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High mitochondrial accumulation of new gadolinium(III) agents within tumour cells†

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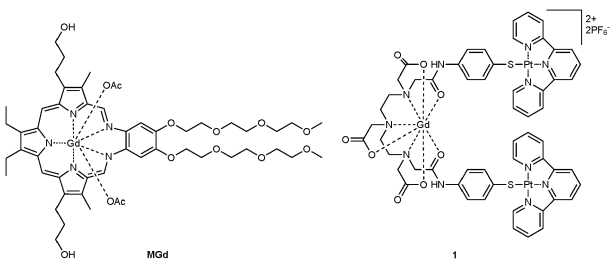
The first bifunctional Gd^{III} complexes covalently bound to arylphosphonium cations and the first tumour-cell selective mitochondrial agents designed for potential application in binary cancer therapies are reported. The highest *in vitro* cellular uptake for any Gd complex reported to date has been described, with levels exceeding 10^{10} Gd atoms per tumour cell.

Gd^{III} complexes have found extensive use in medicine and are primarily employed as water relaxation agents to improve the contrast in magnetic resonance imaging (MRI).[1–3] More recently, Gd^{III} complexes have also shown some promise as therapeutic agents. The macrocyclic texaphyrin derivative known as Motexafin-Gd (MGd) has been to date the only clinically assessed therapeutic agent containing Gd which has been used as a radiosensitiser for conventional whole-brain radiotherapy,[4,5] particularly in the treatment of brain metastases arising from non-small cell lung cancer.[6–9] MGd possesses favourable properties for uptake by tumour cells, with De Stasio *et al.*[6] reporting that up to 90% of glioblastoma cell nuclei were found to contain Gd. More recently, Crossley *et al.*[10] reported the first example of a DNA metallointercalator (**1**) which has the capacity to deliver Gd to A549 lung tumour cell nuclei in a selective manner.



MGd and **1** present new opportunities for cutting-edge cancer therapies, notably neutron capture therapy (NCT)[10–14] and photon activation therapy (PAT),[4,5] in which the development of suitable Gd agents possessing a capacity to deliver considerable quantities of Gd to tumour sites (*ca.* 100 ppm) with low host toxicity is necessary for a therapeutic effect to be realised.[6] In GdNCT, the high linear-energy transfer Auger Coster-Krönig (ACK) electrons represent the main therapeutic entity derived from the thermal neutron capture reactions of the naturally occurring, non-radioactive ^{157}Gd isotope, which has the highest neutron-capture cross-section of all stable nuclides (2.55×10^5 barns).[10–14] Emission of ACK electrons from high-Z atoms can also be achieved by means of X-ray photons due to the photoelectric effect and, unlike NCT, is independent of the isotope. This process is the basis of synchrotron stereotactic radiotherapy (SSR), a variant of PAT employing high-energy, monochromatic synchrotron radiation.[6] *In vivo* data obtained using a rat tumour model have clearly demonstrated the potential of SSR when heavy atoms such as Pt and I were used as radiosensitisers, but no *in vivo* studies employing Gd have been reported to date.[15]

Both NCT and SSR rely upon the production of damaging ACK electrons with an extremely short path-length (*ca.* 12 nm).[16] A critical aspect of both NCT and SSR is the development of tumour-selective agents which can localise near important sub-cellular components such as DNA or mitochondria and lead to a therapeutic effect upon thermal neutron or X-ray photon irradiation, respectively.

Cancer cells typically possess an elevated mitochondrial membrane potential compared to that of normal, healthy cells (*ca.* 60 mV), which results in a highly selective accumulation of delocalised lipophilic cations (DLCs) such as phosphonium salts.[17] Herein we present the synthesis, *in vitro* cellular uptake and biodistribution of the Gd^{III}–triarylphosphonium salts as the first examples. For the first time, a feasibility assessment of tumour-cell selective, mitochondrially-targeted Gd agents with exceptional tumour-cell uptake can be realised for potential application in either NCT or SSR.[6,12–14]

Novel Gd^{III} complexes **2** and **3** (Scheme 1) were synthesised in order to demonstrate the importance of lipophilicity in terms of cytotoxicity, cellular uptake, and intracellular localisation predominately within the mitochondria. Both complexes **2** and **3** (Scheme 1) were prepared by using a three-step synthetic procedure.[18] First, triarylphosphines were reacted with *para*-dibromoxylene in toluene

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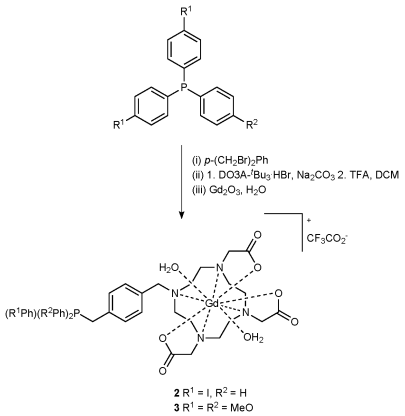

