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Definition of the uptake mechanism and sub-cellular localisation profile of emissive lanthanide complexes as cellular optical probes

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Experimental

Synthesis

The synthesis and characterisation of the Eu and Tb complexes of ligands **L**[1], **L**[2a, 2b] [22a], **L**[3] [21a], **L**[4],**L**[5],**L**[6] [21b] and **L**[7] [26] have been reported elsewhere.

Cellular studies

Three cell lines were selected for cellular studies: CHO (Chinese Hamster Ovary), NIH 3T3 (mouse skin fibroblast) and HeLa (human endothelial carcinoma cells). Cells were maintained in exponential growth as monolayers in F-12 (Ham) medium, DMEM (Dulbecco's Modified Eagle Medium) and RPMI 1640 medium respectively. For each cell line, the medium was supplemented with 10% foetal bovine serum (FBS) and 1% (v/v) penicillin and streptomycin. Cells were grown in plastic culture flasks, with no prior surface treatment. Cultures were incubated at 37 °C, 20% average humidity and 5% (v/v) CO_2. Cells were harvested by treatment with 0.25% (v/v) trypsin solution for 5 min at 37 ºC. Cell suspensions were pelleted by centrifugation at 1000 rpm for 3 min, and were re-suspended by repeated aspiration with a glass pipette.

In order to determine cell number, cells were detached from the flask by treatment with trypsin. Cells were then pelleted and re-suspended in 4 mL medium, and an aliquot of the cell suspension injected into a haemocytometer (Fisher). The number of cells in a grid of volume 100 nL was counted using a light microscope, and the values for four separate grids measured to give an average cell count.

Microscopy

Cells were seeded in 12-well plates on glass cover-slips and allowed to grow to 40% – 60% confluence, at 37 ºC in 5% CO_2. At this stage, the medium was replaced and cells were treated with drugs and complexes as appropriate. For NIH 3T3 and HeLa cells, DMEM lacking phenol red was used from this point onwards. Following incubation, the cover-slips were washed with phosphate-buffered saline (PBS; pH 7.5), mounted on slides and the edges sealed with colourless, quick-dry nail varnish to prevent drying out of the sample.

Epifluorescence images were taken on a Zeiss Axiovert 200M epifluorescence microscope with a digital camera. G365 filters (Zeiss) were employed for excitation of the complexes, 546±12 and 575-625 filters (Comar) used for emission of Tb and Eu respectively and the FITC emission and excitation filter sets were selected for visualisation of LysoTracker and MitoTracker Green. Images were processed, given false colour and overlaid using Zeiss Axiovision and ImageJ software.

Uptake studies

Cells were seeded in 6-well plates and allowed to grow to 80% – 100% confluence, at 37 ºC in 5% CO_2. At this stage, the medium was replaced and cells treated with drugs at the following concentrations: 50 mM sucrose, 50 μM chlorpromazine, 1 mg/mL filipin, 300 nM wortmannin, 3 mM amiloride, 50 ng/mL phorbol 12-myristate-13-acetate, 500 ng/mL 1,2-dipalmitoyl-*rac*-diacylglycerol, 2 μM monensin, 10 μM chloroquine and 0.01% poly-L-lysine (from a 10% solution). Complexes (50 μM) were added 30 min later and the cells incubated for a further 4 h. Following incubation, the medium was removed and the cells washed three times with PBS. 500 μL of lysis buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% protease inhibitor cocktail) was then added to each well, and the cells incubated at 5 °C for fifteen minutes. 3 x 25 μL aliquots of the supernatant were taken

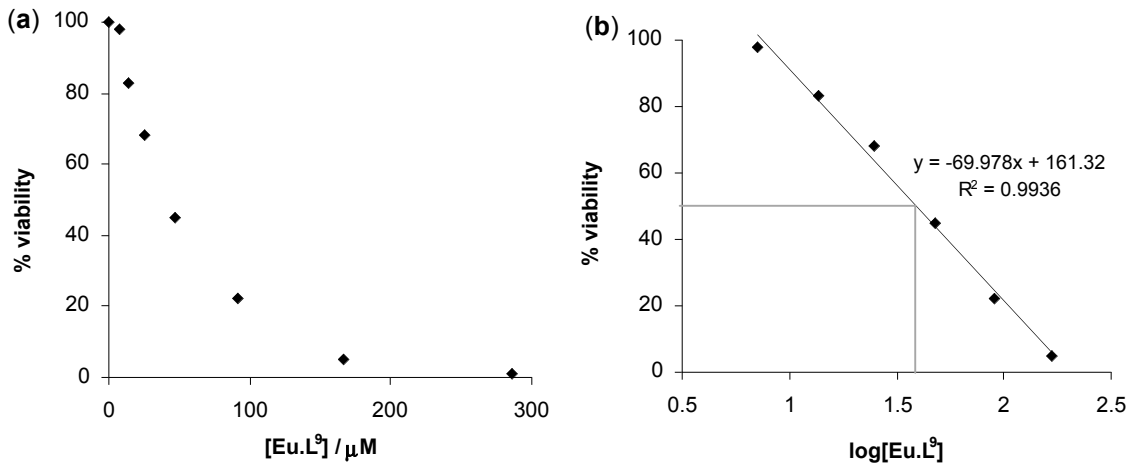
from each well for the BCA assay, as described below, and 400 μL was submitted for ICP-MS analysis.

