Radiochim. Acta **99**, 43–51 (2011) / **DOI** 10.1524/ract.2011.1791

Comparison of different phosphorus-containing ligands complexing ^{68}Ga for PET-imaging of bone metabolism

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(Received June 22, 2009; accepted in final form August 19, 2010)

Ga-68 / *Macrocylic ligands* / *Phosphonates* / *Complex formation* / *Bone metastases*

Summary. ^{99m}Tc-phosphonate structures are well established tracers for bone tumour imaging. Our objective was to investigate different ^{68}Ga-labelled phosphonate ligands concerning labelling kinetics, binding to hydroxyapatite and bone imaging using μ-PET. Seven macrocyclic phosphorus-containing ligands and EDTMP were labelled in nanomolar scale with n.c.a. ^{68}Ga in Na-HEPES buffer at pH ∼ 4. Except for DOTP, all ligands were labelled with > 92% yield. Binding of the ^{68}Ga-ligand complexes on hydroxyapatite was analysed to evaluate the effect of the number of the phosphorus acid groups on adsorption parameters. Adsorption of ^{68}Ga-EDTMP and ^{68}Ga-DOTP was > 83%. For the ^{68}Ga-NOTA-phosphonates an increasing binding with increasing number of phosphonate groups was observed but was still lower than ^{68}Ga-DOTP and ^{68}Ga-EDTMP. μ-PET studies *in vivo* were performed with ^{68}Ga-EDTMP and ^{68}Ga-DOTP with Wistar rats. While ^{68}Ga-EDTMP-PET showed uptake on bone structures, an excess amount of the ligand (> 1.5 mg EDTMP/kg body weight) had to be used, otherwise the $^{68}Ga^{3+}$ is released from the complex and forms gallium hydroxide or it is transchelated to ^{68}Ga-transferrin. As a result, the main focus of further phosphonate structures has to be on complex formation in high radiochemical yields with macrocyclic ligands with phosphonate groups that are not required for complexing ^{68}Ga.

1. Introduction

Besides lung and liver, the bones are most frequently affected by metastases. About 60–80% of these metastases are caused by breast or prostate carcinoma. However, their symptoms (bone pain, pathological fracture, spinal cord compression, hypercalcaemia, bone marrow suppression) are recognized rather late [1, 2]. Hence a diagnosis of bone metastases in an early state together with a subsequent therapy is of great importance for patients.

^{99m}Tc-phosphonates are well established tracers for the diagnosis of bone metastases using planar imaging or single photon emission tomography (SPECT) [3]. However, due to the higher spatial resolution of positron emission tomography (PET), adequate pharmaceuticals with positron emitters would be of great potential. The superior imaging quality in the case of PET/CT imaging is clearly demonstrated by using ^{18}F-fluoride [4]. However, non-cyclotron dependent PET-tracers, *i.e.* radionuclide generator-based derivatives, would provide the required availability for instant tracer synthesis and PET/CT diagnosis. For this attempt the Germanium-68/Gallium-68 generator with the positron emitter ^{68}Ga ($T_{1/2}$ = 67.7 min) represents a promising system. Using our recently developed generator post-processing, it is an excellent source for synthesizing and evaluating new tracers [5].

In the context of imaging bone metastases, several papers have recently discussed the use of ^{68}Ga-EDTMP as a chemical analogue of *e.g.* ^{153}Sm-EDTMP, utilized for the palliative treatment of bone diseases [6–9]. In this study, we investigated several macrocylic phosphonate ligands in terms of ^{68}Ga-ligand complex formation and binding behaviour to hydroxyapatite. The aim of the study was also to systematically study the efficiency of $^{68}Ga^{3+}$ labelling of a series of phosphonate ligands of different structural characteristics (linear and triaza or tetraza macrocyclic ligands). *In vivo* bone uptake of some of those ^{68}Ga-phosphonates was also evaluated in a preliminary μ-PET imaging study on healthy Wistar rats.

2. Materials and methods

2.1 Generator

Germanium-68 ($T_{1/2}$ = 270.8 d) provides the positron emitter Gallium-68 ($T_{1/2}$ = 67.7 min; 89% positron branching) as an easily available and relatively inexpensive source of

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a PET nuclide. A Cyclotron Obninsk Ltd. Co. generator was used, with Germanium-68 fixed on a solid phase of modified titanium dioxide. Gallium-68 is eluted from the generator with 10 mL HCl and is on line-immobilized on a strong acidic cation exchanger. Impurities such as zinc, iron and titanium as well as ^{68}Ge generator breakthrough are further removed by 1 mL of a mixture of hydrochloric acid and acetone (N1: 0.15 M HCl/80% acetone). Subsequently, ^{68}Ga is eluted quantitatively in 400 µL of a second mixture of HCl and acetone (N2: 0.05 M HCl/97.6% acetone) from the cation exchange resin [5]. This fraction serves as an ideal low-volume, low-acidic and chemically highly pure source of ^{68}Ga for subsequent labelling.

2.2 Phosphonates

The following macrocyclic tri- and tetraaza based phosphonate chelators have been used (see Fig. 1 for their chemical structures): H_8DOTP (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(methylenephosphonic acid)), and H_4DO3A-P^{ABn} (1,4,7,10-tetraazacyclododecane-4,7,10-triacetic-1-{methyl[(4-aminophenyl) methyl)]phosphinic acid}) and triazacyclononane (TACN) derivates such as H_4NO2AP (1,4,7-triazacyclononane-1-(methylene phosphonic acid)-4,7-diacetic acid), H_5NOA2P (1,4,7-triazacyclononane-1,4-bis(methylene phosphonic acid)-7-acetic acid) and H_6NOTP (1,4,7-triazacyclododecane-1,4,7-tri(methylene phosphonic acid). For comparison, non-phosphonate ligands H_3NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) and H_4DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) were also tested. The linear phosphonate ligand H_8EDTMP (ethylenediamine-tetra(methylene phosphonic acid)) was evaluated for comparison.

