

Synthesis and stability studies of Ga-67 labelled phosphonium salts

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Abstract

Delocalised lipophilic cations such as tri- and tetra-arylphosphonium are able to diffuse across the mitochondrial membrane which allows them to selectively accumulate in cells with a high transmembrane potential ($\Delta\Psi_m$). The mitochondrial membrane potential of cancer cells and cardiomyocytes has been reported to be significantly higher than that of normal epithelial cells. This feature can be exploited for the selective accumulation of phosphonium derivatives for the purposes of molecular imaging using radionuclides. Four structurally-related, Ga(III)-phosphonium salts were synthesized and fully characterized, and found to be modest in toxicity toward T98G human glioblastoma cells ($IC_{50} > 4$ mM). High-activity (100 MBq) analogues containing Ga-67 were also synthesized and their stabilities in PBS buffer and human serum were determined.

Introduction

Delocalised lipophilic cations (DLCs) such as tri- and tetra-arylphosphonium are able to diffuse across the mitochondrial membrane as its membrane potential is negative and the process occurs without the aid of endogenous transporters.¹⁻⁶ DLCs have been shown to selectively accumulate in cells with high transmembrane potential ($\Delta\Psi_m$).⁷⁻⁹ For example, the difference in $\Delta\Psi_m$ between the colon carcinoma cell line CX-1 and the control green monkey kidney epithelial cell line (CV-1) is reported to be approximately 60 mV (163 mV in tumor cells vs. 104 mV in normal cells).⁹ By exploiting the difference in $\Delta\Psi_m$ between the two mammalian cell lines, several researchers have reported the synthesis of several Cu-64 labelled phosphonium complexes for PET imaging, some of which have shown a high selectivity for tumors.¹⁰⁻¹²

The incorporation of radiolabels such as H-3, C-11, F-18 and I-125 into phosphonium salts have also proved useful for medical (PET and SPECT) imaging. For example, tritiated phosphonium cations such as [³H]-tetraphenylphosphonium (TPP) have been widely used as *in vitro* tumor probes,¹³ and [¹¹C]-triphenylmethylphosphonium has been used to determine the kinetics and tumor selectivity in canine brain glioma by means of PET imaging.¹⁴ 4-([¹⁸F]-fluorophenyl)triphenylphosphonium is another probe developed as a potential myocardial blood flow agent for PET imaging.¹⁵ More recently, [¹²⁵I]-*p*-iodobenzyl triphenyl phosphonium, [¹²⁵I]-*p*-iodobenzyl dipropylphenyl phosphonium and [¹²⁵I]-*p*-iodobenzylmethyl diphenylphosphonium were also investigated as potential myocardial SPECT imaging agents, and all these agents showed good heart tissue uptake.¹⁶

Most phosphonium compounds have utilized several synthetic steps following radiolabelling, an inefficient strategy when the short half-lives of many radioisotopes are considered.¹⁷ This reduces the overall radiochemical yield and limits potential radionuclides to those with longer half-lives.

Previously, our research group has investigated similar Gd(III) phosphonium salts as potential tumor mitochondrial targeting agents for neutron capture and photon activation therapies.^{18,19} *In vitro* studies

of these compounds confirmed their modest toxicity ($IC_{50} > 1$ mM), high uptake by T98G human glioblastoma cells (> 1500 ng Gd/mg protein) and high selectivity of tumor to normal cells compared to the control non-cancerous SVGp12 fetal glial cell line. All these factors make the phosphonium compounds good potential imaging agents with radiometals such as Ga-67. The primary aim of this study was to develop a rapid method for the synthesis of structurally related Ga-67 phosphonium-based agents in high radiochemical yield and purity for PET imaging. Herein we report the synthesis of four structurally-related, Ga(III)-phosphonium salts and radiolabeled analogues containing Ga-67. Their stabilities in PBS buffer and human serum were also determined.

Results and Discussion

Syntheses

Four structurally-related phosphonium ligands **L1** – **L4** containing a phosphonium centre, a xylyl linker and the macrocycle DO3A (1,4,7,10-tetraazacyclododecane,-*N,N',N''*-triacetic acid) were synthesized^{18,19} according to **Scheme 1** and purified by means of reverse phase HPLC on a C18 column. The ‘cold’ Ga complexes were synthesized in high purity by addition of GaCl₃ to ligands **L1** – **L4** in aqueous solution.

Radiolabelling Studies

Ligands **L1** – **L4** were efficiently complexed with ⁶⁷Ga³⁺ under typical conditions (80 °C, 10 min), giving radiochemical yields (based on radioHPLC) of $> 95\%$ for all complexes (**Table 1**).

The Ga-67 radiolabelled complexes containing **L1** – **L4** were purified by means of a C18 SPE and optimally eluted ($>90\%$ recovery of activity) in a 1:1 mixture of EtOH/0.9% saline solution. Ethanol content of $<40\%$ gave poor recoveries of the more lipophilic complexes ⁶⁷Ga·**L2** and ⁶⁷Ga·**L4**. Removal of the EtOH gave a suitably formulated product for stability and serum protein binding studies. In all cases, except for ⁶⁷Ga·**L3**, the final product purity was $>95\%$, as determined by means

of radioHPLC. An uncharacterised minor impurity (< 2%) in **L3** appears to complex ^{67}Ga preferentially. Modification of reaction parameters (time, temperature, and ligand stoichiometry) had little effect on the product distribution. Attempts to purify $^{67}\text{Ga}\cdot\text{L3}$ by the above method also proved problematic. While adequate separation of the two products was achieved, the overall recovery of injected activity was <30%. Recoveries were significantly improved by the addition of $^{\text{nat}}\text{Ga}\cdot\text{L3}$ to the injections, but this product was unsuitable for the stability studies.

