COMMUNICATION

Significant cell uptake of Gd(III)-diphenylphosphoryl-diphenylphosphonium complexes: Evidence for a new conformationally-dependent tumour cell targeting vector

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Andrew J. Hall,[a] Amy G. Robertson,[a] Robert W. Baker,[a] Leila R. Hill[a] and Louis M. Rendina*[a,b]

The synthesis, characterisation, and tumour cell uptake of six novel Gd(III)-diphenylphosphoryl-diphenylphosphonium complexes are reported. The propyl-linked Gd(III) complexes can accumulate inside human glioma cells at prodigious levels, approaching 1200%, over the parent triphenylphosphonium salts. DFT and quantum chemical topology analyses support a new type of conformationally-dependent tumour cell targeting vector.

Tri- and tetra-phenylphosphonium cations have been extensively investigated for their selective accumulation in the mitochondria of many types of tumours, owing to their elevated mitochondrial membrane potential ($\Delta\Psi_m$).[1,2] Such delocalised lipophilic cations (DLC) as tumour-targeting motifs have a number of advantages relative to other mitochondrial targeting strategies, such as those involving small peptides or nanoparticles, including their stability in biological systems, low biological reactivity and immunogenicity, and relatively straightforward syntheses.[2,3] Tri- and tetra-phenylphosphonium cations have been employed as mitochondrial targeting agents for anti-cancer drugs such as doxorubicin,[4] chlorambucil,[5] and 6-methyl coumarin,[6] the antioxidant ubiquinone to combat mitochondrial disease,[7] and the molecular imaging agent ^{99m}Tc-mercaptoacetyltriglycine for the detection of breast tumours.[8] Furthermore, these structures have been coupled to lanthanoid ion chelators and have demonstrated promise as candidates for the development of Gd(III)-based theranostics.[9-11]

Gd(III) complexes have been used extensively as contrast agents for magnetic resonance imaging (MRI) owing to the highly paramagnetic nature of the lanthanoid ion,[12,13] but these complexes have more recently been highlighted for their potential therapeutic applications.[14] Gd complexes are excellent candidates for potential use in Gd neutron capture therapy (GdNCT) as the naturally-occurring isotope ^{157}Gd (15.7% abundance) possesses the largest thermal neutron capture cross-section of all stable isotopes.[15-17] Cell kill by Gd irradiation can also be achieved by means of photon activation therapy (PAT) or, more specifically, synchrotron stereotactic radiotherapy (SSR), as Gd has an accessible *K*-edge energy of 50.2 keV with each photon activation event yielding Auger and Coster-Kronig electrons capable of depositing *ca*. 7.63 keV[17] of energy inside a tumour cell. Triphenylphosphonium and related analogues are an excellent targeting vector for Gd(III)-based theranostics, as complexes containing these functional groups are able to selectively localise within the mitochondria of tumour cells, wherein the very short path length of the ACK electrons produced in either the NCT or PAT process is integral to their efficacy.[9]

The lipophilic nature of these DLCs enables them to effectively traverse the phospholipid bilayer of cellular and mitochondrial membranes.[18,19] However, molecular agents that are overly lipophilic often experience high non-specific uptake in healthy tissue[20,21] and extensive undesirable hepatobiliary excretion.[22] Wang *et al.* have previously investigated a ^{64}Cu-triphenylphosphonium labelled complex that utilised a 1,4,7,10-tetraazacyclododecane-1,4,7-triacetate (DO3A) chelator for use as a tumour imaging agent, and reported significant hepatic uptake and metabolism in nude mice innoculated with the U87MG glioblastoma cell line.[23] These researchers subsequently found the inclusion of a diphenylphosphoryl functionality reduced the Cu radiotracer's hepatic uptake while maintaining comparable tumour uptake, an improvement in its excretion kinetics, and enhanced metabolic stability.[24]

[a] School of Chemistry, The University of Sydney, Sydney, NSW 2006, Australia. E-mail: louis.rendina@sydney.edu.au.
[b] The University of Sydney Nano Institute, Sydney, NSW 2006, Australia.

