when conjugated to a biomolecule, while ^{166}Ho-TRITP is potentially useful for bone targeting due to its considerable uptake by bone.

Introduction

Targeted radionuclide therapy is an important strategy in the management of cancer. The basis for its success is the selective delivery of a high radiation dose to the disease site while sparing normal cells and normal tissues. Some targeted radiolabeled agents to treat tumours or to relief pain associated with bone metastasis are currently in clinical use or under investigation [1–3].

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The design of an effective radiotherapeutic agent involves the selection of a suitable radionuclide and the use of a tumour or bone seeking carrier molecule to deliver the radionuclide to the desired cell or organ. The choice of an appropriate radionuclide is determined by its physical, chemical and biological properties, as well as by its availability and economic factors [4–7]. Radiolanthanides, decaying by beta particle emission, have been explored for therapeutic applications and/or for bone pain palliation, as they present a wide range of beta energies and half-life, which can be easily matched with the biological vector and/or medical application [3, 5, 7].

Therapeutic radiopharmaceuticals are mainly based on the labelling of tumour specific carrier biomolecules, namely peptide analogues [8–16] or monoclonal antibodies (mAb) [17–27], an approach which normally requires derivatisation of the biomolecule with bifunctional chelating agents (BFC) [28–30]. Acyclic and cyclic polyamines have been adopted as BFC, namely diethylenetriamine pentaacetic acid (DTPA) derivatives and 12-membered tetraazamacrocycles with methylcarboxylate pendant arms (DOTA) [29, 30].

For bone pain palliation ^{153}Sm-ethylenediaminetetrakis-(methylphosphonate) (^{153}Sm-EDTMP), a phosphonate ligand labelled with a β^- emitting radionuclide, is currently used for routine clinical application, being generally accepted that its bone localization involves the chemisorption of the phosphonate group by the mineral bone matrix, hydroxyapatite (HA) [31–34]. ^{166}Ho- and ^{177}Lu-EDTMP have also been reported as having favourable biological and physical characteristics for bone pain palliation [35, 36]. ^{153}Sm-EDTMP, in addition to the high and preferential localization in bone, shows rapid blood and soft tissue clearance [37, 38]. However, this radiopharmaceutical is not kinetically inert, and its preparation requires a large ligand excess (250–300 fold ligand excess), if not a high liver uptake is found due to the formation of ^{153}Sm-hydroxyl species [1]. Tetraazamacrocycles with phosphonate pendant arms form highly stable and kinetically inert complexes with radiolanthanides at a considerable lower ligand : metal ratio (1.5–2 : 1), justifying the interest of these ligands for the development of bone pain palliation agents [1]. From this class of complexes, ^{153}Sm- and ^{177}Lu- 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis-(methylphosphonic acid) (^{153}Sm-DOTP and ^{177}Lu-

DOTP) have shown some potential to relieve pain from bone metastases while ^{166}Ho-DOTP seems to be an effective agent for bone marrow ablation in multiple myeloma patients [39–43].

A new trend in bone targeting came with the development of effective multi-drugs, combining anti-cancer therapy (*e.g.*, a radionuclide) with bone resorption inhibitors (*e.g.*, a bisphosphonate) that specifically target bone resorbing cells [44, 45]. In this context a new macrocyclic DOTA-like ligand containing a bis(phosphonic acid) pendant arm (BPAMD) was recently described. Preliminary HA *in vitro* adsorption studies done with ^{160}Tb-BPAMD seem to indicate that this ligand may be promising for bone targeting [46].

Herein, we review our data on the synthesis of ^{153}Sm and ^{166}Ho complexes with two series of tetraazamacrocycles. We also review the *in vitro* and *in vivo* properties of these complexes as well as their potential interest for tumour targeted therapy and/or for palliative therapy of painful bone metastasis [47–49].

Results and discussion

In order to assess the effect of the stereochemical rigidity and cavity size of the macrocycle and nature of pendant arms on the biological behaviour of Sm and Ho complexes, we studied two series of tetraazamacrocycles [47–51]. One series is formed by 14-membered tetraazamacrocyclic ligands containing a pyridine unit and methylcarboxylate or methylphosphonate pendant arms (Fig. 1a). The second series comprises 12- to 14-membered tetrazamacrocycles with methylcarboxylates and/or methylphosphonates pendant arms (Fig. 1b).