Stability Studies

The stabilities of the Ga-67 radiolabelled complexes containing **L1** – **L4** were examined in Dulbecco's phosphate-buffered saline (dPBS). Data for all the time points examined in this study are presented in **Table 2**. All of the radiolabelled complexes demonstrated good stability in dPBS. HPLC chromatograms indicated minor loss of $^{67}\text{Ga}^{3+}$ from the complexes as well as the formation of polar fragments, presumably formed by the cleavage of the $^{67}\text{Ga}\cdot\text{DO3A}\cdot\text{xylyl}$ moiety from the phosphonium salt.

Studies involving human serum allowed for the collection of two sets of data: serum protein binding (**Table 3**) and stability in human serum (**Table 4**). Complexes $^{67}\text{Ga}\cdot\text{L1}$, $^{67}\text{Ga}\cdot\text{L2}$ and $^{67}\text{Ga}\cdot\text{L4}$ all showed the rapid (> 1 h) association of 5-6% of their activity with serum proteins (**Table 3**). This association increased slightly over time but it appears to be a system of limited capacity. At the 24 h time point, 2-3% of the activity was associated with human serum albumin (HSA) proteins.

The stability of the ^{67}Ga complexes in human serum was examined by removing the majority of proteins (>99 %) by ultracentrifugation by means of a 3000 MWCO filter, providing a filtrate that was suitable for injection on a standard C18 HPLC column. **Table 4** shows the stability of these complexes at all time points. Two of the complexes ($^{67}\text{Ga}\cdot\text{L1}$, $^{67}\text{Ga}\cdot\text{L3}$) showed good stability for up to 2 h. All complexes showed some degradation after 24 h but $^{67}\text{Ga}\cdot\text{L3}$ remained mostly intact.

The metabolic profile of the complexes $^{67}\text{Ga}\cdot\text{L1}$, $^{67}\text{Ga}\cdot\text{L2}$, $^{67}\text{Ga}\cdot\text{L3}$ and $^{67}\text{Ga}\cdot\text{L4}$ were investigated using radioHPLC after 24 h (**Figure 1**). Since the complexes are metabolising in serum, it is unclear if the binding is associated with the breakdown products or the original complexes. The main metabolite of these complexes mainly shows up in two regions (1.5 – 2.3 min and 2.4 – 3.7 minutes) of the HPLC chromatograms. The low retention time points to the metabolites being polar in nature, likely formed from the cleavage of the phosphonium group, presumably at the xylyl-bridging group. The first region in the radioHPLC chromatogram comprises unbound ^{67}Ga and $^{67}\text{Ga}(\text{DO3A})$ and the second region comprises $^{67}\text{Ga}(\text{DO3A})$ -xylyl metabolites.

Conclusion

We have synthesized and evaluated the stability of four new Ga-67-labeled phosphonium complexes ($^{67}\text{Ga}\cdot\text{L1}$, $^{67}\text{Ga}\cdot\text{L2}$, $^{67}\text{Ga}\cdot\text{L3}$ and $^{67}\text{Ga}\cdot\text{L4}$). All complexes were stable in PBS, but showed signs of metabolic degradation after 24 h in human serum. The limited solution stability of the complexes after extended time periods allows for possible future application to the shorter-lived Ga-68 isotope.¹⁷ Positron emission tomography (PET) has several technical merits over SPECT, such as higher spatial resolution and more accurate attenuation correction. Ga-68 is an excellent positron emitter (511-keV annihilation radiation; $t_{1/2} = 68$ min) suitable for PET imaging and is available from a Ge-68/Ga-68 generator, which renders it independent of an on-site cyclotron. The short overall time required for labelling and purification using the method reported here allows for future imaging applications involving selected Ga-68 complexes.

Experimental

Materials and Methods

Distilled water was used for all experiments requiring water. THF and MeCN were dried prior to use by following the methods of Armarego and Chai:²⁰ THF was dried over sodium wire and freshly

distilled from benzophenone ketyl. Anhydrous MeCN was freshly distilled from CaH₂. All other solvents were used without further purification.

All precursor chemicals were commercially available. 1,4,7,10-tetraazacyclododecane (cyclen) was purchased from Nowapharm (China). Gallium(III) chloride was purchased from Strem. All other chemicals were purchased from Sigma-Aldrich Co.

Column chromatography was carried out on Grace Davison LC60A 40-63 μ m silica column. Thin layer chromatography was carried out on Merck Kieselgel 60 F₂₅₄ aluminium back plates. Visualisation of plates was achieved by using a 254 nm light.

