Targeting of anionic membrane species by lanthanide(III) complexes: towards improved MRI contrast agents for apoptosis.

Andrew J Surman,^a Gavin D Kenny,^b D Krishna Kumar,^a Jimmy D Bell,^b Duncan R Casey^a and Ramon Vilar^{*a}

^a *Department of Chemistry, Imperial College London, London, UK, SW7 2AZ . E-mail:*

r.vilar@imperial.ac.uk

^b *Metabolic and Molecular Imaging Group, Imaging Sciences Department, MRC Clinical Sciences Centre, Imperial College London, Hammersmith Hospital, London, UK, W12 0HS.*

In most healthy mammalian cells an uneven distribution of the mixture of the phospholipid species that make up the bilayer cell membrane is maintained between inner and outer layers: anionic species (principally phosphatidylserine, PS) are arranged largely on the inner layer.¹ In some abnormal cells this is not the case and a considerable amount of anionic lipids are displayed on the outer membrane surface; this is known in cells undergoing the early/intermediate stages of apoptosis (programmed cell death),² tumour vasculature,³ bacteria and viruses.⁴

Detection and imaging of apoptotic cells *in vivo* is desirable, as a clinical and research tool: the extent and speed of onset of apoptosis in tumours following a treatment has shown to be a good prognostic indicator of treatment outcome.⁵ *In vitro*, apoptotic cells are typically detected using biomolecules known to bind phosphatidylserine, conjugated with fluorescent moieties; the most extensively used in this context has been Annexin V.⁶ For *in vivo* imaging, Annexin V and others have been modified with various functionalities for imaging, for

example with radioactive,⁷ optical,⁸ and, particularly of interest here, MRI probes.⁹⁻¹² While this approach has proved somewhat successful, bioconjugates are bulky (36 kDa in the case of Annexin V), limiting the density with which the ‘payload’ may be delivered and, ultimately, sensitivity. Small molecule probes are desirable for their ease of synthesis, robustness, long shelf life, fast binding kinetics, fast clearance from blood circulation, and ease of formulation for widespread use.¹³

In recent years, the potential of Zn^{2+} -2,2'-dipicolylamine (ZnDPA) to recognise phosphate moieties, and especially phosphorylated biomolecules, has been increasingly realised. Smith has developed a range of bis-ZnDPA complexes as probes able to recognise apoptotic and bacterial cells, which they have demonstrated with considerable success both *in vitro* (liposomal models and cells)^{14,15} and in small animal tumour and bacterial infection models, using NIR optical imaging.^{16,17}

In a recently-published study we showed that bis-ZnDPA, lanthanide complexes $[\text{Ln}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ ($\text{Ln} = \text{Gd}, \text{Eu}$; see Scheme 1), bind strongly to polyanionic species, including Fmoc-phosphoserine.¹⁸ This prompted us to study whether this complex might also bind bisanionic phosphoserine head groups at membrane surfaces. Here we present a study of the interaction between $[\text{Eu}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ and phosphatidylserine derivatives on the surface of cell membrane models (unilamellar vesicles) and preliminary studies of $[\text{Gd}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ as an MRI contrast agent targeted to anionic membrane species in cell cultures.

Scheme 1 Structures of phospholipids, sensitizer, and $[\text{Ln}(\text{L})\text{Zn}_2]^{4+}$.

Unilamellar vesicles are established models for apoptotic cell membranes for *in vitro* assessment of potential probes, and are produced using standard extrusion procedures from mixtures of commercially-available phospholipids. We have followed protocols adapted from those established by DiVittorio *et al*¹⁵ to study the interaction of $[\text{Eu}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ with DOPS (a synthetic phosphatidylserine derivative with the same polar head group – see Scheme 1), with the addition of 2,3-Naphthalimide (**2,3-Nap**, 8.3 mol%) to the lipid mixtures prior to extrusion.[†] **2,3-Nap** is known to sensitise Eu^{3+} emission when incorporated covalently into Eu-bearing dendrimers,¹⁹ or by hydrophobic interactions into Eu-bearing micelles.²⁰ We propose that intermolecular energy transfer may be used to detect the proximity of $[\text{Eu}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ to a membrane incorporating **2,3-Nap** (by hydrophobic interactions), on reversible recognition of surface species, as schematically shown in Fig 1.

