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Radiolanthanide complexes with tetraazamacrocycles bearing methylphosphonate pendant arms as bone seeking agents

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Aim. **Radiolanthanide complexes with ligands bearing phosphonate groups have demonstrated their usefulness as bone seeking agents. Herein, we report on the synthesis of ^{153}Sm and ^{166}Ho complexes with 12- to 14-membered macrocycles containing different number of methylphosphonate pendant arms and their *in vitro* and *in vivo* evaluation in order to assess the effect of the cavity size and type of appended arms on their biological behavior.**

Methods. **Radioactive macrocycle complexes were prepared by reaction of ^{153}Sm/^{166}Ho nitrates with four different tetraazamacrocycles bearing methylphosphonate groups. Radiochemical behavior, *in vitro* stability and charge of complexes were studied by chromatography and electrophoresis. The lipophilicity, plasmatic protein binding and adsorption onto hydroxyapatite (HA) were evaluated by *in vitro* assays. Biodistribution was assessed in CD-1 mice. Radiolabeling efficiency depends both on radionuclide and ligand structure. All the complexes are hydrophilic with an overall negative charge and relatively low protein binding. High *in vitro* stability in human serum and adsorption onto HA was found for all the complexes.**

Results. **Biodistribution and *in vivo* stability studies have demonstrated promising biological profile for targeted radiotherapy, namely a rapid tissue clearance from most organs and rapid total excretion. Additionally, ^{166}Ho-tritp has a high bone uptake, which led to high bone/blood and bone/muscle ratios.**

Conclusion. **Our results clearly demonstrate that 12- and 13-membered macrocyclic ligands led to stable complexes with biological profile adequate to radionuclide therapy. The favorable *in vivo* behavior highlights the interest to further investigate these or closely related complexes to be used as bone seeking agents.**

KEY WORDS: Bone and bones - Pain - Palliative care - Radiopharmaceuticals - Radionuclides.

The use of therapeutic radiopharmaceuticals has been an important strategy in the management of cancer since they can deliver high radiation doses to specific disease sites in target organs or tissues while sparing healthy cells. Among their applications, the treatment of metastatic bone pain has a great impact in cancer patients. Actually, most malignant tumors, especially breast and prostate carcinomas, frequently metastasize to the bone inducing pain. Thus, bone metastases represent a major health burden because of their frequency and the considerable morbidity associated.[1-3]

The key question to develop an effective radiopharmaceutical for therapy is the selection of the most appropriate radionuclide and carrier ligand. In a number of studies several radiolanthanides have demonstrated usefulness for therapeutic applications owing to their ready availability and suitable emission char-

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TABLE I.—*Decay characteristics and production of ^{153}Sm and ^{166}Ho.*

Radio-lantanide	$t_{1/2}$ (h)	β- MeV	γ keV	Target material	Specific activity (MBq/mg)
^{153}Sm	46.8	0.64 (32%) 0.69 (50%) 0.81 (18%)	103 (29%)	$^{152}Sm\ (NO_3)_3$	110-150
^{166}Ho	26.8	1.85 (51%) 1.77 (48%)	81 (7.5%)	$^{165}Ho(NO_3)_3$	220-260

acteristics. Among them, ^{153}Sm and ^{166}Ho, medium- and high-energy β emitters respectively, possess a range of half-lives and β-energies (Table I) with potential application in nuclear medicine and are expected to have different therapeutic potential as consequence of their decay properties.[4, 5]

Radiolanthanide complexes with different ligands containing phosphonate groups have already proven their efficacy as bone targeting agents. Quadramet®, a ^{153}Sm complex with an acyclic ligand holding methylphosphonate arms, is in clinical use for palliative treatment of painful osseous metastases.[6] Despite its clinical use, this radiopharmaceutical presents some disadvantages namely its low kinetic inertness and the need of a large excess of ligand for its preparation.[7, 8] Furthermore, the complex ^{166}Ho-dotp (dotp = [1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl tetra(methylene)]-tetra phosphonic acid) is an effective agent for bone marrow ablation in multiple myeloma patients.[9, 10] Previous studies on the synthesis of radiolanthanide complexes with different tetraazamacrocycles with methylcarboxylate and/or methylphosphonate pendant arms have demonstrated the ability of ^{166}Ho-tritp ([1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl tetra(methylene)] tetra phosphonic acid) for bone targeting.[11, 12] Therefore, we proceeded our research with the synthesis of novel tetraazamacrocycles namely 2,2'-[4,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl] diacetic acid, *trans*-H_6do2a2p and 2-[4,7,10-tris(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1-yl] acetic acid, H_7doa3p[13]. The structure of these macrocyclic ligands is shown as well as the structures of tritp and tetp for comparison (Figure 1).

The aim of the present study was the synthesis of ^{153}Sm and ^{166}Ho complexes with 12- to 14-membered macrocycles bearing a different number of methylphosphonate appended arms and their *in vitro* and *in vivo* evaluation to assess the effect of the cavity size and the type and number of pendant arms on the *in vitro/in vivo* stability and biological behavior of the radiolanthanide complexes. This study intends to get a better insight on structure/biological activity and to contribute for a rational design of radiopharmaceuticals for bone pain palliation/therapy.