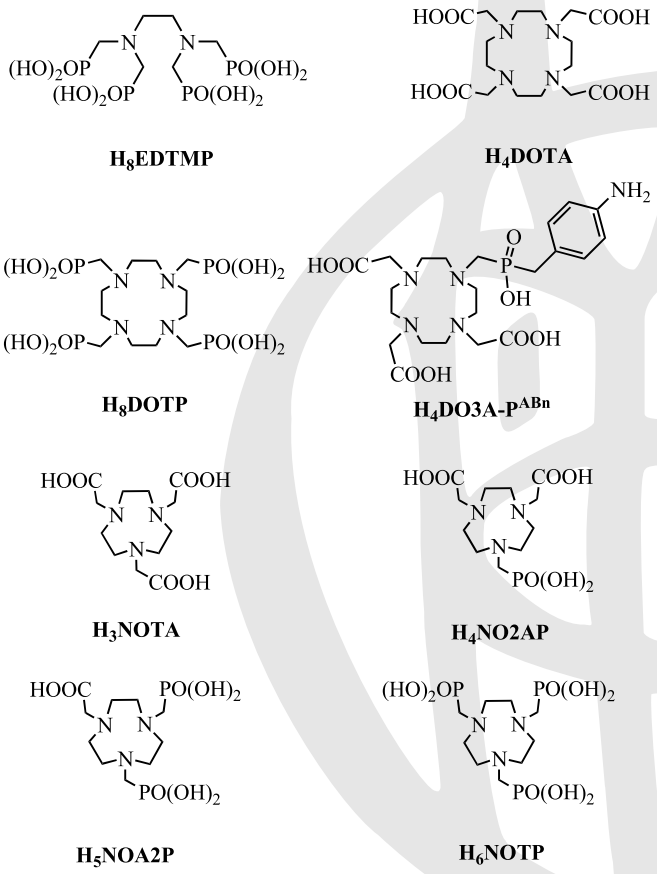

Fig. 1. Chemical structure of the acid forms of the phosphonate and carboxylate ligands used in this work.

2.3 Ligand syntheses

H_8EDTMP was obtained commercially from Sigma-Aldrich. The ligands DOTA [10], DOTP [11], NOTA [12], NOTP [13] and DO3A-P^{ABn} [14] were synthesized according to the literature. The purity of these ligands was checked by ^{1}H NMR spectroscopy, and their chemical shifts were in agreement with the values reported in the literature (DOTA [10], DOTP [11], NOTA [12], NOTP [13] and DO3A-P^{ABn} [14].

Synthesis of H_4NO2AP: 1,4,7-Triazacyclononane-1,4-bis(acetic acid *tert*-butyl ester)-7-(methylene-phosphonic acid diethyl ester) 3

A mixture of 1,4,7-triazacyclononane-1,4-bis(acetic acid *tert*-butyl ester) **2** (1.09 g), triethyl phosphite (0.63 g) and paraformaldehyde (0.96 g) was stirred at room temperature for 4 d. The clear pale yellow oil was kept at 50 °C in high vacuum for 24 h to remove any volatile sideproducts. The yield of the crude ester **3** was 1.64 g. This material contained a small amount of hydroxymethyl phosphonic acid diethyl ester and was used without further purification in the hydrolysis. ^{1}H NMR $CDCl_3$, δ: 4.07 (m, 4 H, $POCH_2$), 3.29 (s, 4H, acetate CH_2), 2.98 (d, $J_{PH} = 8.6$ Hz, 2H, PCH_2), 2.90 (br m, 4H, macrocyclic CH_2), 2.87 (br m, 4H, macrocyclic CH_2), 2.83 (br s, macrocyclic CH_2), 1.40 (s, 18H, *t*-Bu), 1.27 (m, 6H, $POCH_2CH_3$). ^{13}C NMR, $CDCl_3$,

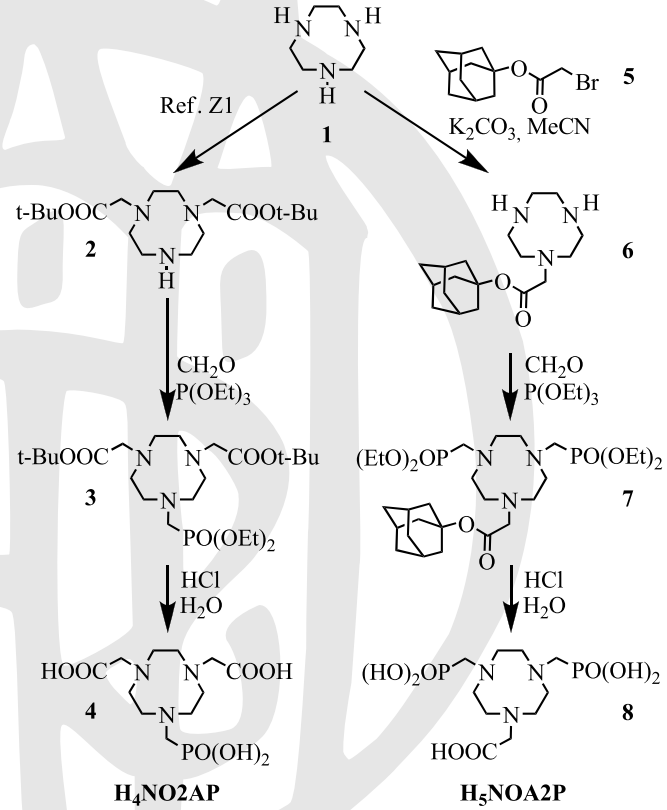

Fig. 2. Synthesis scheme for H_4NO2AP and H_5NOA2P.

δ: 171.08 (CO), 80.71 (*t*-Bu), 61.55 (d, $J_{PC} = 7.0$ Hz, $POCH_2$), 56. 20 (d, $J_{PC} = 5.9$ Hz macrocyclic CH_2) 55.04 (br, macrocyclic CH_2) 55.01 (shoulder, macrocyclic CH_2), 53.10 (d, $J_{PC} = 156.1$ Hz, PCH_2), 28.08 (*t*-Bu), 16.43 (d, $J_{PC} = 5.7$ Hz, $POCH_2CH_3$). ^{31}P NMR, $CDCl_3$, δ: 28.0. ESI-MS (pos) $m/z = 508.27$ $[M+H]^+$.