Table 1 summarizes the stability constants of the Sm and Ho complexes with these two series of macrocyclic ligands, as well as the corresponding pM values calculated at physiological pH (7.4) [47, 48, 51]. Just for comparison, the values described in the literature for DOTA and DOTP are also included [48]. The stability constants of these macrocycles with Sm^{3+} and Ho^{3+} have been determined using a 1 : 2 metal : ligand molar ratio. For the 14-membered tetraazamacrocycles containing a pyridine unit the species distribution diagrams have shown that only mononuclear species were formed, namely ML and MLOH, while for the ligands with methylphosphonate pendant arms protonated species of the type MH_iL ($i = 1$ to 3 or 4) are also formed [47, 51]. The stability constants (K_{ML}) of the complexes formed with ac_3py14 are lower than those formed with MeP_2py14 and P_3py14. A similar trend was also found for the corresponding pM values ($-\log[M]$) (Table 1). The 12- to 14-membered tetraazamacrocycles (DOTA, TRITA,

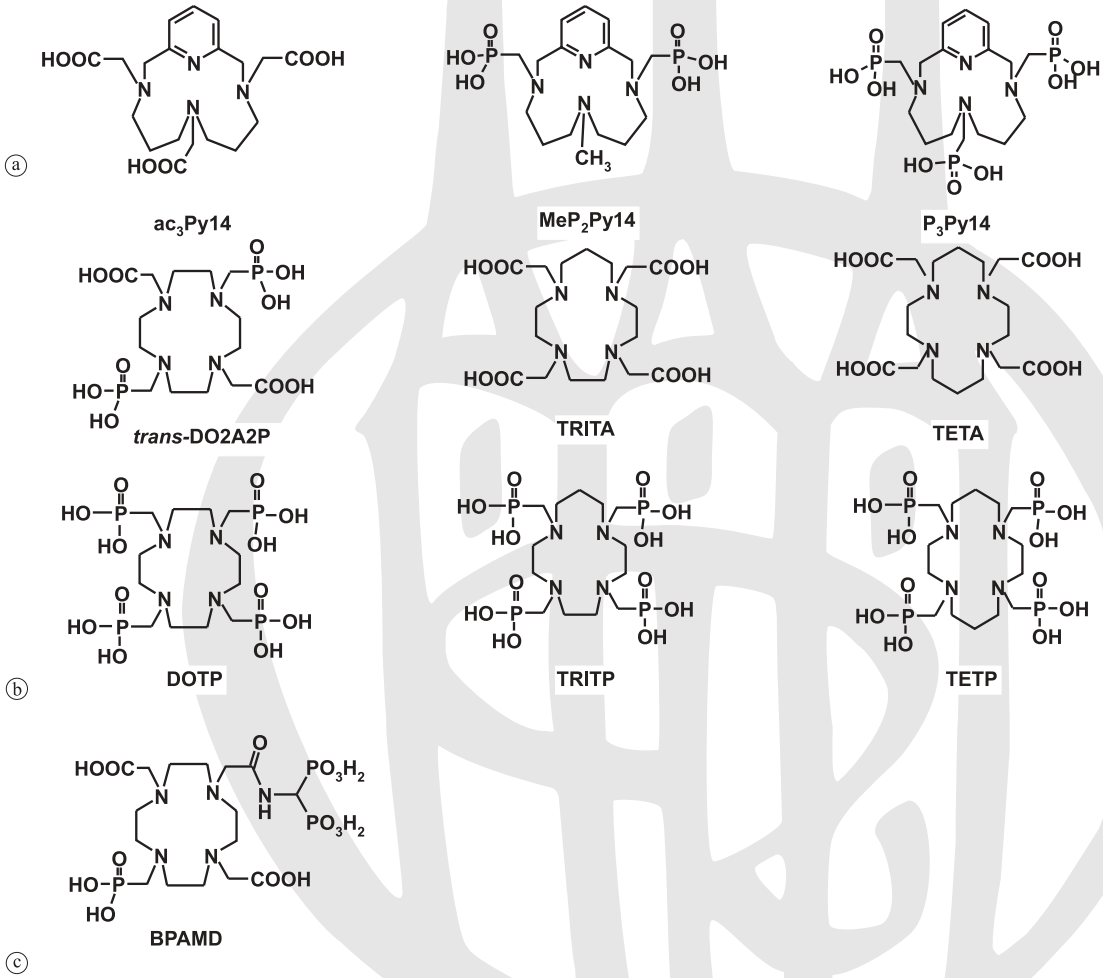

Fig. 1. Structures of the ligands discussed: 14-membered tetraazamacrocycles containing a pyridine unit (**a**); 12- to 14-membered tetraazamacrocycles (**b**); bisphosphonate monoamide analogue of DOTA (**c**).