Total protein content was determined in lysed cells using the bicinchoninic acid protein assay (BCA™ Protein Assay Kit). A BSA standard curve was constructed in the range 0.2 to 1 mg/mL. 25 μL of standards and samples were transferred into triplicate wells of a 96-well plate and 200 μL of BCA reagent mix was added to each well. Absorbance at 540 nm was measured after 10 h incubation at room temperature using a micro-plate reader. Protein concentration for each sample was determined against the standard curve.

Inductively coupled plasma mass spectrometry determinations of europium or terbium concentrations were made by Dr. C. Ottley in the Department of Earth Sciences at Durham University.

Cytotoxicity

Approximately 1 x 10^4 NIH-3T3 cells in 100 μL DMEM were seeded into each well of flat-bottomed 96-well plates and allowed to attach overnight. Complex solutions were added to triplicate wells to give final concentrations over a 2-log range. After 24 h incubation, IC_{50} values were measured using MTT or WST-1. For the MTT method, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT; 1.0 mM) was added to each well and the plates incubated for a further 4 h. The culture medium was removed, and DMSO (150 μL) was added. The plate was shaken for 20 sec and the absorbance measured immediately at 540 nm in a microplate reader against a blank plate containing DMSO. For the WST-1 assay, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1, 10 μL) was added to each well and the plates incubated for a further 30 min. The plate was shaken for 20 sec and the absorbance measured immediately at 450 nm in a micro-plate reader against a blank of DMEM containing 10 μL WST-1 per well. For both methods, IC_{50} values were determined as the drug concentration required to reduce the absorbance to 50% of that in the untreated, control wells, and represent the mean value for data from at least three independent experiments. Example data for [Eu.$\mathbf{L^5}$]Cl_3 are shown below.



Example data from IC_{50} determination for [Eu.**L**^{5}]Cl_3 by the MTT assay (NIH 3T3 cells, 24 h incubation), showing (a) variation of cell viability with concentration on a linear scale and (b) a logarithmic scale, allowing calculation of an IC_{50} value of 41.4 μM.

While the cytotoxicity of all complexes was assessed using the MTT assay, an alternative method was also investigated, utilising WST-1 ((4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulphonate; Roche) which acts by a similar mechanism to MTT. Initial investigations of the assay identified that a final 30 min incubation with the reagent was sufficient to give consistent results. The IC_{50} values obtained by this method for [Eu.**L**^{3}]Cl_3, [Tb.**L**^{4}]Cl_3 and [Eu.**L**^{7}]$(OTf)_3$ were >200, 40 and 59 μM respectively, which are comparable with the values obtained by the MTT assay (Table 1, main paper). The principal advantages of WST-1 over MTT lie in the water-soluble reduction product, which removes the need for a DMSO solubilisation step, and the relatively short incubation time required. WST-1 may therefore be regarded as the reagent of choice for future studies of this type, particularly those that monitor changes in toxicity over time.

Flow cytometric assays

Flow cytometric analysis and sorting was conducted using a Dakocytomation Inc. MoFlo multi-laser flow cytometer (Fort Collins, CO, USA) operating at 60 psi with a 70 μ nozzle. Samples were interrogated with a 100mW 488 nm solid-state laser. Fluorescence signals were detected through interference filters (FL1: 530/40; FL2 or FL4: 670/30 nm) and were collected in the logarithmic mode. Data were analysed using Summit v4.3 software (Dakocytomation). For flow cytometry, cells were grown

to confluence in 6-well plates. Medium was replaced and cells were treated with complex. Cells were detached from the plastic surface with 0.25% (v/v) trypsin solution for five minutes at 37 °C. The resulting cell suspension was pelleted by centrifugation. Immediately prior to flow cytometric analysis, cell suspensions were filtered through a 40 μm filter. For the mitochondrial membrane potential (MMP) assay, cells were incubated with 200 nM MTG and CMXRos for thirty minutes prior to harvesting. Following centrifugation, the cell pellet was re-suspended in 500 μL PBS. Relative mitochondrial membrane potential was calculated by measuring the mean intensity of FL1 (green) and FL4 (red) channels. Carbonyl cyanide *p*-trifluoromethoxylphenyl-hydrazone (FCCP; 20 and 100 μM, 3h) was used as a mitochondrial uncoupler. For the apoptosis-necrosis assay, the cell pellet was resuspended in 500 μL binding buffer (10 mM HEPES at pH 7.5, 0.14 M NaCl and 2.5 mM $CaCl_2$). This suspension was incubated with 0.5 μg/mL annexin-FITC and 2 μg/mL propidium iodide for ten minutes in the dark. Staurosporine (1 μM, 3h) was used for the apoptotic control and ethanol (70%, 5 ºC, 3h) for the necrotic control.

Measurements of Relative Lipophilicity

Lipophilicity was measured as the relative partitioning of the complex between water and 1-octanol. Water was saturated with 1-octanol, and 1-octanol was saturated with water. Complexes were prepared as 100 μM solutions in water and mixed with 1-octanol in 1:2, 1:1 and 2:1 water : octanol ratios. Mixtures were agitated for twelve hours, after which time emission spectra of the water and 1-octanol layers were collected. The complex concentration was calculated at the emission maximum with reference to calibration curves constructed for at least five concentrations between 0 and 100 μM for water and 0 and 40 μM for 1-octanol. For each mixture, the log *P* value was calculated according to the following equation:

$$\log P = \log_{10} \frac{[X]_{\text{1-octanol}}}{[X]_{\text{water}}}$$

Final log *P* values were calculated as the average of at least two replicates of the three solvent mixtures.