1,4,7-Triazacyclononane-1,4-bis(acetic acid)-7-(methylenephosphonic acid) 4

1,4,7-Triazacyclononane-1,4-bis(acetic acid *tert*-butyl ester)-7-(methylenephosphonic acid diethyl ester) **3** [15] (1.5 g) was dissolved in hydrochloric acid (3 M, 50 mL) and the mixture was refluxed for two days. The excess hydrochloric acid was removed by rotary evaporation and the residue was redissolved in water. The yellow solution was treated with activated carbon, filtered and evaporated by rotary evaporation. The residue was redissolved in water (50 mL) and freeze-dried to give crude **4** as a yellow glassy solid (1.20 g), which was redissolved in water (10 mL) and converted to the sodium salt by neutralizing it with NaOH (to pH 11). The solution was concentrated by rotary evaporation and the sodium salt was precipitated by adding ethanol (50 mL) to the residue. It was filtered and dried in nitrogen stream to give a very hygroscopic white solid (1.0 g) This was converted to the free acid form of **4** by loading it onto a Cl-form anion exchange column (DOWEX 1×4-50) and eluting it with HCl (3 M, 500 mL). The fractions containing the product were freeze-dried to give **4** as a pale yellow amorphous solid (0.55 g). ^{1}H NMR, D_2O, δ: 3.73 (s, 4H, acetate CH_2), 3.36 (br 4H, macrocyclic CH_2), 3.25 (br, 4H, macrocyclic CH_2), 3.20 (s, 4H, macrocyclic CH_2), 3.15 (d, $J_{PH} = 11.7$ Hz, PCH_2).^{13}C NMR, D_2O, δ: 170.54 (CO), 56.39 (acetate CH_2), 53.10 (d, $J_{PC} = 140.3$ Hz, PCH_2), 51.51 (d, $J_{PC} = 4.04$ Hz, macrocyclic CH_2), 50.46 (macrocyclic CH_2), 50.22 (macrocyclic CH_2). ^{31}P NMR, D_2O, δ: 12.50. ESI-MS (neg) $m/z = 338.13$ $[M-H]^-$.

Synthesis of H_5NOA2P: 1,4,7-Triazacyclononane-1-mono(acetic acid adamantyl ester) 6

1,4,7-Triazacyclononane **1** (2.60 g) was dissolved in chloroform (30 mL) and the solution was cooled in an ice bath. Bromoacetic acid adamantlyl ester **5** [16] (1.0 g) was added and the mixture was stirred for 30 min. The ice bath was removed and stirring was continued for 1 day at room temperature. The chloroform was removed by rotary evaporation, the residue partitioned between ether (50 mL) and water (20 mL), and the ether layer was washed with water (3 × 20 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated and the residue was dried to a constant mass under vacuum. The oily product slowly turned into a white waxy crystalline solid. Yield: 1.03 g, 78%. This product contained about 5% of the bisalkylated derivative as evidenced by NMR and MS. ^{1}H NMR, $CDCl_3$, δ: 3.23 (s, 2H, acetate CH_2), 2.83, 2.74, 2.68, 2.64, 2.63 (br m, 14H, macrocyclic CH_2 and NH), 2.08 (br s, 3H, adamantyl C3, C5, C7), 2.04 (br s, 6H, adamantyl C2, C8, C9), 1.58 (br s, 6H, adamantyl C4, C6, C10). ^{13}C NMR, $CDCl_3$, δ: 171.10 (CO), 81.04 (adamantyl C1), 57.72 (acetate CH_2), 52.43 (macrocyclic CH_2), 46.26 (macrocyclic CH_2), 45.80 (macrocyclic CH_2), 41.29 (adamantyl C2, C8, C9), 35.95 (adamantyl C4, C6, C10), 30.64 (adamantyl C3, C5, C7). ESI-MS (pos) $m/z = [M+H]^+$ 322.4; 514.53 $[M+H]^+$ for 1,4,7-triazacyclononane-1,4-bis(acetic acid adamantyl ester).

1,4,7-Triazacyclononane-1-mono(acetic acid adamantyl ester)-4,7 bis(methylenephosphonic acid diethyl ester) 7

1,4,7-Triazacyclononane-1-mono(acetic acid adamantyl ester) **6** (0.95 g) was dissolved in triethyl phosphite (1.10 g) and paraformaldehyde (190 mg) was added. The mixture was stirred for 4 d at room temperature and then kept at 50 °C in high vacuum for 24 h to remove any volatile side products. The crude ester **7** (1.7 g) was dissolved in ether (50 mL) and the solution was washed with water (3 × 50 mL). The ether layer was dried over sodium sulfate, filtered and evaporated by rotary evaporation to give **7** as pale yellow oil (1.45 g). ^{1}H NMR, $CDCl_3$, δ: 4.05 (m, 8H, $POCH_2$), 3.22 (s, 2H, acetate CH_2), 2.93 (d, $J_{PH} = 8.9$ Hz, 4H, PCH_2), 2.90 (s), 2.86 (br m), 2.78 (br m) (12H, macrocyclic CH_2), 2.08 (br s, 3H, adamantyl C3, C5, C7), 2.03 (br s, 6H, adamantyl C2, C8, C9), 1.58 (br s, 6H, adamantyl C4, C6, C10), 1.25 (*t*, $J_{HH} = 7.0$ Hz, 12H, $POCH_2CH_3$). ^{13}C NMR, $CDCl_3$, δ: 170.90 (CO), 80.68 (adamantyl C1), 61.50 (d, $J_{PC} = 7.0$ Hz, $POCH_2$), 59.83 (acetate CH_2), 56.55 (d, $J_{PC} = 4.8$ Hz, macrocyclic CH_2), 56.48 (d, $J_{PC} = 4.1$ Hz, macrocyclic CH_2), 55.25 (macrocyclic CH_2), 41.32 (adamantyl C2, C8, C9), 35.99 (adamantyl C4, C6, C10), 30.63 (adamantyl C3, C5, C7), 16.39 (d, $J_{PC} = 5.8$ Hz $POCH_2CH_3$). ^{31}P NMR, $CDCl_3$, δ: 28.0. ESI-MS (pos) $m/z = 622.40$ $[M+H]^+$.