Instrumentation

All ¹H, ¹³C{¹H}, and ³¹P{¹H} NMR spectra were recorded on a Bruker Avance200 (¹H at 200 MHz and ¹³C at 50MHz), Bruker Avance300 (¹H at 300 MHz, ¹³C at 75 MHz, and ³¹P at 121 MHz) or a Bruker AvanceIII500 (¹H at 500 MHz, ¹³C at 125 MHz, and ³¹P at 202 MHz) NMR spectrometer at 300 K. All NMR signals (δ) are reported in ppm. ¹H NMR spectra recorded in CDCl₃ were referenced to TMS and to their residual solvent peaks in all other solvents. ¹³C{¹H} NMR spectra were referenced to their solvent peaks (except for D₂O). ¹³C{¹H} NMR spectra in D₂O were referenced according to an internal standard of TMS (25.145020 MHz). ³¹P{¹H} NMR spectra were referenced according to internal standards of H₃PO₄ (40.480742 MHz). Coupling constants (ⁿJ_{ij}) are reported in Hz. Peak multiplicities have been abbreviated as s (singlet), d (doublet), t (triplet), br (broad) and m (multiplet - unassignable multiplicity).

Low resolution ESI-MS were recorded on a Finnigan LCQ mass spectrometer. High resolution ESI-FT-ICR-MS data were recorded on a Bruker 7.0T mass spectrometer.

HPLC Methods

All HPLC methods used a Waters HPLC system equipped with a UV/vis detector (λ = 254, 230 nm).

Method 1

Preparative: A Waters C18 preparative (19 mm x 150 mm, 5 µm pore size) column was used. The flow rate was 7 mL/min. The mobile phase had a gradient 0-45 min, from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in MeCN) to 0% solvent A and 100% solvent B.

Analytical: A Waters C18 analytical (4.6 mm x 150 mm, 5 µm pore size) column was used. The flow rate was 0.7 mL/min. The mobile phase had a gradient 0-45 min, from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in MeCN) to 0% solvent A and 100% solvent B.

Method 2

Semi-Preparative: An Atlantis T3 Semi-preparative (10 mm x 250 mm, 5 µm pore size) column was used. The flow rate was 3 mL/min. The mobile phase was isocratic 30% MeCN, 60% water and with 10% 0.1M Na₂HPO₄ buffered at pH 7.

Analytical: An Atlantis T3 analytical (4.6 mm x 150 mm, 3 µm pore size) column was used. The flow rate was 0.8 mL/min. The mobile phase was isocratic with 0.01M Na₂HPO₄ in 30% MeCN and 70% water buffered at pH 7.

Method 3

Semi-Preparative: An Atlantis T3 Semi-preparative (410 mm x 250 mm, 5 µm pore size) column was used. The flow rate was 3 mL/min. The mobile phase was isocratic 25% MeCN, 65% water and with 10% 0.1M Na₂HPO₄ buffered at pH 7.

Analytical: An Atlantis T3 analytical (4.6 mm x 150 mm, 3 µm pore size) column was used. The flow rate was 0.8 mL/min. The mobile phase was isocratic with 0.01M Na₂HPO₄ in 20% MeCN and 80% water buffered at pH 7.

***In Vitro* Cytotoxicity Assays**

In vitro cytotoxicity assays were performed using human glioblastoma multiforme (T98G) cells. The cytotoxicity of complexes was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded (density 1×10^4 cells per well) in growth medium (Minimum Essential Medium Eagle supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 units), streptomycin (100 $\mu\text{g}/\text{mL}$) and L-glutamine (2.5 mM; 100 μL) using 96-well plates and were allowed to adhere overnight at 37°C. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in the presence of **1** or the vehicle (control). Serial dilutions of the complex were added to triplicate wells. Maximum concentration (MaxC) for the experiments was 4 mM. After 72 h, the MTT solution in phosphate- buffered saline (PBS; 30 μL , 0.17% *w/v*) was added and the incubation was continued. After a further 4 h, the culture medium and excess MTT solution were removed and the resulting MTT-formazan crystals dissolved by addition of 150 μL DMSO. Cell viability was determined by measuring the absorbance at 600 nm using a Victor3V microplate reader (PerkinElmer). All readings were corrected for absorbance from wells containing the vehicle alone, and the level of MTT was expressed relative to the corresponding vehicle-treated controls as % viability.

Syntheses

General synthetic method 1: bromoxylyl phosphonium salt precursors

A solution of the phosphine in PhMe was added dropwise to a solution of *a,a'*-dibromo-*m*-xylene in the same solvent. For the synthesis of **P3**, the resulting solution was heated at reflux for 4 h, whilst for the synthesis of **P4** the resulting solution was stirred at room temperature for 20 h. The precipitate was filtered off and washed with PhMe and diethyl ether.