Materials and methods

Materials

Chemicals and solvents were of reagent grade and were used without further purification, unless stated otherwise. 1,4,7,10-tetraazacyclododecane (cyclen)

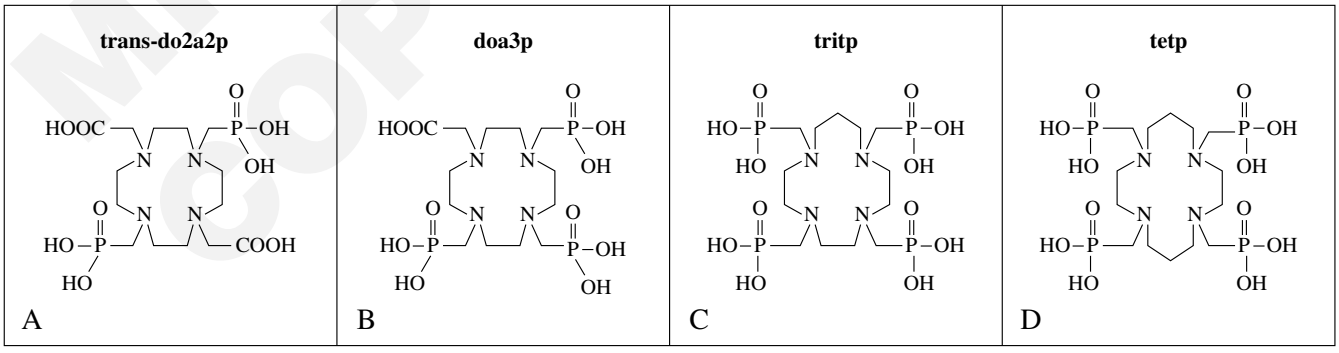

Figure 1.—Structure of the macrocyclic ligands containing methylphosphonate pendant arms: A) 2,2'-[4,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl] diacetic acid (*trans*-do2a2p); B) 2-[4,7,10-tris(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1-yl] acetic acid (doa3p); C) [1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl tetra(methylene)] tetra phosphonic acid (tritp); D) [1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetrayl tetra(methylene)] tetra phosphonic acid (tetp).

and [1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl tetra(methylene)]-tetra phosphonic acid (dotp) were purchased from Strem (New-buryport, MA, USA). Paraformaldehyde, triethyl phosphite, *tert*-butyl bromoacetate, silica gel (200-400 mesh, 60 Å) for column chromatography, D_2O, $CDCl_3$ and all other standard reagents were obtained from Aldrich Chemical Co., Inc.

Enriched Sm_2O_3 (98.4% ^{152}Sm) was obtained from Campro Scientific and natural Ho_2O_3 (99.9%) from Strem Chemicals. Calcium phosphate dibasic (hydroxyapatite, HA) was purchased from Aldrich Chemical Co., Inc. All materials used for radiochemical and biological evaluation of radioactive compounds were reagent grade unless otherwise specified.

Analytical methods

Nuclear magnetic resonance (NMR) spectra of all synthetic intermediates and final products were recorded on a Varian Unit Inova-300 spectrometer. The chemical shift of ^1H (300 MHz) and ^{13}C (75.5 MHz) is relative to tetramethylsilane and to CH_3 of *tert*-butyl alcohol (^1H at 1.24 ppm, ^{13}C at 30.29 ppm, in D_2O), respectively. ^{31}P (121.5 MHz) NMR spectra were recorded in $CDCl_3$ or in D_2O and referenced to external 85% aqueous H_3PO_4 solution (δ = 0 ppm). Carbon, hydrogen and nitrogen elemental analysis was performed on a Perkin-Elmer automatic analyzer.

Mass spectrum was recorded on a Thermo Finnighan LCQ Advantage mass spectrometer equipped with an ESI probe operated under a negative polarity.

The radionuclidic purity of the ^{153}Sm and ^{166}Ho solutions was assessed by ?-ray spectrometry using a Ge (Li) detector coupled to an Accuspec B Canberra multichannel analyzer. Spectra were processed, following efficiency calibration with a ^{152}Eu source. The ^{153}Sm and ^{166}Ho activities produced after irradiation were measured in a dose calibrator (Aloka Curiemeter IGC-3).

Syntheses

The ligands *trans*-H_6do2a2p 2,2'-[4,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl] diacetic acid,[13] tritp [1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl tetra(methylene)] tetra phosphonic acid,[14] tetp [1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetrayl tetra(methylene)] tetra phosphonic acid [14] and the derivative 1,4,7,10-tetraazacyclododecane-1-acetic acid-1,1-dimethylethylester[15] were synthesized according to the procedures described in the literature.

tert-BUTYL {4,7,10-[TRIS(DIETHYLPHOSPHONYL)METHYL]-1,4,7,10-TETRAAZACYCLODODECAN-*1*-YL} ACETATE (1)