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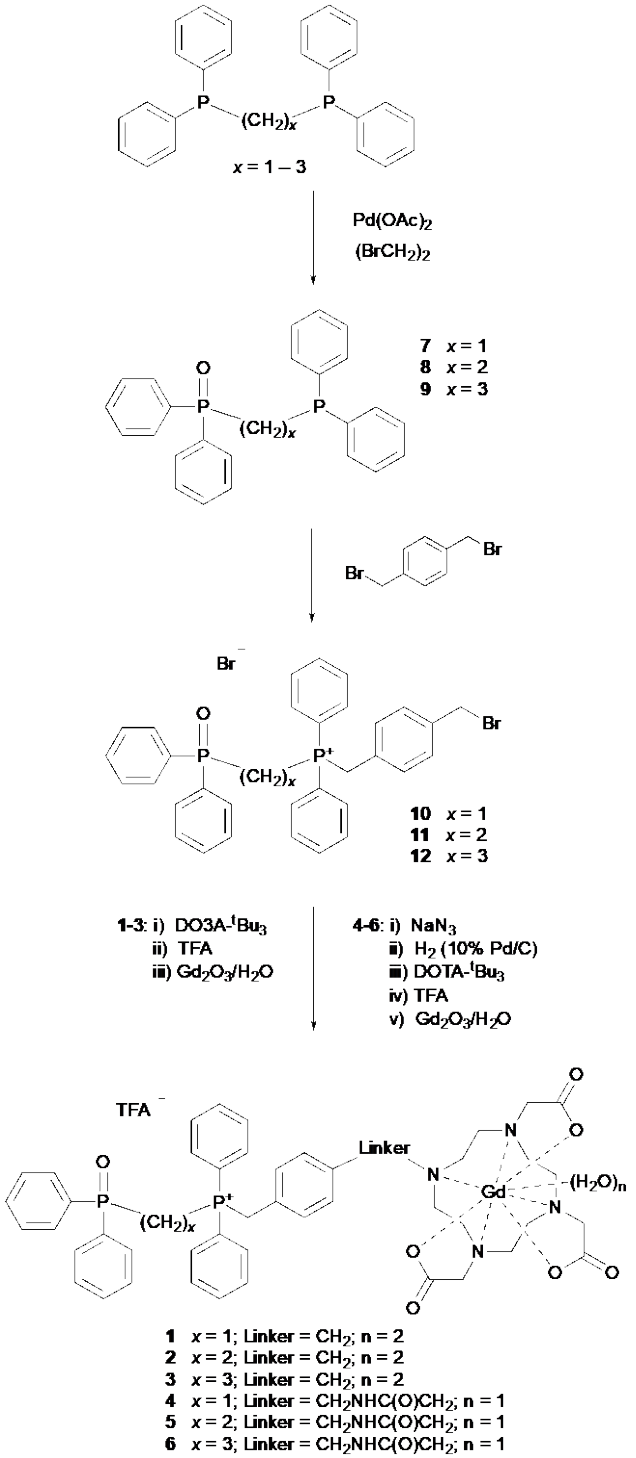

Scheme 1 The synthesis of Gd(III)-diphenylphosphoryl-diphenylphosphonium complexes (**1**–**6**).

We have previously investigated a series of Gd(III)-triphenylphosphonium and -arsonium derivatives for their theranostic potential and synthesised a series of related analogues to probe the impact that the Group 15 element and linker groups had on tumour cell uptake and selectivity.[9-11,25,26] Herein we report the synthesis of a novel series of Gd(III)-diphenylphosphoryl-diphenylphosphonium complexes and examine their uptake in human glial (SVG p12) and human glioblastoma (T98G) cells.

A new series of Gd(III) complexes (**1**–**6**) featuring an alkyl chain of varying lengths between the diphenylphosphonium and diphenylphosphoryl groups were synthesised and fully characterised. The target Gd(III) complexes **1**–**6** (Fig. 1) were prepared by means of a multistep synthesis involving mono-oxidation of various bis(diphenylphosphino)alkanes followed by phosphonium group incorporation at the un-oxidised phosphorus atom, covalently linking this targeting vector to a chelating DO3A moiety and, finally, complexation of the Gd(III) ion (Scheme 1).

Briefly, bis(diphenylphosphino)methane (dppm), 1,2-bis(diphenylphosphino)ethane (dppe), and 1,3-bis(diphenylphosphino)propane (dppp) were each mono-oxidised by means of a biphasic, $Pd(OAc)_2$ catalysed reaction adapted from Grushin,[27] and subsequently isolated by means of flash column chromatography. The purified bis(diphenylphosphino)alkane monooxides (**7**–**9**) then underwent a nucleophilic substitution reaction with one equivalent of α,α'-dibromo-*p*-xylene to afford the corresponding mono-substituted phosphonium salts (**10**–**12**). These phosphonium intermediates were functionalised at the free amine position of the *tert*-butyl triester of DO3A to yield the *tert*-butyl protected pro-ligands of **1**–**3**. Alternatively, the bromomethyl phosphonium salts (**10**–**12**) were converted to the primary amine (*via* their azide intermediates), which could then be coupled to the *tert*-butyl triester of DOTA using the peptide-coupling agent HATU and *N*-methylmorpholine (NMM).[25] All the *tert*-butyl pro-ligands were deprotected with TFA and purified by means of reverse-phase HPLC to give the required free ligands. Complexation of the Gd^{3+} ion was achieved by stirring the free ligand in a suspension of Gd_2O_3 in H_2O to afford the target Gd(III) complexes **1**–**6** in high yield and purity. The purity of each complex (> 95% in the case of complexes **2**, **3**, **5** and **6**; > 90% for complexes **1** and **4**) was determined by means of analytical reverse-phase HPLC, and their identities were confirmed by means of ESI-FTICR-MS or MALDI-TOF-MS.