Scheme 1

to allow for the exclusive formation of the mono-substituted phosphonium salt intermediate. Subsequent *N*-alkylation of the tri-*tert*-butyl ester of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid hydrobromide (DO3A-^{t}Bu$_3$·HBr), followed by acid deprotection of the ^{t}Bu groups, afforded the free macrocyclic ligands in high yield and purity after purification by means of reverse-phase HPLC. Finally, treatment of the free ligands with a suspension of Gd_2O_3 in H_2O gave the target complexes **2** and **3** in high yield (>70%). The identities of complexes **2** and **3** were confirmed by means of high resolution ESI-FTICR-MS (**2**: $[M–CF_3CO_2]^+$: calculated *m/z* 992.1285; found 992.1291; **3**: $[M–CF_3CO_2]^+$: calculated *m/z* 956.2636; found 956.2646) and their purity (>95%) was confirmed by means of analytical reverse phase HPLC. Both complexes **2** and **3** possessed excellent aqueous stability at physiological pH (7.4) and at pH 5.0 (at 37 °C for 24 h). log *P* values for complexes **2**, **3**, and the parent complex ($R^1 = R^2 = H$) were assessed by means of a reverse-phase HPLC protocol,[19] and these were determined to be 1.44 ± 0.05, 1.86 ± 0.09, and 1.24 ± 0.04, respectively. These values are similar to those determined for simple arylphosphonium cations such as TPP (1.20 ± 0.01), and therefore the lipophilicity of the compounds appears to be largely determined by the nature of the arylphosphonium group.

In vitro cytotoxicities of **2** and **3** were assessed by means of standard MTT assay protocols by using a human glioblastoma (T98G) cell line. IC_{50} values were determined to be 2.2 ± 0.4 mM and 1.2 ± 0.2 mM for **2** and **3**, respectively. These values are significantly higher than those of the DNA-targeted Gd agent **1** and MGd which possess IC_{50} values in the low μM range[8,10] against T98G and A2780 ovarian tumour cell lines, respectively, thus confirming the relatively low *in vitro* cytotoxicity of **2** and **3** towards T98G tumour cells.

The cellular uptake of **2** and **3** was determined by ICP-MS analyses of T98G and primary human carotid artery endothelial cells (HCtAEC) following incubation of the Gd^{III} complexes (100 μM) at 37 °C for 48 h. Complexes **2** and **3** displayed statistically significant greater uptake of Gd by T98G cells (5360 ± 120 and 4800 ± 1000 ng mg^{-1} protein, respectively) when compared to HCtAEC cells (700 ± 140 and 800 ± 170 ng mg^{-1} protein, respectively) with selectivity ratios of 7.7 : 1 and 6 : 1, respectively. The tumour cell selectivities are consistent with the expected mechanism of mitochondrial aggregation for DLCs.[17,18,20] The observed levels of Gd uptake in this study are considerably higher than those levels reported for other potential therapeutic Gd agents, *e.g.* the archetypal Gd^{III}–Pt^{II} complex **1** showed a selective (*ca.* 10 : 1) Gd uptake of only 5 ± 1 ng mg^{-1} of protein.[10] Thus, complexes **2** and **3** have the capacity to deliver up to three orders of magnitude more Gd to tumour cells than **1**, with significantly lower cytotoxicity. A direct comparison of tumour cell uptake can also be made with the clinically-assessed agent MGd,[6] whereby *ca.* 3 × 10^9 Gd atoms per T98G cell were reported at a dosage of 100 μM MGd. The Gd levels determined for **2** and **3** thus represent more than one order of magnitude increase in cellular uptake compared to MGd. This difference in uptake is likely due to differences in uptake mechanisms for the complexes, the exact nature of which will be investigated in due course. The *in vitro* tumour selectivities reported for **2** and **3**, however, are of the same order of magnitude as those reported for both **1** (ref. 10) and MGd.[21]

Mitochondrial and cytosolic fractions were isolated from T98G human glioblastoma cells following their incubation with the Gd complex (100 μM) at 37 °C for 48 h. Subsequent analysis of the lysed cell fractions by means of ICP-MS indicated that a significant amount of Gd was taken up into the mitochondria of treated T98G cells, and that Gd uptake into the mitochondria was generally higher than its uptake into the cytosol. This value can be expressed in terms of the ratio between the mitochondrial and cytosolic fractions (*i.e.* M/C ratio). Complex **2** showed an M/C ratio of 3.4 : 1 while **3** showed an M/C ratio of 5.4 : 1. There exists a firm correlation between the M/C ratio and lipophilicity, which highlights the role of lipophilicity in promoting mitochondrial localisation.