An amount of 0.721 g (2.51 mmol) of 1,4,7,10-tetraazacyclododecane-1-acetic acid-1,1-dimethylethylester was suspended in triethyl phosphite (1.5 mL, 8.75 mmol) and the mixture was immersed in an ice bath. Dry paraformaldehyde 0.249 g (8.2 mmol) was gradually added (2 h). The mixture was then allowed to warm up to room temperature and stirring was continued for 60 h, and finally at 45 °C for 1 day. The product was purified by column chromatography on silica gel (gradient elution: MeOH/aq. NH_3 100/0 – 60/25; Rf = 0.3 in MeOH/aq.NH_3 70/25), giving a colorless oil (1.26 g; yield 68%). ^1H NMR ($CDCl_3$/TMS), δ (ppm):4.09 (pseudo quintet, 12H, P(O)O$CH_2$$CH_3$, $^3J_{HP}$=$^3J_{HH}$=7.2 Hz), 3.25 (s, 2H, NCH_2t-Bu), 2.92 (d, 2H, NCH_2P, $^2J_{HP}$=9 Hz), 2.9 (d, 4H, NCH_2P, $^2J_{HP}$=9 Hz), 2.81-2.73 (br, m, 16H, (NCH_2CH_2N), 1.41 (s, 9H, CH_3 t-Bu), 1.28 (t, 18H, P(O)OCH$_2CH_3$, $^3J_{HH}$=7.5 Hz). ^{13}C NMR ($CDCl_3$, δ (ppm): 170.92 (*C*OOt-Bu), 80.7 ($C(CH_3)_3$), 61.67 (P(O)OCH_2CH$_3$), 56.13 (CH_2COOt-Bu), 53.33 (NCH_2CH_2N), 52 (NCH_2CH_2N) 51.61 (NCH_2CH_2N and CH_2(P(O)OCH$_2$CH$_3$), 49.8 (CH_2(P(O)OCH$_2$CH$_3$), 28.16 (CH$_2$COO C(CH_3)$_3$), 16.54 (P(O)OCH$_2CH_3$). ^{31}P NMR ($CDCl_3$/H_3PO_4), δ (ppm): 26.6, 26.5.

2-[4,7,10-TRIS(PHOSPHONOMETHYL)-1,4,7,10-TETRAAZACYCLODODECANE-1-YL] ACETIC ACID (doa3p)

Compound 1 (0.942 g, 1.28 mmol) was dissolved in HCl (20%, 40 mL) and the mixture was refluxed for 48 h. The reaction was filtered and the filtrate was evaporated to dryness in vacuum. The clear oil obtained was then dissolved in H_2O and vacuum dried. This procedure was repeated until a pale brown powder was obtained. The crude product was purified by recrystallization from ethanol/H_2O (25 mL/5 mL). Removal of the solvent yielded a pale yellow solid (0.238 g; 34%). ^1H NMR (D_2O-KOD/TMS); pD=6.2) δ (ppm): 3.63 (s, 2H, NCH_2COOH), 3.3, 3.09, 2.85 (br, m, 22H, overlapping resonances of NCH_2CH_2N and NCH_2P). ^{13}C

NMR (D_2O-KOD/tert-butyl alcohol, pD=6.2), δ (ppm): 176.13 (*C*OOH), 58.89 (*C*H_2COOH), 54.37, 53.83, 53.35, 53.23, 52.95, 52.66, 52.52. ^{31}P NMR (D_2O-KOD/H_3PO_4, pD=7.6), δ (ppm): 15.9, 7.17. MS [(ESI) m/z]: calcd for doa3p: 512.33; found: 511.1 [M-H]$^-$ (100%). Anal. calcd for $C_{13}H_{31}N_4O_{11}P_3.2H_2O$: C, 28.47; H, 6.43 ; N, 10.22; found C, 28.14; H, 6.57; N, 10.34.

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

Samarium-153 and ^{166}Ho were produced in the ITN Portuguese Research Reactor (RPI) by thermal neutron bombardment as previously described.[16] Decay characteristics of these radionuclides, target materials used in the production and the specific activities achieved after 3 h irradiation and at end of bombardment (EOB) can be found in Table I. The radionuclidic purity of ^{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.

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

Radiolabeling of the macrocycles (*trans*-do2a2p, doa3p, tritp, tetp) was performed by dissolving of the ligands (5 mg) in H_2O followed by addition of an adequate amount of ^{153}Sm or ^{166}Ho nitrate solutions to achieve a 1:2 (metal:ligand) molar ratio in a final ligand concentration of 17-19 mM. The experimental labeling conditions were optimized by changing the pH (5-10) using 1M NaOH solution and the incubation temperature (room temperature, 40 ºC, 60 ºC and 70 ºC). Labeling efficiency, chelation kinetics and stability of the radiolanthanide complexes were assessed by ascending chromatography, instant thin-layer chromatography silica gel strips (ITLC-SG) (Polygram, Macherey-Nagel) developed with the mobile phase MeOH:H_2O:NH_4OH (2:4:0.2). In this system the ^{153}Sm/^{166}Ho complexes migrate with R_f=1, 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. These species can be quantified by ascending instant thin layer chromatography using silica gel TLC strips developed with saline. In this system both, the radiolanthanide complexes and ^{153}Sm/^{166}Ho$(NO_3)_3$ migrate with R_f=1.

Similar radiolanthanide complexes with dotp were also prepared and further studied in the same *in vitro* and *in vivo* models for comparison.

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 ºC, at various time points (up to 4 days). Typically, 50 μL of each ^{153}Sm- or ^{166}Ho-complexes solution were added to 100 μL of the different solutions and stored at 37 ºC. Daily, an aliquot of each mixture was taken and evaluated by ITLC analysis, as described above. The percentage of the radiochemical impurities was then calculated.

