ESI

Two-photon microscopy study of the intracellular compartmentalisation of emissive terbium complexes and their oligo-arginine and oligo-guanidinium conjugates.

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1. **Figure S1: plot of absorbance versus emission intensity for selected complexes**
2. **Experimental section giving synthesis, characterisation, cell culture, toxicity and microscopy details**

1.

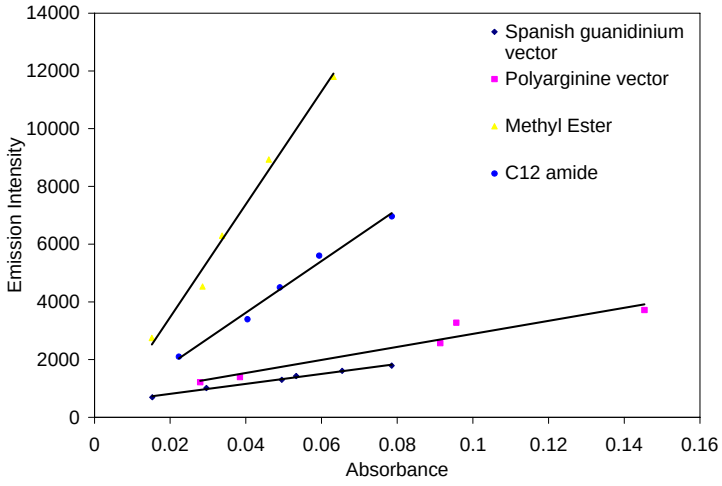

Figure 1: plot of absorbance versus emission intensity for the cited complexes showing the differing sensitivities of the conjugates to static quenching

2. Experimental

General methods

Thin-layer chromatography was carried out on neutral alumina plates (Merck Art 5550) or silica plates (Merck 5554) and visualised under UV (254 nm). Preparative column chromatography was carried out using neutral alumina (Merck Aluminium Oxice 90, activity II-III, 70-230 mesh), pre-soaked in ethyl acetate, or silica (Merck Silica Gel 60, 230 ± 400 mesh). 1H and 13C NMR spectra were recorded on a Varian Mercury 200 (1H at 199.975 MHz, 13C at 50.289 MHz), Varian Unity 300 (1H at 299.908 MHz, 13C at 75.412 MHz), Varian VXR 400 (1H at 399.968, 13C at 100.572 MHz), or a Bruker AMX 500 spectrometer. Chemical shifts are reported relative to TMS and were referenced using the residual protio solvent resonances. Chemical shifts are reported in ppm and coupling constants in Hz. Splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). Mass spectra with electrospray ionisation (ES) were recorded on a VG Platform II (Fisons instruments) or a Thermo Finnigan LTQ instrument operating in positive or negative ion mode, with methanol as the carrier solvent. Accurate masses (MALDI and ESI) were recorded on the Thermo Finnigan LTQ instrument. MALDI-TOF mass spectra were recorded using an Applied Biosystems Voyager DE STR instrument with ditranol as the matrix. Melting points were measured using a Reichart-Köfler block and are uncorrected.

UV/Vis absorbance spectra were recorded on a Perkin Elmer Lambda 900 UV/Vis/NIR spectrometer. Emission Spectra and Lifetimes were measured on a Perkin Elmer LS55 luminescence spectrometer and Instruments SA Fluorolog 3-11 spectrometer and DataMax v2.1 for Windows. Phosphorescence emission spectra were recorded at 77 K using an Oxford Instruments optical cryostat and LS 55B spectrometer, with EPA (diethyl ether, isopentane and ethanol, 5:5:2) as solvent.

The HPLC analysis and separation was carried out on a Perkin Elmer system comprising of Perkin Elmer Series 200 Pump, Perkin Elmer Series 200 Autosampler, Perkin Elmer Series 200 Diode array detector and Perkin Elmer Series 200 Fluorescence detector. GILSON-FC203B fraction collector was used in separation procedures. The stationary phase used was the Phenomenex Synergi 4µ Fusion-RP 80, and the columns used came in two different sizes; 150x4.6 mm (flow rate

1ml/min) and 250x10 mm (flow rate 5 ml/min). The gradients used are described in the Appendix.

Lifetime values were measured as described in references 7a and 10. Single photon epifluorescence images were obtained using a Zeiss Axiovert 200M epifluorescence microscope with a digital camera. Inductively coupled plasma mass spectrometry determinations of europium, gadolinium and terbium concentrations were made by Dr. C. Ottley in the Department of Earth Sciences at Durham University.