Table 1. Thermodynamic stability constants (K_{ML}) and pM values (pH = 7.4) of Sm^{3+} and Ho^{3+} complexes with tetraazamacrocyclic ligands, at $I = 0.10$ M in NMe_4NO_3, 25 °C.

Ligand	log K_{ML} [a]		pM [b]	
	Sm^{3+}	Ho^{3+}	Sm^{3+}	Ho^{3+}
ac_3py14 [c]	9.78	10.31	6.84	7.42
MeP_2py14 [c]	17.26	16.84	11.97	11.37
P_3py14 [c]	18.87	19.16	13.57	13.89
DOTA [d]	23.0	24.8	16.25	17.75
TRITA [e]	16.69	17.38	10.50	11.20
TETA [e]	14.15	15.78	8.71	10.67
DOTP [e]	28.1	29.2	14.76	16.49
TRITP [e]	23.83	24.07	13.51	13.95
TETP [e]	19.11	20.03	10.65	11.99

a: $K_{ML} = [ML]/[M][L]$;
b: values calculated for 100% excess of free ligand at pH = 7.4, $C_L = 2C_M = 2.0 \times 10^{-5}$ M;
c: Refs. [47, 51]; d: $I = 1$ M in NaCl at r.t. and Refs. [48, 51];
e: Refs. [48, 51].

TETA, DOTP, TRITP and TETP) form complexes with high thermodynamic stability constants, the values being with Ho^{3+} higher than those with Sm^{3+} [48]. The K_{ML} and pM values determined for the Sm^{3+} and Ho^{3+} complexes with methylphosphonates are higher than the corresponding values for the methylcarboxylates derivatives [48].

Maximum complexation of ^{153}Sm and ^{166}Ho with ac_3py14, MeP_2py14 and P_3py14 was achieved at room temperature, using a 1 : 2 metal : ligand molar ratio and pH values in the range 7-9, in agreement with solution studies [47]. The ^{153}Sm and ^{166}Ho complexes prepared with these ligands have a neutral overall charge, are hydrophilic and present a significant human serum protein binding. As an example, some of the results obtained for ^{153}Sm are shown in Table 2.

Using the same $M : L$ molar ratio (1 : 2), all the 12- to 14 membered tetraazamacrocycles coordinate to ^{153}Sm and ^{166}Ho. With TRITA and TETA, maximum complexation (> 98%) was achieved at room temperature at pH 6–7. Relatively to ^{153}Sm/^{166}Ho-TRITP and ^{153}Sm/^{166}Ho-*trans*-DO2A2P the kinetics of the complexation was slower and a quantitative yield was only obtained at 70 °C, and at pH 8–9. ^{153}Sm/^{166}Ho-TETP complexes could never be prepared quantitatively, 80% being the maximum yield achieved (pH 7–8). The *in vitro* and *in vivo* evaluation of these complexes was not done, as all the attempts to obtain radiochemically pure species were unsuccessful. The ^{153}Sm/^{166}Ho-*trans*-DO2A2P/TRITA/TRITP/TETA complexes are hydrophilic, present a negative overall charge and a low to medium human serum protein binding (Table 2) [47–49].

The *in vitro* stability of these two series of complexes was evaluated in physiological media (saline, phosphate buffer and human serum) over a five-day period at 37 °C. For the ^{153}Sm/^{166}Ho-py14 macrocyclic complexes no radiochemical impurities were found by incubation in saline or phosphate buffer. However, in human serum the complexes decompose over time (Fig. 2).

^{153}Sm/^{166}Ho-DO2A2P, ^{153}Sm/^{166}Ho-TRITA and ^{153}Sm/^{166}Ho-TRITP complexes are stable in all physiological media but ^{153}Sm/^{166}Ho-TETA complexes are unstable in phosphate buffer (Fig. 2) [48, 49].

Table 2. Human serum protein binding, lipo-hydrophilic character (log P) and overall charge of ^{153}Sm-tetraazamacrocycles [47–49].