1,4,7-Triazacyclononane-1-mono(acetic acid)-4,7-bis (methylenephosphonic acid) 8

1,4,7-Triazacyclononane-1-mono(acetic acid adamantly ester)-4,7-bis(methylene phosphonic acid diethyl ester) **7** (1.0 g) was dissolved in HCl (3 M, 20 mL) and the solution refluxed for two days. 1-Adamantanol got separated in the form of white crystals. The mixture was allowed to cool to room temperature and then extraction was done with ether (3 × 20 mL). The aqueous layer was evaporated by rotary evaporation, redissolved in water (25 mL) and freeze-dried to give a colorless glassy solid (0.93 g). This was redissolved in water (5 mL) and the product separated as a white solid on standing. It was filtered, washed with water (3 × 3 mL) and dried by air suction to give **8** as a white solid (0.58 g). ^{1}H NMR D_2O, NH_3, δ: 3.34 (s, 2H, acetate CH_2), 3.23 (br s, 4H, macrocyclic CH_2), 3.10 (br, 4H, macrocyclic CH_2), 2.94 (d, 4H, $J_{PH} = 10.1$ Hz, PCH_2), 2.85 (br t, 4H, macrocyclic CH_2). ^{13}C NMR, D_2O, NH_3, δ: 179.36 (CO), 58.37 (acetate CH_2), 52.84 (d, $J_{PC} = 132.8$ Hz PCH_2), 51.37 (d, $J_{PC} = 3.6$ Hz, macrocyclic CH_2), 49.59 (d, not resolved, macrocyclic CH_2), 48.38 (s, macrocyclic CH_2). ^{31}P NMR, D_2O, NH_3, δ: 11.59. ESI-MS (neg) $m/z = 374.13$ $[M-H]^-$.

2.4 Labelling with ^{68}Ga(III)

Labelling with ^{68}Ga was performed in 400 µL 0.12 M HEPES sodium salt buffer (Merck KGaA) by adding the 400 µl ^{68}Ga

fraction of N2. Through variation of reaction time (1 to 10 min), temperature (r.t. to 75 °C) and different amounts of the complex ligands (10 to 50 nmol), optimum reaction parameters for ^{68}Ga complex formation were deduced.

2.5 Analysis

Determination of radiochemical labelling yield and complex formation kinetics were carried out by paper chromatography on cellulose sheets (Schleicher & Schuell 589/5) using two solvent systems (A = water : ethanol : pyridine – 4 : 2 : 1; B = isotonic saline) and were measured on a Canberra Packard Instant Imager. Due to the migration of free gallium(III) on cellulose under both conditions, the pH had to be adjusted to 13 by addition of 60 µL 1 M NaOH prior to spotting the probe.

NOTP was used as proof of concept for developing a HPLC method as comparison to radio-TLC.

For this purpose a strong anion exchange column (Partisil 10 SAX 250 × 4 mm) with following conditions was used: 75% 0.75 M phosphate buffer, pH = 3/25% 1.0 M sodium citrate, 1 mL flow; radiodetector Raytest Gabi Star.

2.6 Purification

Complexes with yields lower than 95% had to be purified prior to their use in binding studies. Therefore the reaction mixture was passed over a cation exchanger (50 mg Bio-Rad AG 50W-X8). The non-complexed gallium is immobilized on the exchanger while the ^{68}Ga ligand complexes pass the resin.

2.7 Binding studies

Binding studies on synthetic hydroxyapatite (Hap) were done to simulate the binding of the different ^{68}Ga ligand complexes to bone structures. For this purpose, 20 mg Hap were incubated in 1 mL isotonic saline for 24 h. The test itself was performed by the addition of 50 µL of the ^{68}Ga-ligand complexes to the Hap fraction. After vortexing for 10 seconds, the probes were incubated for 10 min at ambient temperature. The samples were centrifuged and the supernatant was removed. The Hap fraction was washed with 0.5 mL saline. This solution contained less than 2% of the overall ^{68}Ga radioactivity.

The ^{68}Ga radioactivity in combined liquids and the Hap fraction was measured in a curiemeter (Aktivitätsmessgerät M2316, Messelektronik Dresden GmbH). ^{68}Ga complex binding to Hap was determined as percent of ^{68}Ga absorbed to Hap. For comparison of all species possibly formed in the reaction mixture and the investigated ^{68}Ga-complexes, the behaviour of free $^{68}Ga^{3+}$ was analysed similarly. The aim was to validate whether there is any binding of free Ga^{3+} itself to hydroxyapatite.

2.8 µ-PET experiments

In vivo studies were only performed with ^{68}Ga-EDTMP.

The ^{68}Ga complex was prepared with 50 nmol ligand under the described conditions. After purification, a solution was obtained ready for injection. For animal µ-PET imaging, the effect of the overall amount of EDTMP present in the injected ^{68}Ga-EDTMP fraction was varied. It was 22 µg originating from the synthesis protocol in one case. In another experiment, 1.7 mg EDTMP was added to the purified ^{68}Ga-EDTMP fraction and the overall amount of EDTMP injected was 1.7 mg.

Healthy male Wistar rats weighting 500–700 g were used. The complexes were injected through the tail vain (30–73 MBq). Anaesthesia was carried out using chloral hydrate. All experiments were approved by the German law on animal experiments. A Siemens µ-PET Focus 120 Scanner was used. For each study a dynamic scan was performed from 0 to 60 min p.i., followed by a whole body scan at 60–75 min p.i. µ-PET image quantification was not applied.

3. Results

3.1 Generator elution and complex formation

The elution of $^{68}Ga^{3+}$ from the generator and the on line-processing of the eluate are performed within less than five minutes. Figs. 3 and 4 compare typical labelling reactions for the ligands EDTMP, DOTP and DO3A-P^{ABn} at 25 °C (room temperature) and 75 °C.

Fig. 5 summarizes the labelling yields for the triazacyclononane derivatives and DOTA at room temperature and 75 °C after 10 min reaction.

