High Mitochondrial Accumulation of New Gadolinium(III) Agents Within Tumour Cells

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The first bifunctional GdIII 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 is described, with levels exceeding 10^10 Gd atoms/tumour cell.

GdIII complexes have found extensive use in medicine and are primarily employed as water relaxation agents to improve contrast in magnetic resonance imaging (MRI). More recently, GdIII complexes have also shown some promise as therapeutic agents. The macrocyclic tetraxiphyrin derivative known as Motexafin-Gd (MGd) is to date the only clinically-assessed therapeutic agent containing Gd which has been used as a radiosensitiser for conventional whole-brain radiotherapy, particularly in the treatment of brain metastases arising from non-small cell lung cancer. MGD possesses favourable tumour cell uptake properties with De Stasio et al. reporting that up to 90% of glioblastoma cell nuclei found to contain Gd. More recently, Crossley et al. 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 I present new opportunities for cutting-edge cancer therapies, notably neutron capture therapy (NCT) and photon activation therapy (PAT), 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. 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 possesses the highest neutron-capture cross-section of all stable nuclides (2.55 x 10^7 barns). 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 isotope. This process is the basis of synchrotron stereotactic radiotherapy (SSR), a variant of PAT employing high-energy, monochromatic synchrotron radiation. In vivo data obtained using a rat tumour model have clearly demonstrated the potential of SSR when heavy atoms such as Pt and I are used as radiosensitisers but no in vivo studies employing Gd have been reported to date.

Both NCT and SSR rely upon the production of damaging ACK electrons with an extremely short path-length (ca. 12 nm). A critical aspect of both NCT and SSR is the development of tumour-selective agents which can localise near important subcellular 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 a mitochondrial membrane potential compared to that of normal, healthy cells (ca. 60 mV), which results in a high selective accumulation of delocalised lipophilic cations (DLCs) such as phosphonium salts. Herein we present the synthesis, in vitro cell uptake and biodistribution of the first examples of GdIII-arylphosphonium salts. 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.

The novel GdIII 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. First, triarylphosphines were reacted with para-dibromomethylene in toluene 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-tetraacazacyclodecane-1,4,7,10-tetra-acetic acid hydrobromide (DO3A-\textbf{Bu}_3HBr), followed by acid deprotection of the \textbf{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_{2}O_{3} in H_{2}O 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: \text{[M-CF}_{3}C_{6}O]^{+}, calculated m/z 992.1285; found 992.1291; 3: \text{[M-CF}_{3}C_{6}O]^{+}; 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). LogP values for complexes 2, 3, and the parent complex (R^1 = R^2 = H) were assessed by means of a reverse-phase HPLC protocol, 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 the DNA-targeted Gd agent 1 and MGd which possess IC_{50} values in the low \mu M 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 complexes (100 \mu M) at 37°C for 48 h. Complexes 2 and 3 displayed statistically significant greater uptake of Gd in T98G cells (5360 ± 120 and 4800 ± 1000 ng/mg protein, respectively) when compared to HCTAEC cells (700 ± 140 and 800 ± 170 ng/mg 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. 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 showed selective (ca. 10:1) Gd uptake of only 5 ± 1 ng/mg of protein. Thus, complexes 2 and 3 have the capacity to deliver up to three orders of magnitude greater 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, whereby ca. 3 x 10^6 Gd atoms per T98G cell was reported at a dosage of 100 \mu M 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 and MGd.

Mitochondrial and cytosolic fractions were isolated from T98G human glioblastoma cells following incubation of the Gd complex (100 \mu M) 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). Figures 1 and 2 show elemental maps of a single T98G cell incubated with 100 \mu M of 2 and 3 for 48 h and 24 h, respectively; XRF quantitation of intracellular Gd content is summarised in Table 1. Statistically significant (single-tailed Mann-Whitney U test, p < 0.05) levels of Gd were present in the cells after only a 1 h incubation period 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 mitochondrial uptake of the complexes due to the key role the mitochondria play in cellular Fe regulation, metabolism (e.g. heme synthesis and Fe–S cluster assembly), and storage due to mitochondrial ferritin (MtFe). The elemental density maps of those cells dosed with 2 also showed negative correlation to areas of high intensity in the P and Zn 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. 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 cellular biodistribution between 2 and 3.

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

<table>
<thead>
<tr>
<th>Complex</th>
<th>Incubation Time (h)</th>
<th>Gd density /cell&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gd atoms × 10&lt;sup&gt;6&lt;/sup&gt;/cell&lt;sup&gt;b&lt;/sup&gt;</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>0.16 ± 0.01</td>
<td>0.35 ± 0.10</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>0.23 ± 0.06</td>
<td>5.3 ± 0.9</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>1.35 ± 0.46</td>
<td>42 ± 6</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>3.88 ± 1.64</td>
<td>74 ± 8</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SD in µg cm<sup>2</sup>. <sup>b</sup>Mean ± SD. Efflux experiment in which cells were incubated in the presence of 2 for 24 h, followed by incubation for a further 24 h in absence of Gd complex.

Figure 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<sup>-2</sup>) for each element are presented at the top right of each map.

Figure 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<sup>2</sup>) for each element are presented at the top right of each map.

In conclusion, we have developed the first examples of Gd<sup>III</sup> 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 x 10<sup>10</sup> Gd atoms/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. The observed tumour selectivity also presents an opportunity for significantly increased tumour : tissue contrast in MRI, which only employs 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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Notes and references