Ligand	^{153}Sm complexes		
	Protein binding (%)	log P	Distance [a,b] (cm)
ac_3py14	63	−1.51	0
MeP_2py14	51	−1.27	0
P_3py14	43	−1.17	0
DO2A2P	1.5	−1.93	3.5
TRITA	7.0	−1.93	4.3
TRITP	14	−1.48	2.5
TETA	7.8	−1.75	2.0

a: Paper electrophoresis in Tris-HCl buffer (pH 7.4), 1 h at 300 V;
b: Migration towards anode.

Using hydroxyapatite (HA) as a model of bone surface, the degree of adsorption of the ^{153}Sm/^{166}Ho complexes with methylphosphonate ligands onto HA was studied. As shown in Table 3, the values found for ^{153}Sm/^{166}Ho-TRITP are encouraging (90%) and comparable to the values found with other promising complexes. For ^{166}Ho-DO2A2P the HA binding is lower when compared with the other complexes, clearly showing the importance of the number and/or nature of the phosphorus containing pendant arms on the HA binding (Table 3) [49].

The biological behaviour of ^{153}Sm/^{166}Ho-py14 macrocyclic complexes was assessed in CD-1 mice at different times after administration (30 min, 2 h and 24 h). As an example, Fig. 3 shows the biological profile obtained for ^{153}Sm/^{166}Ho-ac_3py14 and ^{153}Sm/^{166}Ho-P_3py14. All the complexes present a similar biodistribution profile: a slow

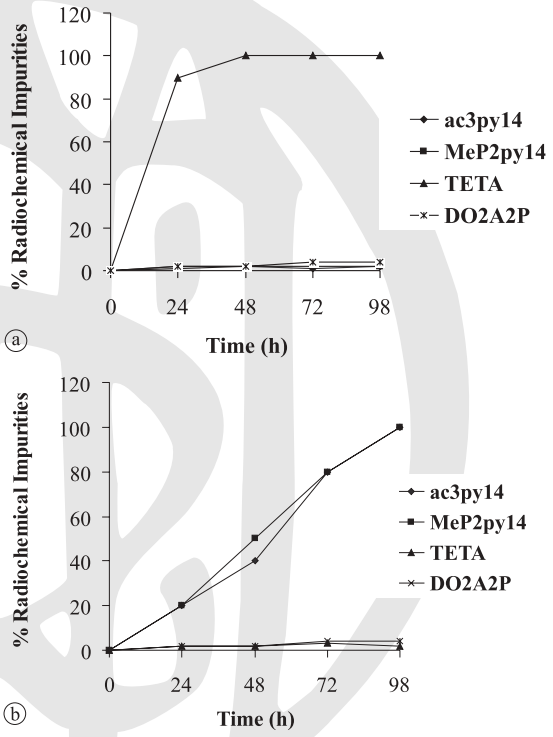

Fig. 2. *In vitro* stability of ^{153}Sm/^{166}Ho-complexes in phosphate buffer (pH 7.4) (**a**) and in human serum (**b**).

Table 3. Binding of radiolanthanide complexes with phosphonate ligands to 50 mg of HA.

^{153}Sm-DOTP[a]	^{153}Sm/^{166}Ho-TRITP[b]	^{166}Ho-DO2A2P[c]	^{160}Tb-BPAMD[d]
5 μM, > 95%, 2 h	0.3 mM, ~ 90%, 1 h	0.4 mM, > 35%, 1 h	0.2–2 mM, > 95%, 1 h

a: Ref. [39]; b: Ref. [48]; c: Ref. [49]; d: Ref. [46].

clearance from organs like blood and muscle, a high and rapid uptake by liver which increases over time and a very slow rate of total radioactivity excretion from the whole animal body (less than 12% of total injected dose at 24 h after administration) [47]. As we discussed previously, the significant spleen and lung uptake may suggest the presence of radioactive colloidal and/or polymeric forms [47, 52]. To get a better insight into this biological profile, metabolic studies were performed by analysis of urine and blood. These studies together with the biological profile of ^{153}Sm/^{166}Ho nitrate solutions, in the same animal model, clearly indicate that ^{153}Sm/^{166}Ho-py14 macrocyclic complexes decompose *in vivo*, these results being consistent with the *in vitro* instability found for these complexes in human serum [47].