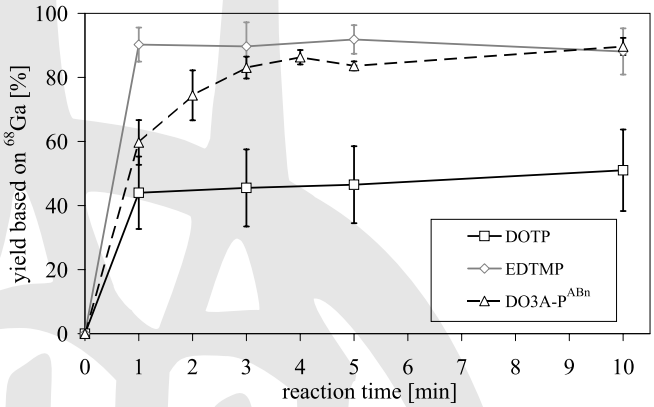

Fig. 3. $^{68}Ga^{3+}$ complex formation for DOTP, EDTMP and DO3A-P^{ABn} at 25 °C (50 nmol of each ligand, pH ~ 4).

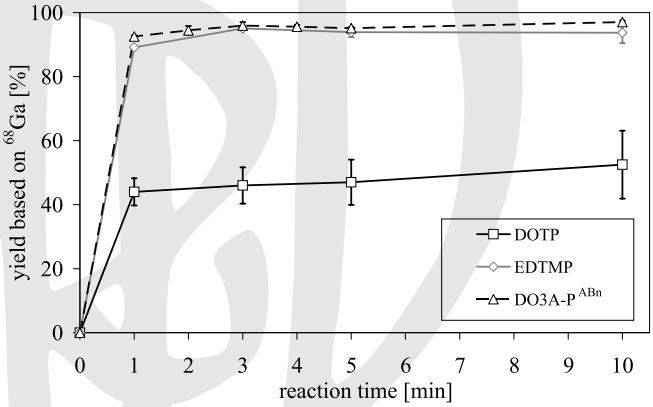

Fig. 4. $^{68}Ga^{3+}$ complex formation for DOTP, EDTMP and DO3A-P^{ABn} at 75 °C (50 nmol of each ligand, pH ~ 4).

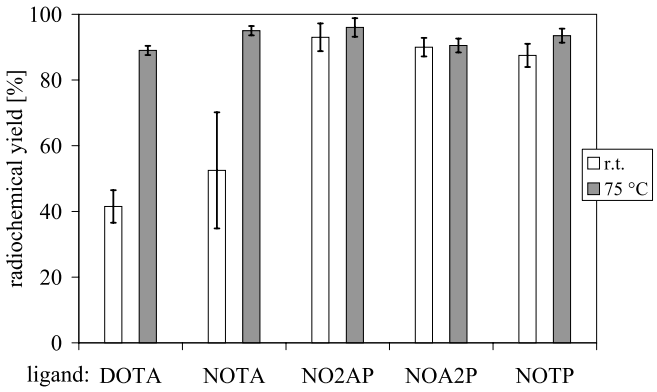

Fig. 5. Yields of the $^{68}Ga^{3+}$ labelling of DOTA, NOTA, NO2AP, NOA2P and NOTP after 10 min at room temperature (r.t.) and 75 °C (50 nmol of each ligand, pH ~ 4).

Labelling finally proceeds at temperatures between 25 and 75 °C within 2 to 10 min in a total volume of 800 µL and at optimal pH of 4.

3.2 HPLC analysis

The ^{68}Ga-NOTP complex was synthesized as described and used for HPLC analysis. The HPLC chromatogram showed 98% radiochemical yield (compared to 95% for radio-TLC for this reaction). The other fraction at 2.1 min retention time is uncomplexed ^{68}Ga, *cf*. Fig. 6.

3.3 Hydroxyapatite binding

^{68}Ga complexes with DOTA and NOTA showed the expected results of almost any binding to hydroxyapatite (Hap), which is 7.6% for the ^{68}Ga-DOTA complex and 6.3% for ^{68}Ga-NOTA. The ligand ^{68}Ga-DO3A-P^{ABn} also has negligible binding to hydroxyapatite, as the adsorption experiments show a 5.4 ± 0.4% ^{68}Ga content in the final Hap fraction. In terms of quantification of Hap binding, these amounts of non-phosphonate ^{68}Ga-complexes still measurable in the washed Hap fraction are in the following taken as "unspecific binding". For unspecific binding, therefore, an average value of 6 ± 1% was adopted.

Interestingly, free $^{68}Ga^{3+}$ is significantly taken up by Hap with 84.4 ± 2.9%. However, this is interpreted as chemisorption of ^{68}Ga hydroxide, formed in the saline medium.

The percentage of ^{68}Ga-phosphonate complexes bound to Hap is summarized in Fig. 7. The uptake illustrated has been corrected by an average of 6% of "unspecific binding". The results of the hydroxyapatite binding assays indicate strongest binding of ^{68}Ga-EDTMP and ^{68}Ga-DOTP.

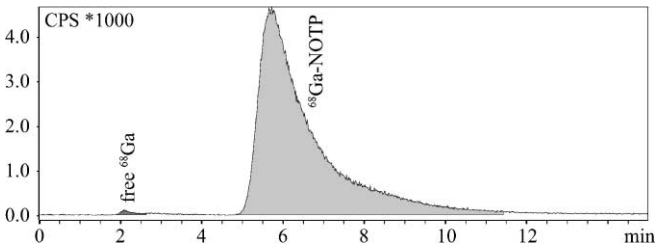

Fig. 6. HPLC chromatogram of the reaction mixture of ^{68}Ga-NOTP.

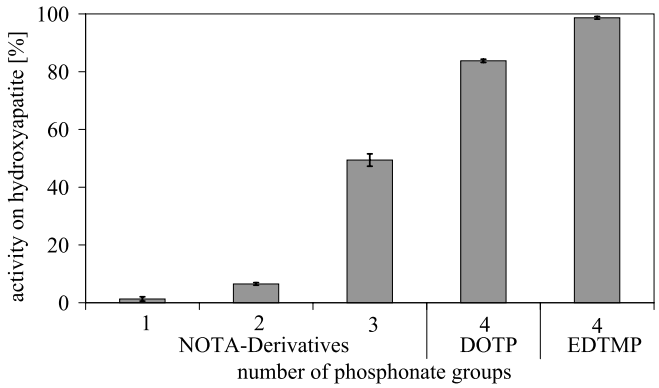

Fig. 7. Binding efficacies of ^{68}Ga-phosphonate complexes on 20 mg of Hap at 25 °C.