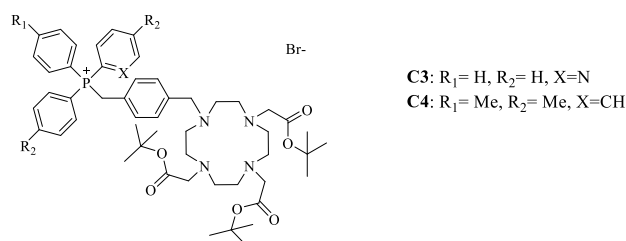
(4-(Bromomethyl)benzyl)diphenyl(pyridin-2-yl) phosphonium bromide (P3)

Yield: 1.46 g (72.4%). ESI-MS: m/z 447.93 ($[M - Br]^+$). 1H NMR ($CDCl_3$) δ 8.93 (d, 1H, Ph, $^2J_{HP} = 4.5$ Hz), 8.39-8.34 (m, 1H, Ph), 8.09-8.07 (m, 1H, Ph), 7.80-7.74 (m, 11H, Ph), 7.15-7.07 (m, 4H, Ph), 5.48 (d, 2H, Ph, $^4J_{HP} = 15$ Hz), 4.38 (s, 2H, CH_2). ^{13}C NMR ($CDCl_3$) δ 152.0 (d, Ph, $^1J_{CP} = 105$ Hz), 144.8 (s, Ph), 143.3 (s, Ph), 138.4 (d, Ph, $^3J_{CP} = 21$ Hz), 138.1 (d, Ph, $^3J_{CP} = 15$ Hz), 135.2 (s, Ph), 134.6 (d, Ph, $^2J_{CP} = 39$ Hz), 131.1 (d, Ph, $^1J_{CP} = 93$ Hz), 131.6 (d, Ph, $^2J_{CP} = 39$ Hz), 130.1 (d, Ph, $^1J_{CP} = 48$ Hz), 129.5 (s, Ph, $^3J_{CP} = 12$ Hz), 128.3 (s, Ph), 127.5 (d, Ph, $^2J_{CP} = 33$ Hz), 117.1 (s, Ph), 115.9 (s, Ph), 32.8 (s, CH_2), 29.5 (d, CH_2 , $^1J_{CP} = 183$ Hz). ^{31}P NMR ($CDCl_3$) δ 19.5 (s).

(4-(Bromomethyl)benzyl)tri-*p*-tolylphosphonium bromide (P4)

Yield: 0.89 g (94.9%). ESI-MS: m/z 535.00 ($[M - Br]^+$). 1H NMR ($CDCl_3$) δ 7.62 (m, 9H, Ph), 7.60 (m, 6H, Ph), 7.58 (m, 1H, Ph), 7.55 (m, 1H, Ph), 7.37 (s, 4H, Ph), 7.05 (d, 1H, Ph, $^4J_{HP} = 1.16$ Hz), 5.15 (d, 2H, CH_2 , $^2J_{HP} = 14.52$ Hz), 3.90 (s, 2H, CH_2), 3.90 (s, 9H, CH_3). ^{13}C NMR ($CDCl_3$) δ 146.3 (s, Ph), 137.9 (d, Ph, $^3J_{CP} = 15$ Hz), 134.8 (s, Ph), 134.1 (d, Ph, $^2J_{CP} = 39$ Hz), 131.7 (d, Ph, $^3J_{CP} = 21$ Hz), 130.8 (d, Ph, $^1J_{CP} = 54$ Hz), 129.2 (d, Ph, $^3J_{CP} = 9$ Hz), 127.5 (d, Ph, $^2J_{CP} = 36$ Hz), 118.2 (s, Ph), 117.0 (s, Ph), 114.0 (s, Ph), 112.8 (s, Ph), 32.7 (s, CH_2), 30.3 (d, CH_2 , $^1J_{CP} = 189$ Hz), 21.7 (s, CH_3). ^{31}P NMR ($CDCl_3$) δ 21.2 (s).

General synthetic method 2: *t*-butyl-protected phosphonium-DO3A ligands



A solution of DO3A-*t*Bu₃HBr, phosphonium salt and Na₂CO₃ in MeCN was stirred at reflux for 20 h. The mixture was filtered, the solvent was removed *in vacuo*, and the residue was recrystallised from acetone/diethyl ether to yield a colourless solid.

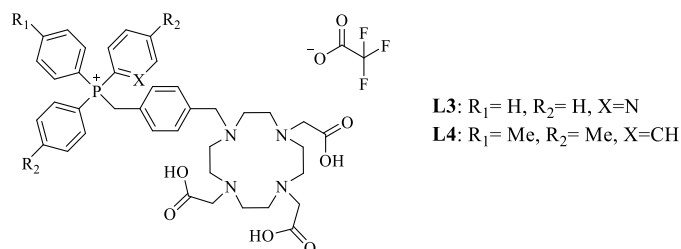
Diphenyl(pyridin-2-yl)(4-((4,7,10-tris(2-(tert-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium bromide (C3)

Yield: 1.24 g (84.6%). ESI-MS: m/z 880.52 ($[M - Br]^+$). 1H NMR ($CDCl_3$) δ 8.17-8.07 (m, 2H, Ph), 7.78-7.64 (m, 2H, Ph), 7.53-7.42 (m, 8H, Ph), 7.22-7.19 (m, 2H, Ph), 7.10-7.07 (m, 2H, Ph), 5.35 (br, 2H, CH_2), 4.12 (s, 9H, CH_3), 3.59 (s, 2H, CH_3), 3.03-2.23 (br m, 25H, CH_2), 1.46 (s, 18H, CH_3), 1.45 (s, 9H, CH_3). ^{13}C NMR ($CDCl_3$) δ 173.5 (s, C=O), 172.6 (s, C=O), 134.7 - 128.5 (m, Ph), 83.0 (s, C), 82.8 (s, C), 56.1 (m, CH_2), 49.7 (m, CH_2), 28.1 (s, CH_3), 28.0 (s, CH_3). ^{31}P NMR ($CDCl_3$) δ 19.7 (s).