Quantitative uptake and biodistribution of **2** and **3** within individual T98G cells were determined by means of synchrotron XRF experiments (Table 1). Fig. 1 and 2 show elemental maps of a single T98G cell incubated with 100 μM of **2** and **3** for 48 h and 24 h, respectively; XRF quantitation of the intracellular Gd content is summarised in Table 1. Statistically significant (single-tailed Mann–Whitney *U* test, $p < 0.05$) levels of Gd were observed in the cells after an incubation period of only 1 h with **2**. For both complexes **2** and **3**, the synchrotron XRF elemental density maps for Gd showed a strong correlation to regions of high intensity in the Fe elemental density maps at all incubation times. This elemental correlation is consistent with the mitochondrial uptake of the complexes due to the key role the mitochondria plays in cellular Fe regulation, metabolism (*e.g.* heme synthesis and Fe–S cluster assembly), and storage due to mitochondrial ferritin (MtFe).[22] The elemental density maps of the cells dosed with **2** also showed

Table 1 Mean Gd uptake by individual T98G human glioblastoma cells following treatment with complexes **2** and **3**, as determined by synchrotron X-ray fluorescence (XRF)

Complex	Incubation time (h)	Gd density per cell[a]	Gd atoms × 10^9 per cell[b]	*N*
2	1	0.16 ± 0.01	0.35 ± 0.10	4
2	6	0.23 ± 0.06	5.3 ± 0.9	5
2	48	1.35 ± 0.46	42 ± 6	5
2	48[c]	0.28 ± 0.11	5.7 ± 1.4	4
3	6	0.50 ± 0.29	17 ± 2	5
3	24	3.88 ± 1.64	74 ± 8	5

[a] Mean ± SD in μg cm^{-2}. [b] Mean ± SD. [c] Efflux experiment in which cells were incubated in the presence of **2** for 24 h, followed by incubation for further 24 h in the absence of the Gd complex.

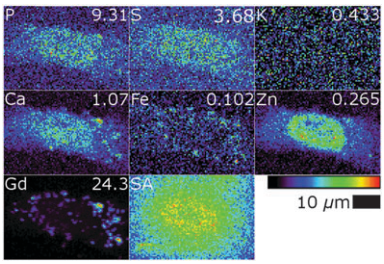

Fig. 1 XRF elemental distribution maps of a single T98G human glioblastoma cell dosed with 100 μM of **2** for 48 h showing P, S, K, Ca, Fe, Zn, Gd, and scattered X-ray (SA). Maximal area densities (μg cm^{-2}) for each element are presented at the top right of each map.

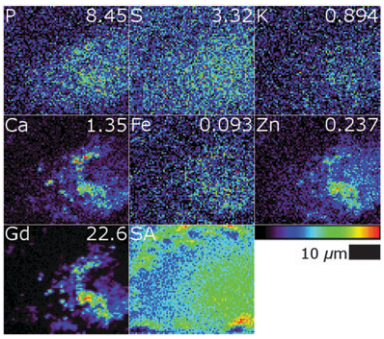

Fig. 2 XRF elemental distribution maps of a single T98G human glioblastoma cell dosed with 100 μM of **3** for 24 h showing P, S, K, Ca, Fe, Zn, Gd, and scattered X-ray (SA). Maximal area densities (μg cm^{-2}) for each element are presented at the top right of each map.

negative correlation to areas of high P and Zn intensity with the Gd/Fe regions; regions of high P and Zn density are indicative of the nucleus due to the chromosomal DNA and Zn finger proteins, respectively.[10] However, the elemental density maps of cells incubated with **3** showed regions of high P and Zn intensity co-localised with the Gd/Fe regions. This observation represents a major change in the distribution of endogenous elements within the cell in response to **3**, as the control cells show a clear distinction between areas of high intensity in the Fe and Zn/P maps. Further work is required to unravel the reasons for the differences observed in the cellular biodistribution between **2** and **3**.

In conclusion, we have developed the first example of Gd^{III} complexes which include a triarylphosphonium functionality for tumour-cell targeting of mitochondria. We have also demonstrated their favourably low *in vitro* cytotoxicity, excellent *in vitro* tumour:-healthy cell selectivity, preferential localisation within the mitochondria of treated cells, and a capacity to deliver remarkably high levels of Gd into T98G tumour cells (up to 7×10^{10} Gd atoms per cell, or *ca.* 3000 ppm) by means of both ICP-MS analysis and synchrotron XRF quantitation. Such Gd levels far exceed those determined for the tumour cell uptake of Gd-containing DNA-binding agents by greater than three orders of magnitude, and lie well above the Gd threshold calculated for efficacious GdNCT and GdSSR.[6] The observed tumour selectivity also presents an opportunity for the significantly increased tumour:tissue contrast in MRI, which employs only non-tumour selective Gd contrast agents. Both *in vitro* and *in vivo* GdNCT, GdSSR, and MRI experiments with selected complexes are planned and the results of these studies will be reported in due course.

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