Adsorption studies

Adsorption of the ^{153}Sm and ^{166}Ho complexes onto HA was determined by incubation with different amounts of HA, as already described.[11, 12]

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.[11, 12]

Lipophilicity was assessed by determination of the partition coefficient (*P*) *n*-octanol/saline and expressed as log *P*, according to the previously described method.[11, 12]

Plasmatic protein binding was determined by gel filtration on Sephadex G-25 using saline or phosphate buffer (pH 7.4) as eluent after 1 h incubation of the radiolanthanide complex (100 μL) with 1 mL of human blood plasma, as previously reported.[11, 12]

In vivo *studies*

BIODISTRIBUTION STUDIES

The *in vivo* behavior of the radioactive complexes was evaluated in groups of 4-5 female CD-1 mice (randomly bred, from Charles River Laboratories, Spain) weighing approximately 20-22 g. Animals were injected intravenously 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 postinjection, according to a previously described method.[11, 12] Results were expressed as percentage of injected dose per gram of organ (% I.D./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 (% I.D.).

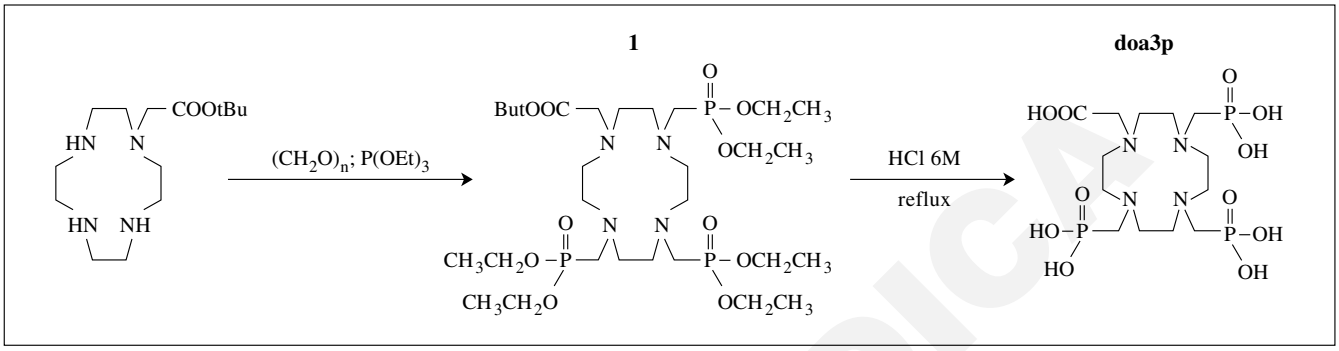

Figure 2.—Synthesis of the ligand H_7doa3p.

TABLE II.—*Labeling experimental conditions and efficiency of ^{153}Sm and ^{166}Ho complexes.*

Ligand	^{153}Sm complexes		^{166}Ho complexes	
	Labeling conditions	Labeling efficiency (%)	Labeling conditions	Labeling efficiency (%)
trans-do2a2p	0.5 h; 60/70 °C; pH 8-9 2 h; r.t.; pH 8-9	>98	0.5 h; 60/70 °C; pH 8-9 2 h; r.t.; pH 8-9	>98
doa3p	15 min; r.t.; pH 8-9	>98	24 h; r.t.; pH 8-9 15 min; 70 °C pH 8-9	>98
dotp	1 h; 60/70 °C; pH 8-9	>98	1 h; 60/70 °C; pH 8-9	>98
tritp	2 h; 60/70 °C; pH 9	>98	2 h; 60/70 °C; pH 9	>98
tetp	2.5 h; 60/70 °C; pH 7	>80	2.5 h; 60/70 °C; pH 8	>80

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 guidelines on the use of living animal in scientific investigation, and followed the principles of laboratory animal care.

Results

Synthesis of the Tetraazamacrocycles

The ligands *trans*-H_6do2a2p, tritp and tetp were prepared as described in the literature. [13, 14] The novel ligand doa3p was prepared by using the synthetic route shown in Figure 2. Briefly, the monoalkylated cyclen was converted to the intermediate phosphonate 1 using triethylphosphite and paraformaldehyde in a Mannich-type reaction. The product was purified by column chromatography on silica gel (MeOH- 25% aq.NH_3). The purification by the standard distillation techniques did not result as the excess of triethylphosphite and other volatile impurities could not be eliminated, even after keeping the crude product under high vacuum (50-60 ºC) for several hours.

Conversion of the intermediate phosphonate 1 into the phosphonic acid doa3p was achieved by refluxing in conc. HCl. The ligand was purified by recrystallization from ethanol/water. Complete details on the synthesis, characterization and structural studies of doa3p can be found in Campello MP, Santos I. Manuscript in preparation.