Tri-*p*-tolyl(4-((4,7,10-tris(2-(tert-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium bromide (C4)

Yield: 0.89 g (85.0%). ESI-MS: m/z 922.00 ($[M - Br]^+$). 1H NMR ($CDCl_3$) δ 7.58-7.52 (m, 6H, Ph), 7.46-7.42 (m, 6H, Ph), 7.07-7.05 (m, 4H, Ph), 5.18 (br, 2H, CH_2), 3.69 (s, 9H, CH_3), 3.72 (s, 2H, CH_3), 3.05-2.28 (br m, 25H, CH_2), 1.49 (s, 18H, CH_3), 1.47 (s, 9H, CH_3). ^{13}C NMR ($CDCl_3$) δ 173.4 (s, C=O), 172.5 (s, C=O), 146.2 (s, Ph), 135.8 (s, Ph), 134.1 (d, Ph, $^3J_{CP} = 39$ Hz), 131.3 (s, Ph), 130.9 (d, Ph, $^3J_{CP} = 54$ Hz), 130.6 (s, Ph), 127.0 (d, Ph, $^2J_{CP} = 33$ Hz), 114.9 (s, Ph), 113.7 (s, Ph), 82.8 (s, C), 82.4 (s, C), 58.0 (s, CH_2), 56.6 (s, CH_2), 55.8 (s, CH_2), 50.8 (m, CH_2), 49.1 (m, CH_2), 30.9 (d, CH_2 , $^1J_{CP} = 195$ Hz), 28.0 (s, CH_3), 27.8 (s, CH_3), 21.8 (s, CH_3). ^{31}P NMR ($CDCl_3$) δ 22.9 (s).

General synthetic method 3: phosphonium-DO3A ligands



The coupled ligand **C3** or **C4** (0.5 g) was dissolved in trifluoroacetic acid (5 ml) and stirred at room temperature for 24 h. The solvent was removed *in vacuo* and the crude residue dissolved in H_2O (100

ml) and extracted with CHCl_3 (3 x 20 ml). The aqueous layer was reduced *in vacuo* to afford an off-white solid. This solid was then purified by reverse-phase HPLC (Method 1) and the product fractions were lyophilised to afford a fluffy white solid.

Ligands **L1** and **L2** were prepared as reported previously.^{18,19}

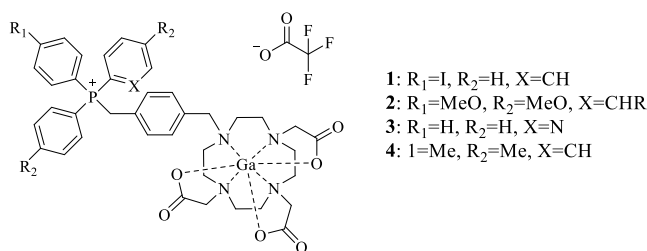
Diphenyl(pyridin-2-yl)(4-((4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium trifluoroacetate (L3)

Yield: 0.37 g (86.5%). HPLC Retention Time (Method 1) = 16.32 min. ESI-MS: m/z 836.93 ($[\text{M} - \text{Br}]^+$). ^1H NMR (D_2O) δ 7.90-7.87 (m, 2H, Ph), 7.80-7.75 (m, 2H, Ph), 7.61-7.50 (m, 8H, Ph), 7.38-7.36 (m, 2H, Ph), 7.27-7.21 (m, 2H, Ph), 7.02-7.00 (m, 2H, Ph), 4.72 (d, 2H, CH_2 , $^2J_{\text{HP}} = 14.7$ Hz), 4.35 (br s, 2H, CH_2), 4.03 (br s, 2H, CH_2), 3.4-3.1 (br m, 20H, CH_2). ^{13}C NMR (CDCl_3) δ 162.1 (s, C=O), 162.6 (s, C=O), 152.0 (d, Ph, $^1J_{\text{CP}} = 75$ Hz), 143.7 (s, Ph), 142.2 (s, Ph), 138.6 (d, Ph, $^2J_{\text{CP}} = 42$ Hz), 134.2 (d, Ph, $^2J_{\text{CP}} = 39$ Hz), 130.0 (s, Ph, $^1J_{\text{CP}} = 51$ Hz), 118.2 (s, Ph), 116.4 (s, Ph), 115.2 (s, Ph), 114.4 (s, Ph), 110.5 (s, Ph), 57.1 (s, CH_2), 55.0 (s, CH_2), 53.2 (s, CH_2), 50.8 (m, CH_2), 48.5 (m, CH_2), 28.7 (d, CH_2 , $^1J_{\text{CP}} = 192$ Hz). ^{31}P NMR (D_2O) δ 22.8 (s).