The ^{68}Ga complexes with the triazacyclononane derivatives show an increase of binding to Hap with rising number of phosphonate groups, though still much lower than in the case of ^{68}Ga-EDTMP or ^{68}Ga-DOTP.

3.4 µ-PET studies

Initial *in vivo* experiments using 55 MBq ^{68}Ga-EDTMP containing 50 nmol (22 µg) EDTMP revealed no uptake on bone structures of the healthy rats. The main activity was found in the bladder, *cf*. Fig. 8.

However, applying much higher amounts of the free aliphatic ligand EDTMP (1.7 mg), a significant uptake of ^{68}Ga-EDTMP (73 MBq injected) on the skeleton of healthy rats was obtained (Figs. 9, 10). In coronal slices (Fig. 9) the image shows clearly each rib and the spine. The only other organ showing high ^{68}Ga activity was the bladder, *cf*. Fig. 10c.

4. Discussion

4.1 Synthesis of H_4NO2AP and H_5NOA2P

The synthesis of H_4NO2A was straightforward and started from 1,4,7-triazacyclononane-1,4-bis(acetic acid *tert*-butyl

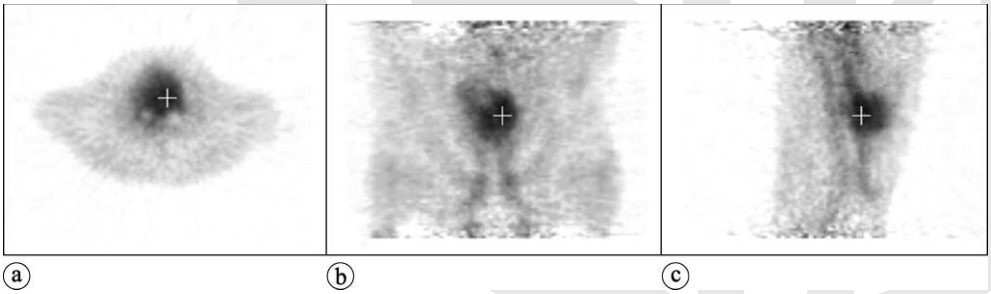

Fig. 8. ^{68}Ga-EDTMP distribution measured *in vivo*. Transversal (**a**), coronal (**b**) and sagittal (**c**) µ-PET images 30–60 min p.i., Wistar rat, 55 MBq ^{68}Ga-EDTMP injected containing 22 µg EDTMP.

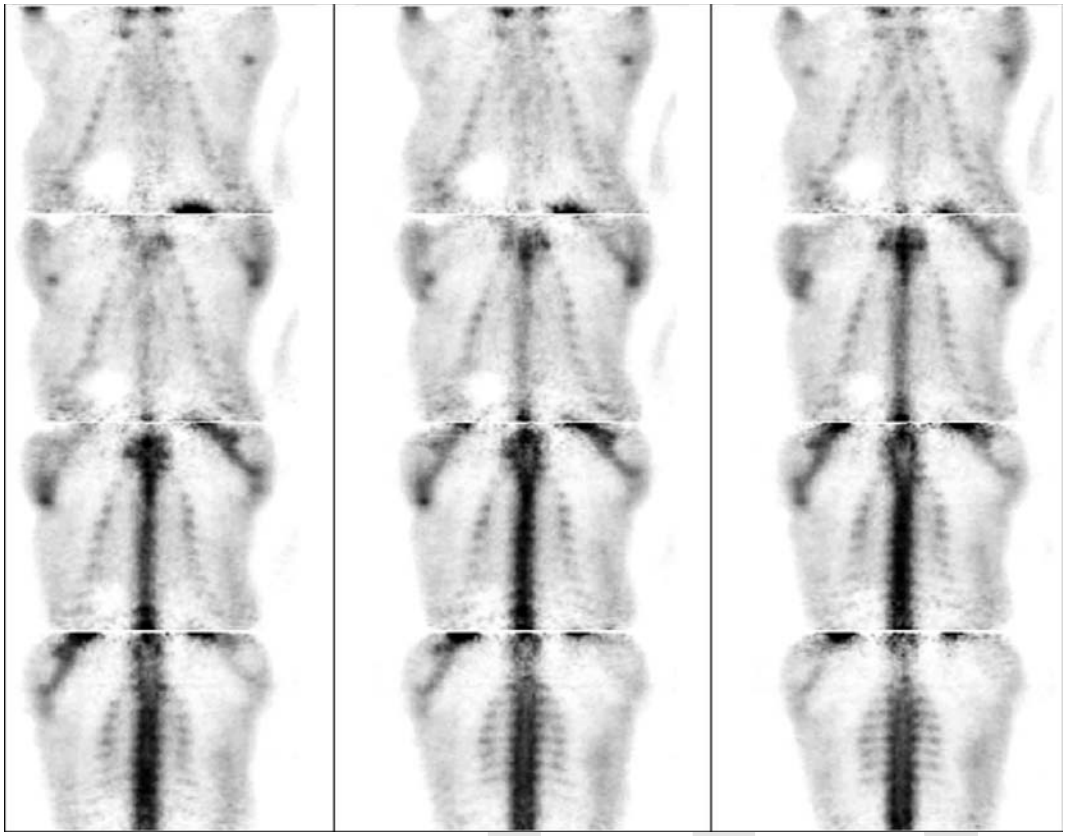

Fig. 9. ^{68}Ga-EDTMP distribution measured *in vivo*. Coronal μ-PET images 30–60 min p.i., Wistar rat, 73 MBq ^{68}Ga-EDTMP injected containing 1.7 mg EDTMP.