Figure 1 Schematic representation of sensitisation of Eu^{3+} -centred emission by naphthalimide on recognition of membrane species.

A series of vesicles were produced to demonstrate this, and the emission spectra ($\lambda_{\text{ex}} = 335$ nm) resulting from their interaction (or lack of it) with $[\text{Eu}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ are summarised in Fig 2: solutions contained $[\text{Eu}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ along with vesicles formed from lipid mixtures with either 1:1 DOPS:DOPC (**1**), 1:1 DOPS:DOPC with **2,3-Nap** (**1-S**) or DOPC with **2,3-Nap** (**2-S**) (see Scheme 1 for chemical structures). Strong characteristic Eu-centred emission bands were only observed in solution **1-S**, where vesicles contained both **2,3-Nap** and DOPS; in the others; where vesicles lacked this combination, and in solutions of the complex alone, little emission was observed. This suggests that only on selective recognition of the anionic DOPS headgroup by $[\text{Eu}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ is the Eu^{3+} centre brought sufficiently close to the bilayer-incorporated **2,3-Nap** for energy transfer to occur (as in Fig 1). This mechanism is further corroborated by the dramatic decrease of Eu-centred emission on addition of pyrophosphate (previously shown is very strongly bound by $[\text{Eu}(\text{L})\text{Zn}_2]^{4+}$),¹⁸ demonstrating the reversible nature of the recognition by an intact complex, as opposed to dissociation of the Eu^{3+} cation induced by the anionic DOPS head group (see Fig. S2 in the ESI). To confirm

this further we added the zinc-free $[\text{Eu}(\text{L})]$ complex to **1-S** and recorded the emission. As can be seen in Fig. S3, the emission in this case was much lower than when $[\text{Eu}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ was used (the emission recorded likely be due to a small degree of non-specific binding by $[\text{Eu}(\text{L})]$).

Figure 2 Results from interaction of $[\text{Eu}(\text{L})\text{Zn}_2]^{4+}$ with a variety of vesicles ($\lambda_{\text{ex}} = 335$ nm; excitation and emission slit width are 5 nm and 1 nm respectively). Notable sensitisation only takes place in DOPS/naphthalimide -bearing vesicles (**1-S**), where recognition by $[\text{Eu}(\text{L})\text{Zn}_2]^{4+}$ may occur (spectrum of highest intensity, coloured red).

With the reasonable presumption of an even distribution of DOPS between inner and outer leaflets of the membrane bilayer in vesicles of this size,²¹ it was possible to construct a Job's plot consistent with 1:1 binding (Fig. 3, inset). DOPS-containing vesicles (**1-S**) were then titrated with $[\text{Eu}(\text{L})\text{Zn}_2](\text{NO}_3)_4$,[†] with the resulting variation in emission at 614 nm shown in Fig 3; an association constant (K_a) of $1.81 \times 10^4 \text{ M}^{-1}$ for recognition of the available (outer surface) DOPS by $[\text{Eu}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ was estimated (fitting data to a standard 1:1 model –

Fig. 3 and ESI).

Figure 3 Emission data (614 nm) from titration; line indicates non-linear curve fitting. The inset shows Job plot indicating a 1:1 interaction between complex and DOPS exposed on the surface of vesicles (**1-S**).

Given these encouraging results showing recognition of the PS head group on the surface of cell models, we proceeded to a preliminary MRI study, using $[\text{Gd}(\text{L})\text{Zn}_2](\text{NO}_3)_4$ as a contrast agent for imaging of cells in which apoptosis had been induced. In a protocol similar to that previously reported to assess apoptosis-targeting contrast media,^{9,22} a murine lymphoma (EL4) cell culture was grown, split in two, and apoptosis induced in one population by incubation in etoposide (15 μM). Proportions of early-stage apoptosis, necrosis, and viable cells in both populations were determined by flow cytometry (double-staining with propidium iodide and annexin V-FITC; Fig. S4 in ESI). In the untreated cells, 84% remained viable, with 11% necrotic and negligible amounts found to be early apoptotic; by contrast, only 38% of etoposide-treated cells remained viable, with 19% necrotic and 19% found to be