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

All radiolanthanide complexes were obtained by reacting ^{153}Sm/^{166}Ho$(NO_3)_3$ with the macrocyclic ligands, in a 1:2 metal to ligand molar ratio. Radiolabeling experimental conditions were optimized for each ligand to obtain high radiochemical purity (Table II). It was observed that the highest yields for complex for-

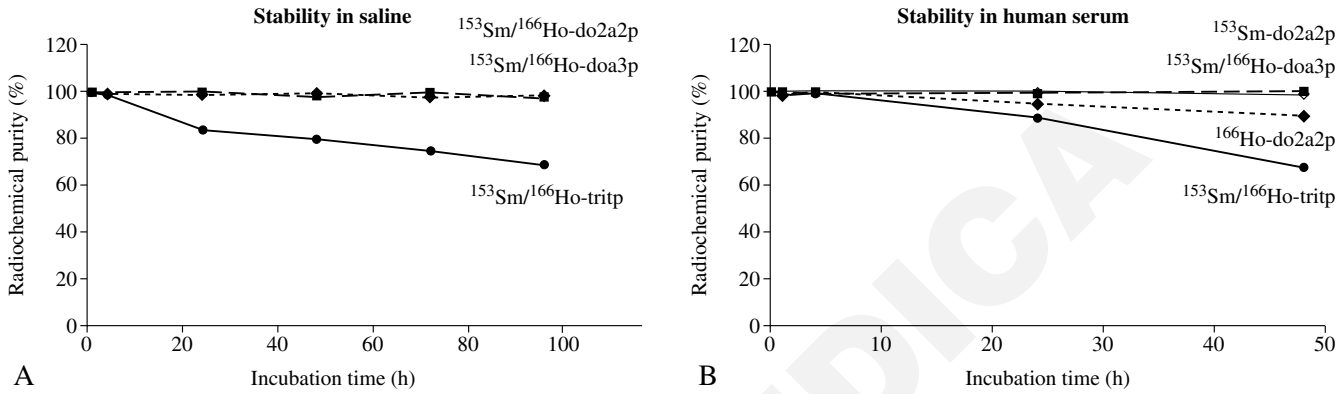

Figure 3.—Stability of $^{153}Sm/^{166}Ho$ complexes in saline (A) and human serum (B) at 37 ºC up to 4 days.

TABLE III.—*Plasmatic protein binding and lipo-hydrophilic character (log P) of $^{153}Sm/^{166}Ho$ complexes.*

Ligand	^{153}Sm complexes		^{166}Ho complexes	
	Protein binding (%)	Log *P*	Protein binding (%)	Log *P*
trans-do2a2p	15	-1.93	15	-1.32
doa3p	1.3	-1.68	1.2	-1.57
tritp	14	-1.48	2	-1.08

mation were obtained at pH 8-9 for *trans*-do2a2p, doa3p and dotp, while complexation with tritp occurred preferentially at pH 9. For the ligands *trans*-do2a2p and doa3p maximum labeling efficiency was accomplished at room temperature whereas for dotp and tritp the labeling yields were improved by incubation at 60/70 ºC. Most of the radioactive complexes could be obtained with high labeling efficiencies (>98%), except the radiolanthanide complexes with tetp. With this ligand the maximum labeling yield achieved was about 80% and due to such result no evaluation of $^{153}Sm/^{166}Ho$-tetp was carried out.

The stability studies were undertaken up to 4 days in different physiological solutions and assessed by ITLC and cellulose acetate electrophoresis. Results indicated that all the complexes are stable in phosphate buffer (pH 7.4) and in 0.1 M Tris-HCl buffer (pH 7.4). After 24 h of incubation 84% and 89% of the $^{153}Sm/^{166}Ho$-tritp complexes remain intact in saline and human serum, respectively, whereas $^{153}Sm/^{166}Ho$-doa3p and ^{153}Sm-*trans*-do2a2p do not reveal any radiochemical impurities (Figure 3). After 24 h of incubation in human serum, ^{166}Ho-*trans*-do2a2p presents some degradation that increases slightly over time, probably related to trans-chelation with serum proteins.

All the $^{153}Sm/^{166}Ho$ complexes migrate as single radiochemical species have a negative charge, at pH 7.4, and exhibit hydrophilic character. Data from plasmatic protein binding and lipophilicity are summarized in Table III. The lowest percentage of protein binding was found for complexes with doa3p (1.2 and 1.3) although all the other complexes also show a relatively low protein binding.

Adsorption studies onto HA, a model of the extracellular matrix of bone, have been suggested as an *in vitro* method to anticipate the *in vivo* stability of the radiolanthanide complexes [17] and to predict their ability to be taken by the bone tissue. For that reason, HA binding studies of the $^{153}Sm/^{166}Ho$ complexes were carried out in order to evaluate their potential as bone-seeking agents.

Analysis of the adsorption curves has indicated that both complexes with tritp, a 13-membered macrocycle containing 4 methylphosphonate groups, reach a maximum binding of 90% onto 10 mg of HA (Figure 4). For the same amount of HA, a significantly binding decrease was observed among complexes with ligands containing only 2 or 3 methylphosphonate groups. The complexes with the macrocycle bearing 2 *trans*-phosphonate pendant arms presented the lowest absolute binding. It was also observed that ^{166}Ho complexes with the 12-membered macrocycles bound more significantly to HA than the corresponding ^{153}Sm complexes.

The biodistribution profiles of ^{153}Sm- and ^{166}Ho-

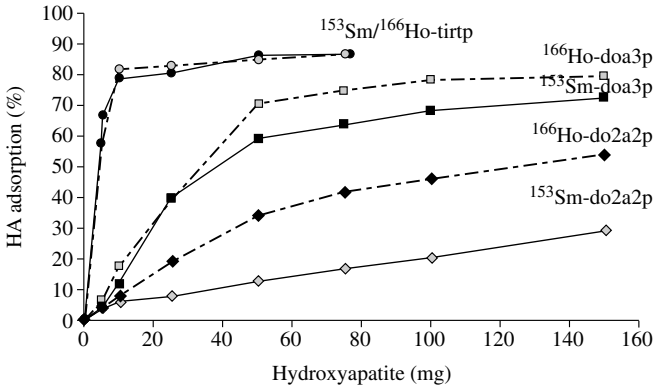

Figure 4.—Hydroxyapatite adsorption of $^{153}Sm/^{166}Ho$-complexes (%) as function of the hydroxyapatite amount. HA: hydroxyapatite.

complexes in normal CD-1 mice are presented in Figures 5 and 6, respectively. Whole animal body radioactivity excretion is graphically represented in Figure 7.