Tri-*p*-tolyl(4-((4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphonium trifluoroacetate (L4)

Yield: 0.32 g (79.9%). HPLC Retention Time (Method 1) = 21.17 min. ESI-MS: m/z 753.73 ($[\text{M} - \text{Br}]^+$). ^1H NMR (D_2O) δ 7.36-7.24 (m, 14H, Ph), 6.97-6.95 (m, 2H, Ph), 4.56 (d, 2H, CH_2 , $^2J_{\text{HP}} = 15$ Hz), 4.25 (br s, 2, CH_2), 4.02 (br s, 2, CH_2), 3.4-3.1 (br m, 20H, CH_2), 2.27 (s, 9 H, CH_3). ^{13}C NMR (D_2O) δ 162.7 (s, C=O), 162.2 (s, C=O), 146.9 (s, Ph), 133.5 (d, Ph, $^2J_{\text{CP}} = 39$ Hz), 132.0 (s, Ph), 131.3 (s, Ph), 130.6 (d, Ph, $^1J_{\text{CP}} = 51$ Hz), 122.1 (s, Ph), 118.2 (s, Ph), 114.3 (d, Ph, $^2J_{\text{CP}} = 54$ Hz), 113.0 (s, Ph), 110.5 (s, Ph), 57.2 (s, CH_2), 54.7 (s, CH_2), 53.0 (s, CH_2), 51.1 (m, CH_2), 49.5 (m, CH_2), 48.4 (m, CH_2), 29.7 (d, CH_2 , $^1J_{\text{CP}} = 198$ Hz), 20.8 (s, CH_3). ^{31}P NMR (D_2O) δ 21.3 (s).

General synthetic method 4: 'cold' Ga(III) complexes



Equimolar amounts of the deprotected ligand **L1 – L4** (50 mg) and GaCl₃ was stirred in water (5 mL) at room temperature for 72 h. The reaction mixture was then purified by reverse-phase HPLC (Method 1) and the product fractions were lyophilised to yield the product as a fluffy white powder.

4-Iodophenyl)diphenyl(4-((4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl) phosphoniumgallium(III) trifluoroacetate (1)

Yield: 11.8 mg (24.7%). HPLC Retention Time (Method 1) = 19.38 min. ESI-FT-ICR-MS for [M - CF₃CO₂]⁺ Calculated *m/z* 903.12935; Found 903.12859. ¹H NMR (D₂O) 8.08-8.06 (m, 2H, Ph), 7.91-7.90 (m, 2H, Ph), 7.71-7.69 (m, 8H, Ph), 7.35-7.31 (m, 4H, Ph), 7.07-7.06 (m, 2H, Ph), 4.84 (m, 2H, CH₂), 4.06-3.94 (m, 8H, CH₂), 3.57-3.54 (m, 6H, CH₂), 3.42-3.32 (m, 8H, CH₂), 2.98 (d, 2H, CH₂, ²J_{HH} = 11.5 Hz). ¹³C NMR (D₂O) δ 173.8 (s, C=O), 173.6 (s, C=O), 163.0 (d, Ph, ¹J_{CP} = 145 Hz), 139.1 (s, Ph, ²J_{CP} = 50 Hz), 135.0 (d, Ph, ³J_{CP} = 40 Hz), 134.0 (d, Ph, ³J_{CP} = 40 Hz), 132.0 (d, Ph, ⁵J_{CP} = 15 Hz), 131.3 (d, Ph, ⁴J_{CP} = 25 Hz), 130.8 (d, Ph, ⁴J_{CP} = 15 Hz), 130.0 (d, Ph, ²J_{CP} = 50 Hz), 128.8 (d, Ph, ³J_{CP} = 35 Hz), 119.8 (s, Ph), 117.5 (s, Ph), 117.1 (d, Ph, ¹J_{CP} = 55 Hz), 116.4 (d, Ph, ²J_{CP} = 50 Hz), 115.2 (s, Ph), 112.9 (s, Ph), 103.4 (d, Ph, ⁴J_{CP} = 15 Hz), 64.2 (s, CH₂), 63.3 (s, CH₂), 59.4 (s, CH₂), 57.3 (s, CH₂), 57.0 (s, CH₂), 54.5 (s, CH₂), 54.1 (s, CH₂), 29.1 (d, CH₂, ¹J_{CP} = 195 Hz). ³¹P NMR (D₂O) δ 23.2 (s).

Tris(4-methoxyphenyl)(4-((4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl)phosphoniumgallium(III) trifluoroacetate (2)

Yield: 28.3 mg (58.5%). HPLC Retention Time (Method 1) = 20.22 min. ESI-FT-ICR-MS for [M - CF₃CO₂]⁺ Calculated *m/z* 867.26440; Found 867.26308. ¹H NMR (D₂O) δ 7.49-7.42 (m, 6H, Ph), 7.29-7.26 (m, 2H, Ph), 7.13-7.12 (m, 6H, Ph), 7.03-7.00 (m, 2H, Ph), 4.62 (d, 2H, CH₂, ²J_{HP} = 14.7 Hz), 4.07-4.03 (m, 2H, CH₂), 3.94 (s, 6H, CH₃), 3.89 (s, 9H, CH₃), 3.5-3.3 (m, 14H, CH₂), 2.99 (d, 2H, CH₂, ²J_{HH} = 10.2 Hz). ¹³C NMR (D₂O) δ 173.7 (s, C=O), 164.3 (s, Ph), 163.1 (s, Ph), 162.6 (s, Ph), 135.8 (d, Ph, ²J_{CP} = 45 Hz), 131.8 (s, Ph), 131.2 (s, Ph), 130.5 (s, Ph), 129.4 (s, Ph), 118.3 (s, Ph), 115.6 (d, Ph, ¹J_{CP} = 54 Hz), 114.4 (s, Ph), 108.8 (s, Ph), 107.6 (s, Ph), 59.4 (s, CH₂), 57.3 (s, CH₂), 57.0 (s, CH₂), 55.8 (s, CH₂), 54.5 (s, CH₂), 54.0 (s, CH₂). ³¹P NMR (D₂O) δ 20.5 (s).