early apoptotic. Samples of each population were incubated with varying contrast agent concentrations (0, 100, 300 and 600 μM) at room temperature for 30 min; they were collected by centrifugation, the unbound contrast agent removed by washing with saline and the cell pellet re-suspended in saline (with 1% agarose to stop cell sedimentation) and loaded into 250 μl PCR tubes for MRI imaging. Relaxation rates ($1/T_1$) and image intensity (T_1 -weighted image) are shown in Figs. 4 and 5, respectively, with selected T_1 -weighted images inset of Fig. 5. Considerable differences in T_1 between the etoposide-treated and untreated populations were observed, even when incubated with low concentrations of $[\text{Gd}(\text{L})\text{Zn}_2](\text{NO}_3)_4$. As a consequence, large differences between the two populations were clearly observable in T_1 -weighted images (both by inspection and quantification of intensity) in the presence of 300 μM or greater $[\text{Gd}(\text{L})\text{Zn}_2](\text{NO}_3)_4$, with negligible enhancement of intensity in the untreated populations, even after incubation with 600 μM $[\text{Gd}(\text{L})\text{Zn}_2](\text{NO}_3)_4$.

Figure 4 MR relaxation rate ($1/T_1$) of Etoposide-treated and untreated cells with varying concentrations of the contrast agent: the Etoposide-treated cells manifest increased binding of $[\text{Gd}(\text{L})\text{Zn}_2]^{4+}$ in a greater increase in relaxation rate ($1/T_1$) than untreated cells as the concentration of contrast agent is increased. (* = $p < 0.05$).

Figure 5 MR signal intensity in T₁-weighted (T_R = 0.7 s) image of Etoposide-treated and untreated cells with varying concentrations of the contrast agent: Etoposide-treated cells show a considerable increase in signal intensity as the concentration of contrast agent is increased, while that of untreated cells is very small by comparison. (* = p < 0.05).

Taken together these results make it clear that the treated cells interact with [Gd(L)Zn₂](NO₃)₄ considerably more strongly, despite only relatively small differences in cell viability (only *ca.* 1/5 being identified as early apoptotic in treated population). While there is some measurable 1/T₁ increase in the untreated population on interaction of [Gd(L)Zn₂](NO₃)₄, non-specific binding is unsurprising in mixed cell populations (>10% necrotic) and the enhancement may stem from a number of mechanisms:[‡] it is not sufficient to prevent clear differentiation between increasing concentrations on inspection of their images. This selectivity is consistent with the high selectivity for apoptotic cells previously reported in a wide and structurally diverse range of bis-(Zn-DPA) complexes.^{14,15,17} As such we feel that these positive results should encourage the development of small Gd³⁺-bis(Zn-DPA) complexes as MRI probes for apoptosis, as an alternative to the continued development

of bioconjugate-based probes. Although neutral Gd-DTPA-bis(amide) complexes have been used clinically for some time, their *in vivo* stability has been recently questioned,²³ especially when interacting with cell surfaces.²⁴ Were this to prove problematic, macrocyclic Gd³⁺ complexes with two pendant Zn-DPA moieties are likely to prove more robust *in vivo*.

In conclusion, we have demonstrated *in vitro* and *in cellulo* the ability of [Ln(L)Zn₂](NO₃)₄ to target clinically important anionic membrane species, both in synthetic cell models and in cell cultures, and shown it possible to use [Gd(L)Zn₂](NO₃)₄ as a contrast agent for the differentiation of apoptotic cultures in MRI images. Furthermore, we have demonstrated a novel method for studying the interaction of Ln³⁺ complexes with membrane species, which may be more broadly applicable either as demonstrated, using different membrane-bound targets, or in an inverse configuration with energy transfer to amphiphilic lanthanide(III) species bound in a membrane.²⁰

This work was supported by the Medical Research Council (MRC), the European Commission-Marie Curie IIF and the Engineering and Physical Sciences Research council (EPSRC). We thank Mr. Kogularamanan Suntharalingam for his help with cytotoxicity assays.

Notes and references

† Electronic Supplementary Information (ESI) available: preparation of [Ln(L)Zn₂](NO₃)₄, preparation of liposomes, luminescence experiments, preparation of cell cultures, and MRI imaging of cell samples. See DOI: XXX

‡Interaction with the untreated cell population may be due to non-specific binding of viable cells (not unlikely for a positively-charged complex in saline, an extremely uncompetitive medium, but less likely *in vivo*), incomplete removal of unbound complex, binding of the tiny population of apoptotic cells present, induction of apoptosis, dissociation of Gd^{3+} from the ligand, or binding of necrotic cells. Differentiation between these possibilities is beyond the scope of this preliminary study.