The significant bone uptake found for ^{153}Sm/^{166}Ho complexes with MeP_2py14, P_3py14 and ac_3py14 may be explained by the *in vivo* metal release, which mimics Ca^{2+} interaction with the mineral bone matrix hydroxyapatite. Our studies are in accordance with others previously described for ^{153}Sm-ac_3py12 [53].

As an example, Fig. 4 shows the biodistribution profile of ^{166}Ho-TRITA and ^{166}Ho-TETA, and, just for comparison, we also show the profile obtained for ^{166}Ho-DOTA in the same animal model [48].

As shown in Fig. 4, the *in vivo* results for DOTA and TRITA complexes are quite similar, demonstrating a rapid clearance from most organs, including blood and soft tissues and a very high rate of total radioactivity excretion (> 90% at 2 h) [48]. However, complexes with TETA show a remarkably slow clearance from main organs and also a low rate of total body excretion (> 80% for ^{166}Ho-TETA and 40% for ^{153}Sm-TETA). Metabolic studies have demonstrated that ^{153}Sm/^{166}Ho-TRITA and ^{166}Ho-TETA are stable *in vivo*, as well as ^{153}Sm/^{166}Ho-DOTA [48]. However ^{153}Sm-TETA is instable. These results clearly show that 13-membered tetraazamacrocyles with methylcarboxylate pendant arms lead to complexes which present a DOTA-like biological profile, being interesting for labelling tumour targeting biomolecules.

As can be seen in Fig. 5, the complexes ^{153}Sm/^{166}Ho-DO2A2P and ^{166}Ho-TRITP have a DOTA-like profile, namely high *in vivo* stability and rapid clearance from tissues and whole body excretion.

The main differences observed in biodistribution studies in mice between these complexes with methylcarboxylates and/or methylphosphonates are mostly related to the degree of bone uptake. The ^{153}Sm/^{166}Ho-TRITP complexes have considerable accumulation and long residence in the bone, especially ^{166}Ho-TRITP. Nevertheless, ^{153}Sm-TRITP reveals a slower blood clearance and significantly higher liver uptake, associated to radioactivity retention, than the ^{166}Ho complex, which suggest the presence of radiochemical impurities. Thus, ^{166}Ho-TRITP has the best biological profile for therapeutic purposes, due to its rapid total excretion and washout from all organs, which led to favourable radioactivity ratios for bone/non target organs.

In spite of the heterogeneity in the animal models, Table 4 presents some relevant parameters related with bone uptake for ^{166}Ho-TRITP and for other complexes described as good bone agents or in clinical use [31, 32, 39, 48, 49]. Although the bone/blood and bone/muscle ratios are lower than those described for ^{153}Sm-EDTMP [31], the analysis of