In vitro cytotoxicities were determined for complexes **1**–**6** by means of a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay[28] over a concentration range of 62.5 μM – 2 mM. A summary of the results (Table S1) reports the half-maximal inhibitory concentration (IC_{50}) of each complex for the human glial (SVG p12) and human glioblastoma (T98G) cell lines. Complexes **1**–**6** were all found to exhibit IC_{50} values in the low mM range for both cell lines, and these results are in agreement with those previously-reported for related DO3A complexes.[9-11]

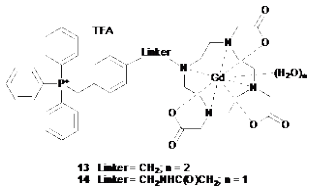

Figure 1 The structures of parent Gd(III)-phosphonium complexes.[10,25]

Table 1 Lipophilicities, Gd uptake data in human glial SVG p12 and human glioblastoma T98G cells, and tumour : normal (T:N) uptake ratios of complexes **1**–**6**.

Com-plex	Log*P* [a]	Conc (μM)	SVG p12 uptake (ng Gd / mg protein)	T98G uptake (ng Gd / mg protein)	T:N
1	1.32 ± 0.11	62.5	130.5	240.0	1.8
		125	942.3	2271.1	2.4
		250	968.4	2523.1	2.6
		500	1918.1	2715.4	1.4
2	1.28 ± 0.12	62.5	131.9	258.9	2.0
		125	920.6	2984.9	3.2
		250	941.2	1727.7	1.8
		500	2436.3	2154.2	0.9
3	1.34 ± 0.19	62.5	716.9	1852.2	2.6
		125	1475.4	3397.9	2.3
		250	3257.4	5109.8	1.6
		500	3904.1	8974.5	2.3
4	1.46 ± 0.23	62.5	216.0	293.3	1.4
		125	2045.1	5389.9	2.6
		250	2362.8	3576.7	1.5
		500	2014.2	3976.8	2.0
5	1.54 ± 0.29	62.5	162.0	289.0	1.8
		125	2064.8	3826.0	1.9
		250	1468.1	2559.8	1.7
		500	2252.1	2640.0	1.2
6	1.54 ± 0.29	62.5	557.6	2003.7	3.6
		125	1716.6	2500.3	1.5
		250	6334.4	8271.2	1.3
		500	5861.7	9232.0	1.6

[a] Mean ± SEM

In vitro cellular uptake studies were performed for complexes **1**–**6** using both SVG p12 and T98G cell lines at four different dosing concentrations (62.5, 125, 250, and 500 μM). The harvested cells were analysed for accumulated Gd by means of ICP-MS and these values were normalised to protein content, as determined by means of a modified Lowry protein assay. Representative cell uptake results and tumour-to-normal (T:N) cell uptake ratios are presented in Table 1.

When compared to the parent Gd(III)-triphenylphosphonium complexes,[9-11,25] it is clear that complexes **1**–**3** and **4**–**6**, respectively, are capable of accumulating inside tumour cells at a much higher level than the parent complexes **13** and **14**, respectively (NB: complexes **13** and **14** were also run within the *same* set of MTT assays – see ESI). For example, the cell uptake of complex **6** exceeds that of the parent complex **14** by a remarkable *ca*. 700% at the 125 and 500 μM doses, and *ca*. 1200% at 250 μM. In all complexes **1** – **6**, the tumour cell uptake levels were found to be the highest reported for this arylphosphonium class of Gd(III) complexes, but do not exceed the remarkably high intracellular Gd levels recently reported for Gd(III)-triphenylarsonium salts.[26] However, when compared to the parent phosphonium complexes **13** and **14**, the incorporation of a diphenylphosphoryl group into the tumour targeting vector results in a significant enhancement in tumour- and glial-cell uptake of one order of magnitude or greater in some cases. Hence, the diphenylphosphoryl-diphenylphosphonium group must play some important role in facilitating cell uptake. A small increase in lipophilicity for these complexes compared to **13** (log*P* = 1.24 ± 0.02[10,25]) and **14** (log*P* = 1.12 ± 0.20[10,25]) may partly contribute to an enhanced cell uptake but this factor alone cannot account for the dramatic tumour cell uptake of complexes **3** and **6**. Furthermore, the P=O functionality is known to contribute to an increased cell uptake in other systems,[24,29] but it cannot account for the remarkable uptake observed for complexes **3** and **6**, both of which contain a propylene linker between the two phosphorus centres, when compared to their shorter linker analogues **1**, **2**, **4** and **5**, and, indeed, the parent complexes **13** and **14**.