1. J. A. Op den Kamp, *Annu. Rev. Biochem.*, 1979, **48**, 47.
2. R. A. Schlegel and P. Williamson, *Cell Death and Differentiation*, 2001, **8**, 551.
3. S. Ran, A. Downes and P. E. Thorpe, *Cancer Res.*, 2002, **62**, 6132.
4. J. Mercer and A. Helenius, *Science*, 2008, **320**, 531.
5. J. M. Brown and L. D. Attardi, *Nat. Rev. Cancer*, 2005, **5**, 231.
6. H. H. Boersma, B. L. J. H. Kietselaer, L. M. L. Stolk, A. Bennaghmouch, L. Hofstra, J. Narula, G. A. K. Heidendal and C. P. M. Reutelingsperger, *J. Nucl. Med.*, 2005, **46**, 2035.
7. S. Rottey, G. Slegers, S. Van Belle, I. Goethals and C. Van de Wiele, *J. Nucl. Med.*, 2006, **47**, 1813.
8. V. Ntziachristos, E. A. Schellenberger, J. Ripoll, D. Yessayan, E. Graves, A. Bogdanov, Jr., L. Josephson and R. Weissleder, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 12294.
9. A. A. Neves, A. S. Krishnan, M. I. Kettunen, D.-E. Hu, M. M. De Backer, B. Davletov and K. M. Brindle, *Nano Lett.*, 2007, **7**, 1419.
10. M. Zhao, D. A. Beauregard, L. Loizou, B. Davletov and K. M. Brindle, *Nat. Med.*, 2001, **7**, 1241.
11. K. M. Brindle, *Adv. Enzyme Regul.*, 2002, **42**, 101.
12. C. Burtea, S. Laurent, E. Lancelot, S. Ballet, O. Murariu, O. Rousseaux, M. Port, L. Vander Elst, C. Corot and R. N. Muller, *Mol. Pharmaceutics*, 2009, **6**, 1903.
13. R. G. Hanshaw and B. D. Smith, *Bioorg. Med. Chem.*, 2005, **13**, 5035.

14. R. G. Hanshaw, C. Lakshmi, T. N. Lambert, J. R. Johnson and B. D. Smith, *ChemBioChem*, 2005, **6**, 2214.
15. K. M. DiVittorio, J. R. Johnson, E. Johansson, A. J. Reynolds, K. A. Jolliffe and B. D. Smith, *Org. Biomol. Chem.*, 2006, **4**, 1966.
16. W. M. Leevy, S. T. Gammon, H. Jiang, J. R. Johnson, D. J. Maxwell, E. N. Jackson, M. Marquez, D. Piwnica-Worms and B. D. Smith, *J. Am. Chem. Soc.*, 2006, **128**, 16476.
17. A. G. White, N. Fu, W. M. Leevy, J.-J. Lee, M. A. Blasco and B. D. Smith, *Bioconjugate Chem.*, 2010, **21**, 1297.
18. A. J. Surman, C. S. Bonnet, M. P. Lowe, G. D. Kenny, J. D. Bell, E. Toth and R. Vilar, *Chem. Eur. J.*, 2011, **17**, 223.
19. J. P. Cross, M. Lauz, P. D. Badger and S. Petoud, *J. Am. Chem. Soc.*, 2004, **126**, 16278.
20. C. S. Bonnet, L. Pellegatti, F. Buron, C. M. Shade, S. Villette, V. Kubicek, G. Guillaumet, F. Suzenet, S. Petoud and E. Toth, *Chem. Commun.*, 2010, **46**, 124.
21. J. R. Silvius and J. Gagne, *Biochemistry*, 1984, **23**, 3232.
22. H.-i. Jung, M. I. Kettunen, B. Davletov and K. M. Brindle, *Bioconjugate Chem.*, 2004, **15**, 983.
23. P. Yerram, G. Saab, P. R. Karuparthi, M. R. Hayden and R. Khanna, *Clin. J. Am. Soc. Nephrol.*, 2007, **2**, 258.
24. L. Frullano, J. Rohovec, S. Aime, T. Maschmeyer, M. I. Prata, J. J. Pedroso de Lima, C. F. G. C. Geraldles and J. A. Peters, *Chem. Eur. J.*, 2004, **10**, 5205.