Complexes with the 12-membered macrocyclic ligands have been rapidly cleared from the blood stream and major organs. There is no significant accumulation of radioactivity in any major organs except in the kidneys. Most of the injected dose (>93% and >88% of $^{153}Sm/^{166}Ho$-*trans*-do2a2p and ^{153}Sm-doa3p, respectively) was excreted 2 h after administration. Furthermore, ^{153}Sm-doa3p showed a rapid accumulation and long residence in the bone while a moderate bone uptake was found for $^{153}Sm/^{166}Ho$-*trans*-do2a2p, at 30 min after administration, rapidly decreasing over time. $^{153}Sm/^{166}Ho$-dotp complexes, previously described as promising bone agents, were also studied in order to compare the radiochemical and biological behavior. Both complexes have shown bone uptake with high rate of total excretion, rapid blood clearance and no significant uptake in all the other main organs. Tissue distribution data of the $^{153}Sm/^{166}Ho$ complexes with the 13-membered macrocycle indicated a pronounced difference between the two complexes in the organs uptake rate and clearance. ^{166}Ho-tritp has shown rapid total excretion and washout from main organs including blood and soft tissues,

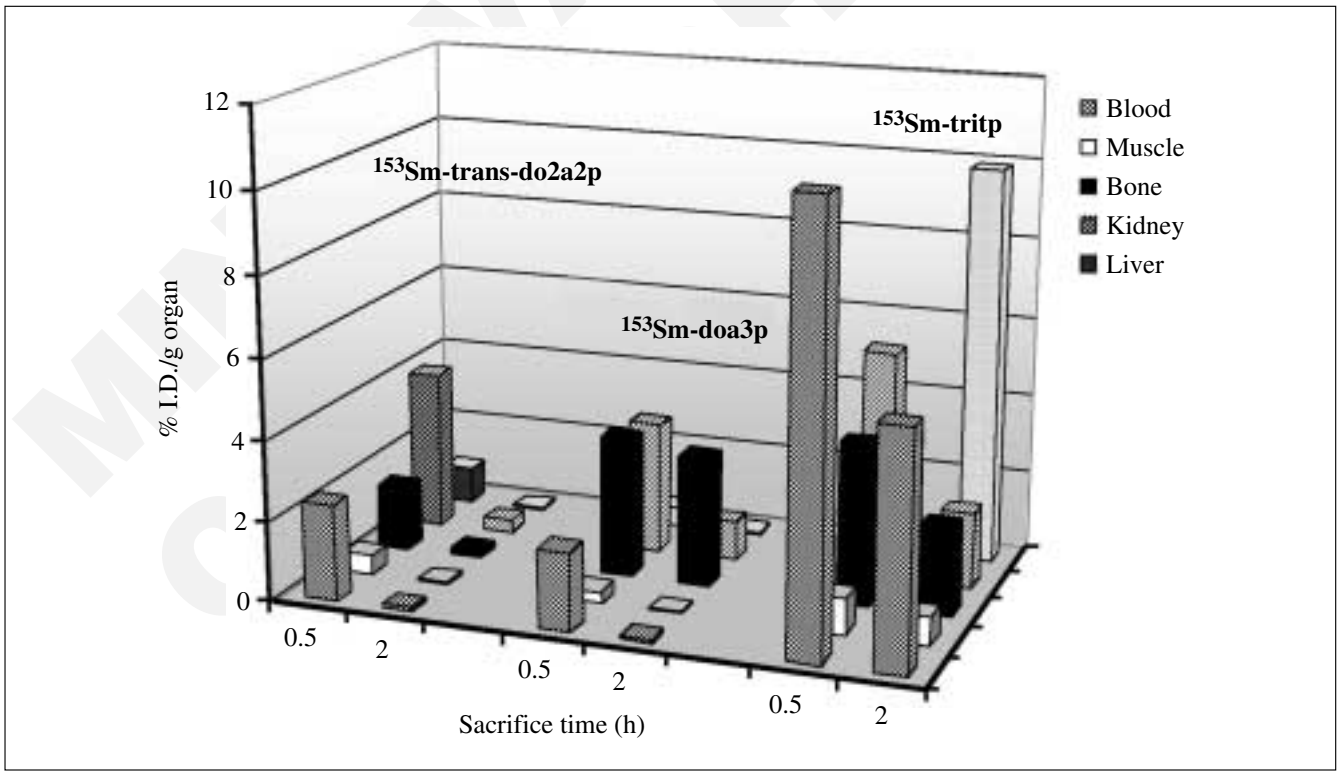

Figure 5.—Biodistribution results (% I.D./g organ) of ^{153}Sm-complexes at 30 min and 2 h after intravenous administration in CD-1 mice (n=4-5).