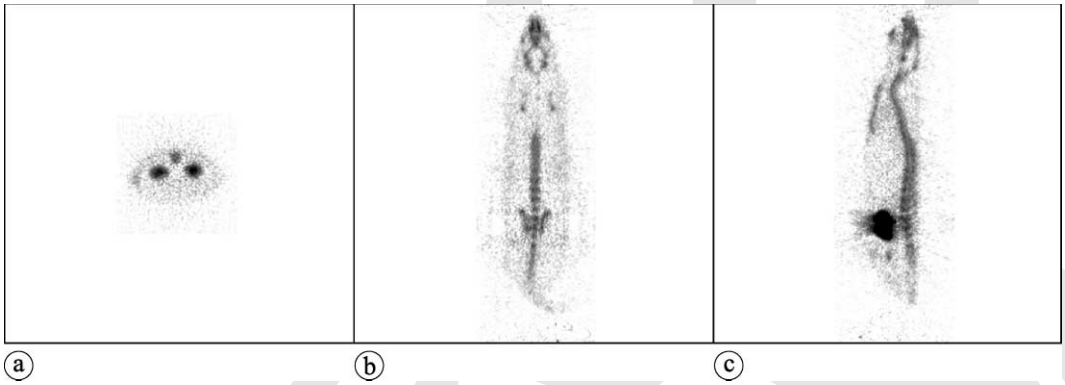

Fig. 10. ^{68}Ga-EDTMP distribution measured *in vivo*. Transversal (**a**), coronal (**b**) and sagittal (**c**) μ-PET images 60–75 min p.i., Wistar rat, 73 MBq ^{68}Ga-EDTMP injected containing 1.7 mg EDTMP.

ester) (**2**), which is easily available from 1,4,7-triazacyclononane (**1**) [15]. **2** was converted to the methylenephosphonate diethyl ester derivative **3** in a Mannich-Arbuzov reaction and the free acid **4** was obtained by the acid hydrolysis of the ester groups (Fig. 2). The synthesis of H_5NOA2P was more challenging. There are several possible synthetic routes to this ligand, all of which involve the selective functionalization of the parent macrocycle **1**. Preliminary experiments suggested that monofunctionalization of 1,4,7-triazacyclononane (**1**) could advantageously be achieved by reacting bromoacetic acid 1-adamantyl ester (**5**) [16] with large excess of **1**. The product 1,4,7-triazacyclononane-1-mono(acetic acid adamantyl ester) (**6**) is sparingly soluble in water in its deprotonated form and therefore, excess **1** can easily be removed by a simple aqueous wash. **6** was then converted to the phosphonate ester derivative **7** and removal of the ester groups by acid hydrolysis gave the desired final product **8** (Fig. 2).

4.2 ^{68}Ga-Labelling

Besides DOTP, all ligands show a very high radiochemical yield of > 92% after 10 min heating at 75 °C and pH about 4, although the ligands are only used in nanomole scale.

Labelling of DOTP with $^{68}Ga^{3+}$ is more difficult when compared to the complexation with lanthanides such as ^{177}Lu [17, 18]. The Ln^{3+} cations are relatively easily complexed at pH 7–9 with DOTP [11, 19, 20]. Due to the formation of insoluble $Ga(OH)_3$ (it has a $K_{sp} = 5 \times 10^{-37}$ which is much lower than that for lanthanides, varying between $La(OH)_3$: $K_{sp} = 1 \times 10^{-19}$ and $Lu(OH)_3$: $K_{sp} = 2.5 \times 10^{-24}$) [21], reactions carried out with Ga^{3+} and DOTP at this alkaline pH are problematic. In the present work reactions were performed at a maximum pH of ∼ 4 in order to suppress the mentioned formation of the ^{68}Ga-hydroxide species and under these conditions only the out of cage complex is probably formed and full complexation may also be hampered by kinetic problems [22].

Interestingly, the labelling yields of the NOTA-based phosphonates are very high already at low temperature. This reflects the favourable thermodynamic and kinetic properties of triazacyclononane-based chelators for $^{68}Ga^{3+}$ labelling.

4.3 HPLC analysis

Initially a HPLC system for the inactive Ga-complexes was developed. This was a strong anion exchange column (Partisil 10 SAX 250 4mm) and a 0.75 M phosphate buffer, pH = 3, (1 mL flow; detection at 200 nm with Dionex UVD170U). In the case of Ga-NOTP, for example, the UV-based chromatograms showed retention times of 4.7 min for the Ga-NOTP complex and 7.3 min for the free ligand. However, these conditions did not work for the radioactive compounds. Possibly because of very low concentration of the radioactive complexes and also due to the use a metal based HPLC system, the retention times could not be reproduced. The radioactive reaction mixture showed intense tailing over many minutes.

Therefore a new solvent system was developed using 25% of 1 M sodium citrate. Other ^{68}Ga-species possibly occurring in the reaction were injected for control. For example, uncomplexed ^{68}Ga is sufficiently separated from the final complex ^{68}Ga-NOTP (*cf*. Fig. 6). HPLC analysis showed similar results compared to TLC. Beside this, a ^{68}Ga-intermediate is formed in the HEPES reaction buffer alone and the very weak Ga-HEPES complex itself [23] appears at 3.5 min retention time.

4.4 Purification

^{68}Ga-ligand complexes obtained in > 95% yield were used directly for further experiments. Due to the lower yield of 51 ± 12% for the ^{68}Ga-DOTP complex, purification was needed in this case. It was carried out by passing the reaction mixture over a cation exchanger. This provides an easy and fast method of purifying this complex from free, *i.e.* uncomplexed $^{68}Ga^{3+}$. Chemical purification was also applied for ^{68}Ga-EDTMP as this complex was obtained for *in vivo* μ-PET studies.

4.5 Binding studies

^{68}Ga-DOTA and ^{68}Ga-NOTA, the chelates of non-phosphonate ligands, showed an unspecific binding to Hap of ∼ 6%. After deduction of this unspecific adsorption for the ^{68}Ga-TACN derivatives, a trend among their binding affinity to Hap with increasing number of phosphonate groups is observed, ranging from NO2AP: $7.6 - 6 \approx 2\%$ to NOA2P: $12.8 - 6 \approx 7\%$ and NOTP: $55.7 - 6 \approx 50\%$.