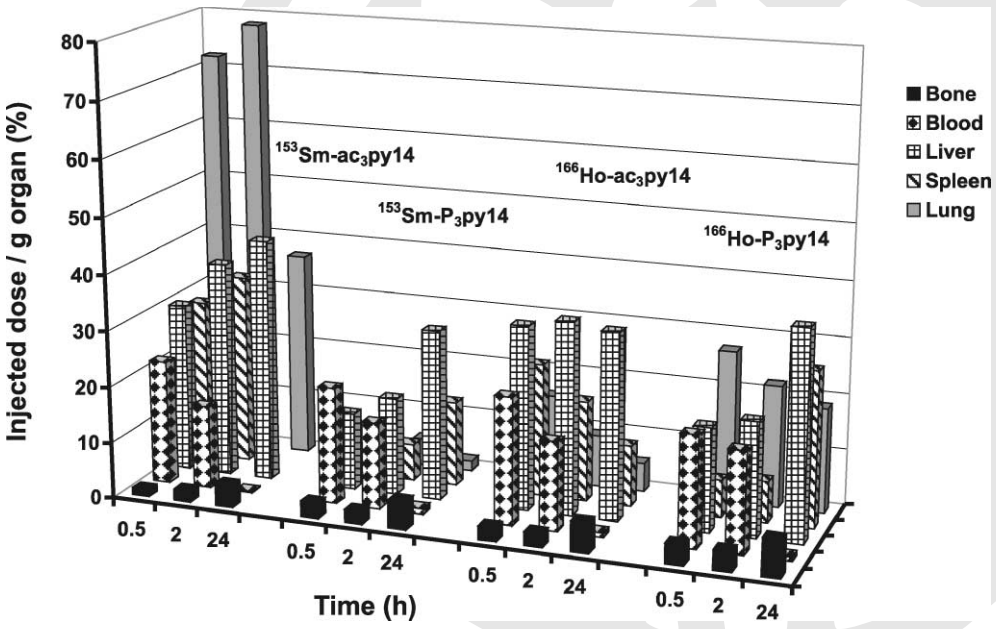

Fig. 3. Biodistribution data expressed as a percent of injected dose per g organ (%ID/g organ ± SD) of ^{153}Sm/^{166}Ho-ac_3py14 and ^{153}Sm/^{166}Ho-P_3py14.

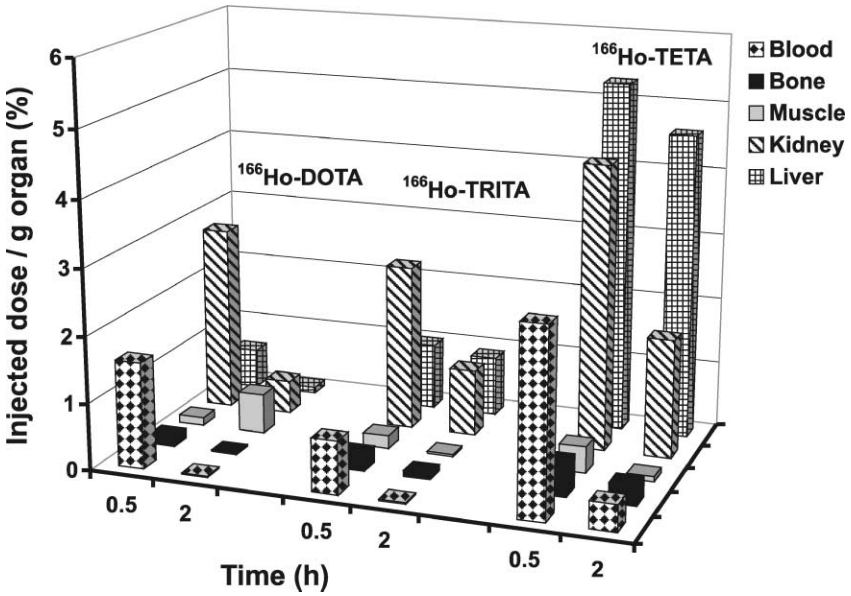

Fig. 4. Biodistribution data expressed as a percent of injected dose per g organ of ^{166}Ho-DOTA, ^{166}Ho-TRITA and ^{166}Ho-TETA [48].

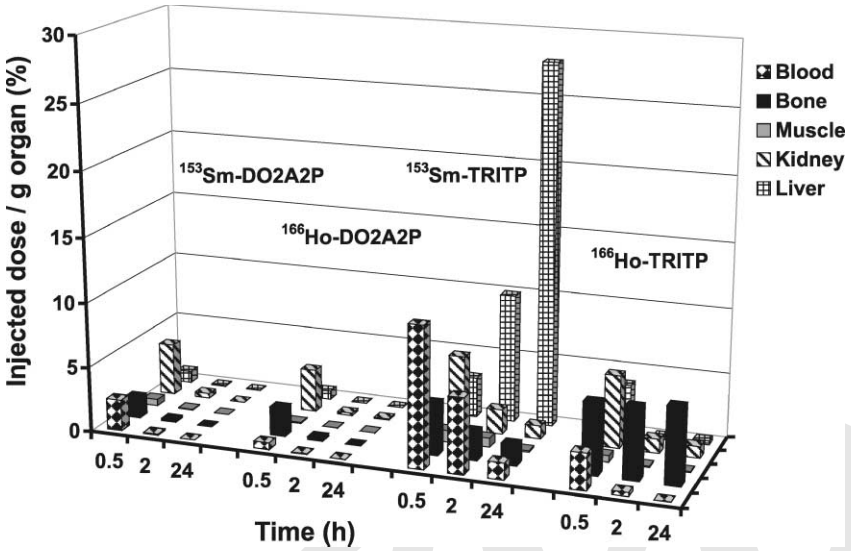

Fig. 5. Biodistribution data expressed as a percent of injected dose per g organ of ^{153}Sm/^{166}Ho-DO2A2P and ^{153}Sm/^{166}Ho-TRITP.

these data clearly demonstrates that ^{166}Ho-TRITP is a good candidate for bone targeting owing to its evident accumulation in bone and rapid clearance from other tissues. Additionally, ^{166}Ho-TRITP has the advantage, when compared with ^{153}Sm-EDTMP, of being prepared with a very low metal : ligand molar ratio (1 : 2), due to its kinetic inertness.