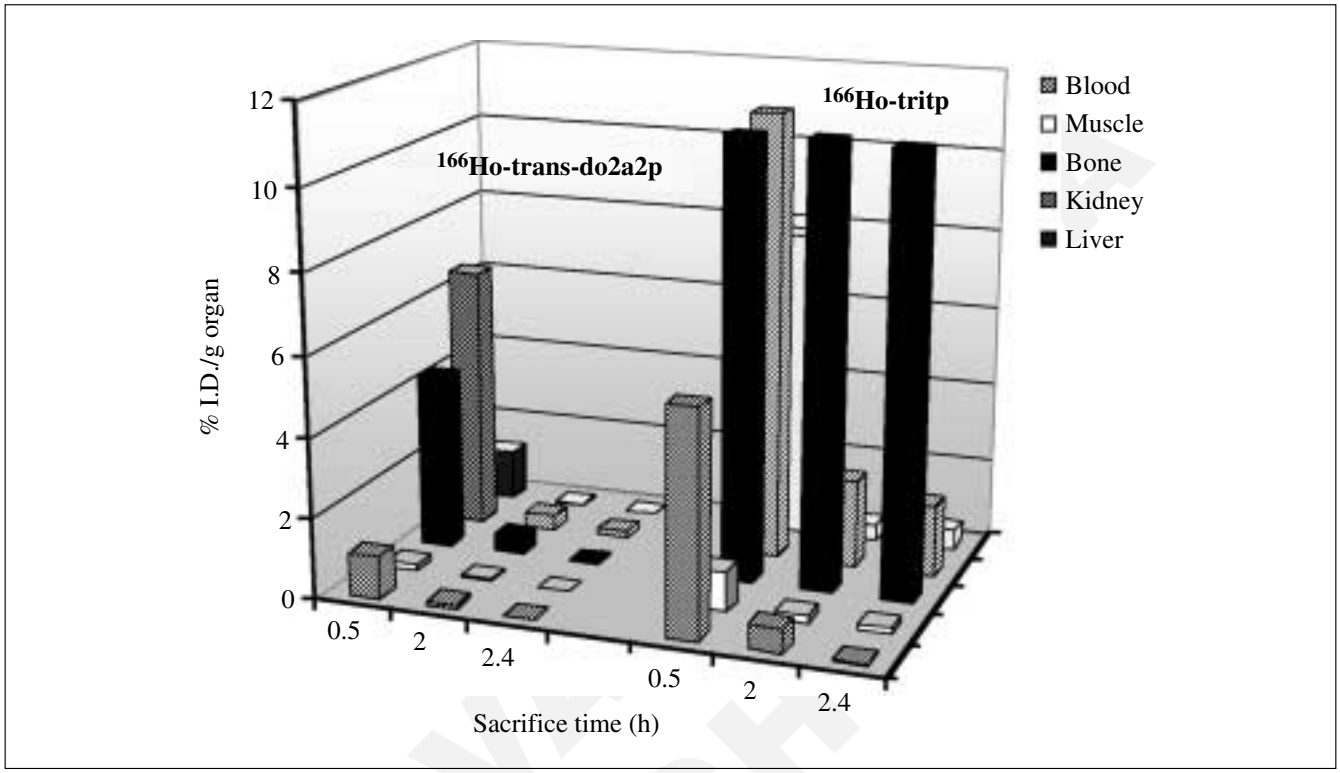

Figure 6.—Biodistribution results (% I.D./g organ) of ^{166}Ho-complexes at 30 min, 2 h and 24 h after intravenous administration in CD-1 mice (*n*=4-5).

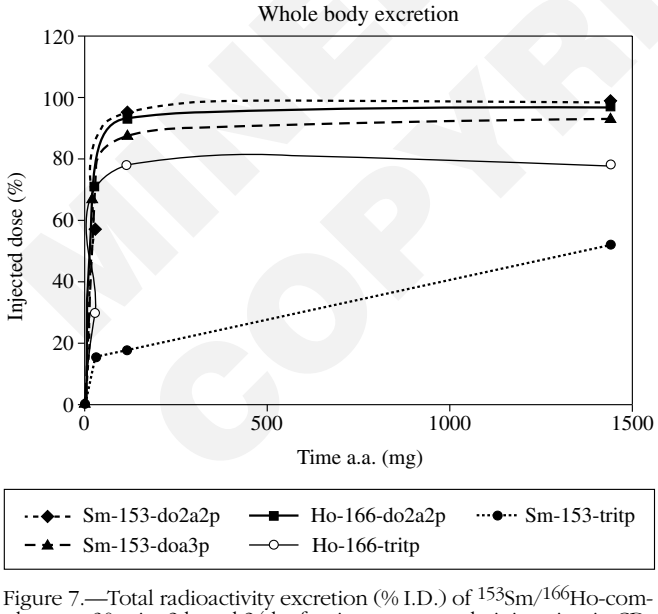

Figure 7.—Total radioactivity excretion (% I.D.) of ^{153}Sm/^{166}Ho-complexes at 30 min, 2 h and 24 h after intravenous administration in CD-1 mice (*n*=4-5).