However, these binding parameters are still much lower than those found for the ^{68}Ga-DOTP and ^{68}Ga-EDTMP complexes. A similarly high binding capacity to Hap was found for the analogous chelate ^{153}Sm-DOTP in the literature [24, 25]. The further increase of Hap binding capacity achieved by four phosphonates, as represented in the ^{68}Ga-DOTP complex, may directly be related to the increase of the number of phosphonate groups per ligand. There may be evidence that enhanced binding to Hap is facilitated by geminal configurations, *i.e.* by the concerted action of two neighbouring phosphonate groups. This is reflected by bisphosphonate structures established for ^{99m}Tc-MDP (methylene diphosphonate) for example, as recently shown for a bisphosphonate mono-amide analogue of DOTA [26]. In that work, (4-{[(bis-phosphonomethyl)carbomoyl]methyl}-7,10-bis-(carboxymethyl)-1,4,7,10-tetraazacyclododec-1-yl)-acetic acid (BPAMD) was labelled with carrier-free ^{160}Tb (^{160}Tb-BPAMD) and strong binding to Hap was observed.

In our comparative study, ^{68}Ga-EDTMP showed excellent binding to Hap. ^{68}Ga-EDTMP seems to behave similarly to the well established ^{153}Sm-EDTMP in terms of binding capacity to bone metastases. Interestingly, experimental data on the tracer's uptake on Hap are difficult to compare for various experimental setups. A recently published study on binding of ^{68}Ga-EDTMP showed 4.3 ± 2.7% on 3 mg Hap after 120 min only [8]. The authors used commercially available multibone kits with 25 mg EDTMP and added 2 mL of a 0.1 M HCl direct generator eluate together with isotonic saline and kept it at ambient temperature for 30 min. Using 20 mg of hydroxylapatite is in our setup a good marker for binding of the phosphonates. Different publications dealing with up to 100 mg of hydroxyapatite [27, 28] in binding studies reveal that these studies are not an optimal solution for binding studies.

The DO3A-P^{ABn} was involved in the present study to investigate whether a phosphorus containing acid group needed for metal coordination or substituted derivatives show affinity to Hap. The DO3A-P^{ABn} contains one phosphinate acid group in the molecule. The low binding of the ^{68}Ga-DO3A-P^{ABn}-complex indicates, that a free phosphonate group is needed for binding to Hap.

4.6 μ-PET measurements

The first attempt of *in vivo* imaging of bone structures of a healthy rat was conducted with ^{68}Ga-EDTMP of 50 nmol EDTMP concentration only. The image showed accumulation of the activity in the bladder, with negligible uptake at the skeleton. This seems to be in analogy to the reported ^{153}Sm-EDTMP characteristics. With 4 μg EDTMP, the ^{153}Sm-EDTMP is stable for 1 h in plasma whereas for 24 h it is stable only when using 50 μg EDTMP [29]. The trivalent metal is released from the complex very fast *in vivo* because of low thermodynamic and kinetic complex stability (Sm-EDTMP: $\log K = 14.44$) [30]. This can be completed by the usage of an excess amount of the ligand. Consequently, a large excess of EDTMP is injected with the ^{153}Sm-EDTMP tracer.

The same strategy seems to be required in the case of ^{68}Ga-EDTMP. Recent work [8, 9] used a large amount of 25 mg EDTMP for complexing $^{68}Ga^{3+}$. Our experiments indicate that at best 1 mg of EDTMP is needed to identify ^{68}Ga-EDTMP bone uptake in rats (which is about > 1.5 mg/kg body weight). If a lower amount of EDTMP is applied, the ^{68}Ga released from the complex is taken up as hydroxide or colloids by the liver and/or is transchelated to the protein transferrin due to the high stability of Ga-transferrin ($\log K_1 = 20.3$, $\log K_2 = 19.3$) [31].

When 1.7 mg EDTMP ligand (3.9 μmol) was co-injected, the promising results shown in Figs. 9 and 10 were obtained. For an additional investigation of the minimum amount of EDTMP required for *in vivo* stabilisation, two other experiments with 1 and 3 mg co-injected ligand were performed. Even with 1 mg there was an accumulation of activity at the bone, but with worse statistics compared to the use of 1.7 and 3 mg EDTMP.

5. Conclusion

Syntheses of no-carrier-added ^{68}Ga phosphonate complexes are performed within 20 min or less after elution of the generator in high yields of $> 92\%$. First evaluations on synthetic apatite show high binding within 10 min for both ^{68}Ga-EDTMP and the macrocyclic ^{68}Ga-DOTP. However due to the limitation of the reaction pH, ^{68}Ga-DOTP could not be synthesized in sufficient radiochemical yields ($51 \pm 12\%$), interesting for imaging purpose.

Preliminary μ-PET imaging on healthy Wistar rats demonstrated bone uptake *in vivo* for ^{68}Ga-EDTMP. To overcome the addition of EDTMP (or MDP in the case of ^{99m}Tc), the attention still would be focused on macrocylic ligands forming thermodynamically and kinetically stable complexes with Ga(III). Here, the macrocyclic ligands are of particular interest, as the complex stability is higher than those for open-chain ligands like EDTMP.

NOTA-based phosphonates clearly show favourable ^{68}Ga-ligand complex formation parameters in terms of high labelling yield at low temperature compared to DOTA-analogues. The lower Hap uptake in the case of macrocylic NOTA-based ligands with 3, 2 or 1 phosphonate groups reflects the impact of the number of phosphonate targeting groups. Interestingly, the non-Hap binding of DO3A-P^{ABn} indicates further requirements to design optimum tracers for imaging bone metabolism.

Nevertheless, further research will focus on new tetraaza- or triaza-macrocyclic chelates with free bisphosphonate structural characteristics, able to complex Gallium and bind to bone structures.

Acknowledgment. This work was carried out in the framework of COST D38 Action "Metal-Based Systems for Molecular Imaging Applications" and EU-FP6 "Network of Excellence" EMIL (No. LSHC-2004-503569) project.

C. F. G. C. Geraldes thanks the Foundation of Science and Technology (F.C.T.), Portugal (project PTDC/QUI/70063/2006) and FEDER. I. Lukeš thanks the Long-Term Research Plan of the Ministry of Education of the Czech Republic (No. MSM0021620857).

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