Diphenyl(pyridin-2-yl)(4-((4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl) phosphoniumgallium(III) trifluoroacetate (3)

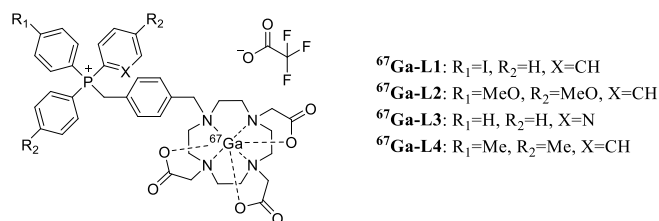
Yield: 4.4 mg (9.2%). HPLC Retention Time (Method 1) = 14.51 min. ESI-FT-ICR-MS for [M - CF₃CO₂]⁺ Calculated *m/z* 778.22795; Found 778.22777. ¹H NMR (D₂O) δ 8.93-8.92 (m, 1H, Ph), 8.11-8.09 (m, 1H, Ph), 7.91-7.86 (m, 4H, Ph), 7.69-7.29 (m, 8H, Ph), 7.16-7.14 (m, 2H, Ph), 7.08-7.05 (m, 2H, Ph), 4.90-4.85 (m, 2H, CH₂), 4.05-3.93 (m, 8H, CH₂), 3.76-3.73 (m, 2H, CH₂), 3.55-3.29 (m, 12H, CH₂), 3.01-2.98 (m, 2H, CH₂). ³¹P NMR (D₂O) δ 19.5 (s).

Tri-*p*-tolyl(4-((4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl)methyl)benzyl) phosphoniumgallium(III) trifluoroacetate (4)

Yield: 26.0 mg (53.7%). HPLC Retention Time (Method 1) = 20.87 min. ESI-FT-ICR-MS for [M - CF₃CO₂]⁺ Calculated *m/z* 819.27965; Found 819.27947. ¹H NMR (D₂O) δ 7.76-7.60 (m, 12H, Ph), 7.39 (br s, 2H, Ph), 7.21 (br s, 2H, Ph), 4.41 (br s, 2H, CH₂), 4.04-3.99 (m, 4H, CH₂), 3.5-3.1 (m, 24H, CH₂). ¹³C NMR (D₂O) δ 163.1 (s, C=O), 162.7 (s, C=O), 133.0 (s, Ph), 131.3 (s, Ph), 131.0 (d, Ph, ²J_{CP} = 36 Hz), 129.0 (d, Ph, ¹J_{CP} = 48 Hz), 128.7 (s, Ph), 118.1 (s, Ph), 114.2 (s, Ph), 57.3 (s, CH₂), 55.1 (s, CH₂), 53.0 (s, CH₂), 51.1 (s, CH₂), 49.4 (s, CH₂), 48.3 (s, CH₂), 29.6 (d, CH₂, ¹J_{CP} = 261 Hz). ³¹P NMR (D₂O) δ 38.2 (s).

Radiolabelling Studies

General synthetic method 5: Ga-67 complexes



⁶⁷GaCl₃ in 0.1 M HCl and HEPES buffer (1.0 M) were mixed in a ratio of 2:1 in an acid-washed 1.5 mL Eppendorf tube. The pH was adjusted with 0.5 M KOH to 4 - 4.5 and 5 nmol of deprotected ligand (**L1 - L4**) was added. The mixture was heated on a heating block at 80°C for 10 min. The crude product was purified by HPLC (Method 2 for ⁶⁷Ga·**L1**, ⁶⁷Ga·**L2** and ⁶⁷Ga·**L4**; Method 3 for ⁶⁷Ga·**L3**). The purified fractions were diluted to 20 mL and loaded onto Oasis HLB (hydrophilic-lipophilic-balanced reversed-phase sorbent) sample extraction product and washed with water (3 x 3 mL). The compound was eluted off with a 1:1 mixture of EtOH/0.9% saline solution which was then removed using a centrifuge evaporator.

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Table 1. Radiochemical yield and purity and SPE purification recovery of Ga-67 complexes containing L1 – L4.

Complex	Radiochemical yield (HPLC)*	Radiochemical purity^{#,*}	SPE purification recovery (%)
⁶⁷Ga-L1	98.3 ± 0.5 (n = 6)	95.6 ± 0.9 (n = 3)	98.2 ± 0.9 (n = 3)
⁶⁷Ga-L2	97.2 ± 0.5 (n = 6)	97.4 ± 1.2 (n = 3)	94.4 ± 2.4 (n = 3)
⁶⁷Ga-L3	97.6 ± 0.4 (n = 3)	91.7 ± 1.8 (n = 3)	95.6 ± 1.4 (n = 3)
⁶⁷Ga-L4	96.9 ± 0.6 (n = 3)	98.5 ± 0.4 (n = 3)	91.5 ± 1.0 (n = 3)

*Based on HPLC peak integrations.