TABLE IV.—*Bone to blood and bone to muscle ratios of radioactivity after administration of ^{166}Ho-tritp.*

	^{166}Ho-tritp		
	30 min	2 h	24 h
Bone/blood	2	23	274
Bone/muscle	10	55	84

whereas ^{153}Sm-tritp has evidenced a slower total excretion and tissue clearance associated to high uptake and retention in liver. Even so, both complexes are rapidly taken by bone, the main difference when compared with the complexes with ligands bearing methylcarboxylate pendant arms. However, a fast radioactivity decrease in bone was observed for ^{153}Sm-tritp. Therefore, ^{166}Ho-tritp have shown significantly higher bone/blood and bone/muscle ratios of radioactivity than any of the other ^{153}Sm/^{166}Ho-complexes under study (Table IV). In the same animal model, ^{166}Ho-tritp has demonstrated a bone uptake and bone/non

target organs ratios comparable to those found for ^{166}Ho-dotp which is under clinical trials.[10, 11]

Chromatographic analysis of blood and urine samples collected at sacrifice time have indicated that all the radiolanthanide complexes, except ^{153}Sm-tritp, were excreted as single radiochemical species presenting a chromatographic behavior analogous to the injected complexes. By contrast, radiochemical impurities were found in the blood analysis of mice injected with ^{153}Sm-tritp.

Discussion

Labeling efficiency and complexation kinetics of the radiolanthanide complexes depend both on the cavity size of the macrocycle and on the number and type of appended arms but also seems to be affected by the relative position of the arms (*trans*/*cis*) and by the metal ion size. While the labeling efficiency is higher than 98% for the 12- and 13-membered macrocycles the maximum yield achieved with the 14-membered macrocycle was only 80%. This finding has clearly evidenced that, for each radiometal the complexation kinetics decreases with the increase of the macrocyclic cavity size. Similar results have also been found for macrocycles bearing methylcarboxylate pendant arms.[11, 12] Moreover, the substitution of methylphosphonate group by methylcarboxylate also increases the kinetics of the reaction. In fact, the radiolabeling of 12-membered mixed ligands, with 1 or 2 acetate groups, has occurred with high labeling efficiency at room temperature. The number of mixed pendant arms and their relative position seems also to play an important role on the reaction rate as well as the metal ion size. Indeed, the ligand containing one acetate group has reacted significantly faster with ^{153}Sm than the ligand with 2 acetate groups, nevertheless the kinetics decreases with the smaller ^{166}Ho. This may result from different conformational rearrangements of the complexes. In order to clarify this point, structural studies in solution and in the solid state are presently underway (Campello MP, Santos I. Manuscript in preparation).

In vitro stability studies have indicated that all complexes are highly or moderately stable under physiological conditions although complexes with 12-membered tetraazamacrocycles are more robust. Even assuming that the radiochemical impurities found were consequence of trans-chelation with serum proteins, the small degree of radioactive complex dissociation in human serum over 48 h indicated that the radiolanthanides did not bind with high affinity to any major serum proteins. This result is in agreement with the low *in vitro* plasmatic protein binding found. Since stability is an important requirement for their potential *in vivo* application, these studies indicated that complexes fulfil the main criteria to pursue with biological studies in animal models.

As expected, HA binding evaluation of the complexes has confirmed that the number of phosphonate groups highly affects the adsorption to the main mineral component of bone, as the bound radioactivity directly increases with the number of phosphonate groups. All the ^{166}Ho complexes bind to a higher extend than the corresponding ^{153}Sm complexes, except ^{166}Ho-tritp. Based on stability studies, these results cannot be explained by complex dissociation and could be ascribed to the formation of complexes with a low coordination number and with more available free phosphonate groups to bind to HA.

The biodistribution profile of the complexes with rapid tissue clearance from most organs, including blood, reflects the low plasmatic protein binding and hydrophilic character found *in vitro*. The kidney uptake observed at lower times after administration is only related with the excretory pathway, as confirmed by the fast whole body excretion. Bone uptake, as expected from the HA binding assays, directly correlates with the number of methylphosphonate pendant arms since the bone accumulation follows the trend tritp>doa3p>do2a2p. The slowest washout from most organs of the ^{153}Sm-tritp resulted from *in vivo* instability of the complex as revealed by the blood chromatographic analysis. This slow clearance in addition to a faster decrease of bone radioactivity accumulation led to a less favorable bone/non target organ ratio of this complex. The absolute bone uptake exhibited by ^{166}Ho-tritp (5.5±1 at 24 h) is comparable to that obtained with the ^{153}Sm/^{166}Ho-dotp (4.1±0.7 and 5.2±1.2 for ^{153}Sm and ^{166}Ho complexes, respectively, at 24 h) in the same animal model.[11] This result as well as the long residence time in bone makes ^{166}Ho-tritp a potential bone seeking agent.

Conclusions

In this study, we compared the *in vitro* and *in vivo* performance of ^{153}Sm/^{166}Ho complexes with 12- to

14-membered tetraazamacrocycles bearing a different number of methylphosphonate pendant arms. Our results have demonstrated that the 12- and 13-membered macrocyclic ligands under study led to stable complexes with suitable physicochemical properties for *in vivo* administration. The *in vitro/in vivo* stability and biodistribution behavior of these radiolanthanide complexes depends not only on the backbone of the ligands and on the radiometal, but also on the type and number of appended arms. In general, the biodistribution profile of these complexes has confirmed to be adequate for targeted radiotherapy, being ^{166}Ho-tritp a potential candidate for bone targeting.

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