To probe potential reasons for the marked dependence of the cell uptake on the alkyl linker length between the diphenylphosphonium and diphenylphosphoryl groups, density functional theory (DFT) calculations were carried out on model compounds $[Ph_2P(O)(CH_2)_xPBnPh_2]^+$, where x = 1–3, corresponding to calculated structures **15**–**17**, respectively. In the gas-phase (approximating behaviour in a lipophilic environment) the modelled compounds all prefer to adopt collapsed structures, minimizing the distances between the O atom and the cationic-P (P^+) of the phosphonium ion, rather than sterically less-demanding extended structures, with extended structures being *ca*. 40-60 kJ mol^{-1} higher in free energy (at the B3LYP-D3/def2-SV(P) level). Also evident was the favourability of conformations featuring close-contacts between the O atom and H atoms of the P^+ substituents. Quantum chemical topology analyses using the quantum theory of atoms in molecules (QTAIM)[30,31] and the B3LYP-DFT extension of the interacting quantum atoms (IQA)[32] approach were carried out to examine further the nature of these interactions (at the B3LYP/def2-TZVP//B3LYP-D3/def2-SV(P) level). IQA interaction energies (E_{IQA}) confirm the favourability of shorter O–P^+ distances; for example, the O–P^+ distance for the global minimum energy conformation of **17** (Fig. 2) is 3.54 Å and the E_{IQA} for the O,P^+-interaction is 210 kJ mol^{-1} lower in energy than for the next lowest free energy conformation (**17b** Fig. S3, ESI), where the O–P^+ distance is 4.29 Å. As illustrated in Fig. 2, the QTAIM analysis also reveals the presence of bond paths for the majority of the energetically favourable O,H-close-contacts. Key QTAIM parameters at the bond critical points for these interactions are all consistent with a closed-shell electrostatic interaction, similar to more familiar hydrogen bonding situations (see ESI for details). The DFT modelling shows that

while the global minimum energy conformations of the methylene- (**15a**, Fig. S1, ESI), ethylene- (**16a**, Fig. S2, ESI), and propylene-linked structures are all supported by three O,H-interactions, the propylene-linker's third O,H-interaction involves the tether methylene attached to the phosphonium ion, rather than an *ortho*-H of the benzyl-substituent as with **15a** and **16a**. Evidently, the linker length can have a significant influence on the conformation, and likely in turn the observed prodigious tumour cell uptake of complexes **3** and **6**.

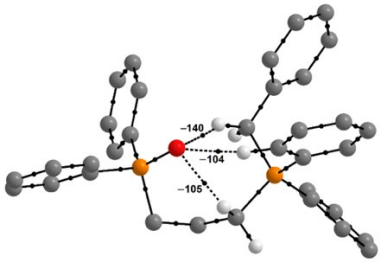

Figure 2 Perspective view of the molecular graph for the global minimum energy conformation of **17** (grey spheres = C, orange = P, red = O, white = H). Black lines depict located bond paths and the black dots indicate located bond critical points (bcp). Dashed lines represent weak bond paths, where the electron density at the bcp is $\rho(r) < 0.025\ e a_0^{-3}$; values for the E_{IQA} (kJ mol^{-1}) for these interactions are shown adjacent to the bcp. Only selected hydrogen atoms and their associated bond paths/critical points are shown, and some weak bond paths have been omitted, for clarity.

In conclusion, complexes **1**–**6** were successfully synthesised in high yields and purity. These complexes were all found to have low (mM) cytotoxicity at therapeutically-relevant concentrations, with significant *in vitro* tumour cell uptake and reasonable tumour cell selectivity at lower concentrations. The increase in tumour cell uptake for these complexes over the parent triphenylphosphonium complexes was found to be remarkably high, approaching 1200% in the case of complex **6**. The related propyl complexes **3** and **6** demonstrate far greater tumour cell uptake relative to other complexes at higher concentrations, and DFT and quantum chemical topology analyses support a new type of conformationally-dependent tumour cell targeting vector. *In vivo* uptake and biodistribution studies involving selected Gd(III) complexes, along with glioma tumour imaging using MRI, are planned and the results of this work will be reported in due course.

Conflicts of interest

There are no conflicts of interest to declare.

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