#After SPE purification.

Table 2. Stability of Ga-67 complexes with **L1 – L4** in dPBS at all timepoints.

Complex	1h	2h	4h	24h
⁶⁷Ga·L1	93.5	94.4	94.0	88.2
⁶⁷Ga·L2	94.7	96.1	95.8	92.5
⁶⁷Ga·L3	100	100	99.8	99.7
⁶⁷Ga·L4	96.9	97.1	96.2	93.9

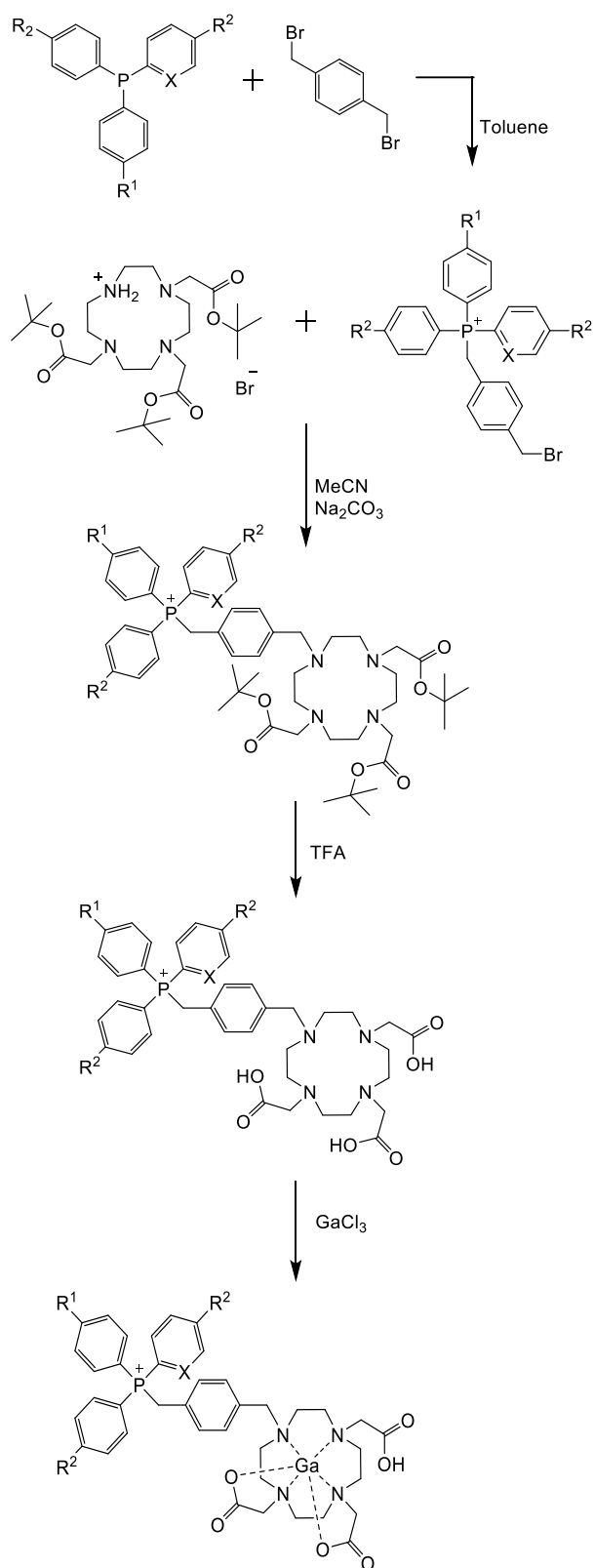
Table 3 Binding of Ga-67 complexes with L1 – L4 to human serum proteins at all timepoints.

Complex	1h		2h		4h		24h	
	HSA	Other	HSA	Other	HSA	Other	HSA	Other
⁶⁷ Ga-L1	0	4.73	0	4.72	0	6.90	1.95	5.44
⁶⁷ Ga-L2	0	5.97	0	6.59	0	6.93	3.08	7.38
⁶⁷ Ga-L3	0	0	0	0	0	0	2.20	0
⁶⁷ Ga-L4	0	5.77	0	5.75	0	6.08	2.98	7.78

Table 4 Stability of Ga-67 complexes with L1 – L4 in human serum at all timepoints.

Complex	1h	2h	4h	24h
⁶⁷ Ga:L1	84.45	76.30	73.19	56.26
⁶⁷ Ga:L2	90.70	49.77	44.56	28.16
⁶⁷ Ga:L3	90.78	92.21	91.06	72.49
⁶⁷ Ga:L4	92.08	54.67	43.04	14.40

Scheme 1



1: $\text{R}^1 = \text{I}$, $\text{R}^2 = \text{H}$, $\text{X} = \text{CH}$; 2: $\text{R}^1 = \text{MeO}$, $\text{R}^2 = \text{MeO}$, $\text{X} = \text{CH}$
 3: $\text{R}^1 = \text{H}$, $\text{R}^2 = \text{H}$, $\text{X} = \text{N}$; 4: $\text{R}^1 = \text{Me}$, $\text{R}^2 = \text{Me}$, $\text{X} = \text{CH}$

Figure 1. RadioHPLC chromatograms of serum ultrafiltrates after 24 h.