Concluding remarks

The reviewed tetrazamacrocycles present high or extremely high thermodynamic stability constants with Sm and Ho. However, the K_{LnL} and pM (Ln = Sm, Ho) values decrease when the cavity size increases, and for the same cavity size and macrocycle backbone the values are lower for ligands with methylcarboxylate pendant arms. In general, for the same macrocycle the K_{LnL} and pM values are higher for Ho than for Sm.

Using a 1 : 2 metal : ligand molar ratio, all the tetraazamacrocycles studied react quantitatively (> 98%) with [^{153}Sm$(NO_3)_3$] and [^{166}Ho$(NO_3)_3$] in the pH range 6–9. ^{153}Sm/^{166}Ho-TETP complexes were the only ones which could not be prepared quantitatively (yield: 80%). The *in vitro* studies demonstrated that ^{153}Sm/^{166}Ho-ac$_3$py14, MeP_2py14 and P_3py14 complexes are unstable in human serum, presenting a significant protein binding. *In vivo* they are not kinetically inert and decompose forming either colloidal species and/or complexes with serum proteins, which certainly justifies the high liver and spleen uptake and the slow rate of total excretion found. ^{153}Sm/^{166}Ho-DO2A2P, TRITA, TRITP and TETA complexes are all formed quantitatively, being stable under physiological conditions, except ^{153}Sm/^{166}Ho-TETA which decomposes in phosphate buffer and also slightly *in vivo*. ^{153}Sm/^{166}Ho-DO2A2P and ^{153}Sm/^{166}Ho-TRITA present a DOTA-like biological profile, being promising for therapy when conjugated to a biomolecule. Otherwise, ^{166}Ho-TRITP could be potentially useful for bone targeting due to its considerable bone uptake and biological profile.

In summary, the increase of the cavity size from 12- to 14- decreases the thermodynamic stability of the complexes and leads to less favourable biological profiles, this

Table 4. Comparative study of the uptake of $^{153}Sm/^{166}Ho$-phosphonate complexes.

Organ	Uptake (% ID/g) of $^{153}Sm/^{166}Ho$ complexes		
	^{153}Sm-EDTMP	^{153}Sm-DOTP	^{166}Ho-TRITP
Blood	0.002 ± 0.002^a 0.0^b	0.18^c 0.001 ± 0.000^d	0.29 ± 0.09^e
Liver	0.027 ± 0.005^a 0.25^b	0.0065^c 0.091 ± 0.052^d	0.23 ± 0.03^e
Muscle	0.003 ± 0.001^a 0.22^b	0.017^c 0.003 ± 0.001^d	0.09 ± 0.01^e
Femur/tibia	3.72 ± 0.26 (femur)^a 2.3 (femur)^b	0.218 (femur)^c 3.94 ± 0.50 (tibia)^d	5.5 ± 0.8^e (femur)
Bone/blood	1833 ± 1274^a $-^b$	1.21^c 3940.0^d	22.7^e
Bone/muscle	1459 ± 505^a 10.5^b	12.8^c 1313.3^d	54.8^e
Excretion	49.11 ± 3.92^a 49.1^b	$-^c$ 40.13 ± 5.92^d	77.8 ± 1.0^e
Time post-injection	2 h	0.5 h^c 3 h^d	2 h^e
Animal strain	Sprague–Dawley rats	Wistar rats	Mice

a: Ref. [31]; b: Ref. [32]; c: Ref. [39]; d: Ref. [40]; e: Ref. [48].

effect being more relevant when a pyridine unit is introduced in a 14-membered tetraazamacrocycle. In fact, the stereochemical rigidity imposed by the pyridine unit in the 14-membered tetraazamacrocycles decreases the kinetic inertness of the lanthanide complexes. Finally, radiolanthanide complexes with 12- or 13- membered tetraazamacrocycles are expected to be useful for medical applications as good agents for tumour or bone targeting, depending on the nature of the pendant arms.

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