Cell culture , toxicity and flow cytometry

Three cell lines were selected for cellular studies, including CHO (Chinese Hamster Ovary) cells and NIH-3T3, mouse skin fibroblast (connective tissue) cells. The latter cells are transformed, and comprise adherent cells, which grow in a monolayer. These cell lines were cultured in a copper jacket incubator at 37°C, 20% average humidity and 5% (v/v) CO_2 in 75 cm3 plastic culture flasks. Cells for microscopy were grown on glass cover slips in 12-well plates DMEM (Dulbecco's Modified Eagle Medium), and F-12(Ham) medium were used for NIH3T3 and CHO cells respectively, each containing 10% (v/v) NCS (Newborn Calf Serum) and 1 % (v/v) penicillin-streptomycin. The HeLa cells were cultured in an RMPI 1640 medium supplemented with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin in 5% CO2. Complexes were loaded onto cells using the appropriate growth medium. Prior to analysis by flow cytometry, cells were detached from the glass surface with 1% (v/v) trypsin solution at 37°C for 5 min.

For the two-photon microscopy work, the cells were grown in 60 x 15 mm culture dishes at 2 ml/dish, and were allowed to attach overnight. The cell medium in each well was changed immediately prior to acquisition of the images. The cells were imaged in a tissue culture chamber (5% CO2, 37^{o}C) and through a Zeiss 510 LSM (upright configuration) confocal microscope with a femtosecond-pulsed Ti:Sapphire laser (Libra II, Coherent). The excitation beam produced by the fs laser, was tunable from 720-900 nm, (λ_{ex} = 720 nm, ~1mW), which was passed through an LSM 510 microscope with HFT 650 dichroic (Carl Zeiss, Inc.) and focused onto the cover-slipadherent cells using a 63 x oil immersion objective.

IC50 values were determined using the MTT assay, as described by Carmichael[14], which makes use of the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan product by the mitochondrial dehydrogenase of viable cells. This insoluble formazan was quantified spectrophotometrically upon dissolution in DMSO. 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. Following 24 h incubation, 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 plates were shaken for 20 seconds and the absorbance measured immediately at 540 nm in a microplate reader. 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 for data from at least three independent experiments.

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 μM nozzle. Samples were interrogated with a 100mW 488 nm solid state laser. Fluorescence signals were detected through interference filters (53040, 670/30 nm) and were collected in the logarithmic mode. Data were analysed using Summit v4.3 software (Dakocytomation).

Synthesis of L^1 and derived terbium complexes and conjugates

10-(7-Methoxycarbonyl-2-)-1,4,7-tris(tertbutoxycarbonyl)-1,4,7,10-tetraazacyclododecane

A solution of 2-bromomethyl-7-methoxycarbonyl-1-azaxanthone (140 mg, 0.402 mmol), 1,4,7-*tris*(*tert*butoxycarbonyl)-1,4,7,10-tetraazacyclododecane (190 mg, 0.402 mmol), K_2CO_3 (220 mg, 1.59 mmol) and a catalytic amount of KI in acetonitrile (15 ml) was heated to reflux overnight. The reaction mixture was filtered and salts were washed with CH_2Cl_2 (3x20 ml). The solvents were removed under reduce pressure and the residue was purified by chromatography on alumina (CH_2Cl_2 to 2% MeOH)

to give the product as a pale yellow solid (200 mg, 0.270 mmol, 67%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.40 (9H, s, CH_3), 1.47 (18H, s, CH_3), 2.81 (4H, br s, ring CH_2), 3.20-3.70 (12H, br m, ring CH_2), 3.97 (3H, s, CH_3), 4.00 (2H, s, CH_2), 7.57 (1H, d, *J* 8, H^3), 7.65 (1 H, d, *J* 8.5, H^9), 8.41 (1H, dd, *J* 8.5, 2, H^8), 8.61 (1H, d, *J* 8, H^4), 8.98 (1H, d, *J* 2, H^6). ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.7 (CH_3), 46.5-50.8 (C ring), 52.7 (OCH_3) 58.5-59 (CH_2AZA), 79.9 (C_q-*tBu*), 115.4 ($C^{4'}$), 119.1 (C^9), 121.6 ($C^{6'}$), 122.0 (C^3), 127.0 (C^7), 129.4 (C^6), 136.2 (C^8), 137.7 (C^4), 158.4 ($C^{9'}$), 159.8 ($C^{1'}$), 160.1 (C^2), 165.9 ($COOCH_3$, COO*t*Bu), 177.1 (C^5). m/z (ES^+) (MH^+) 740.2 (MH^+), 762.2 (MNa^+). HRMS (ES^+) 740.3860 ($C_{38}H_{54}N_5O_{10}$ requires: 740.3865) (MH^+).

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1-(7-Methoxycarbonyl-azaxanthonylmethyl)-1,4,7,10-tetraazacyclododecane

A solution of 1-(2-methyl-7-methoxycarbonyl-1-azaxanthonyl)-4,7,10-*tris*(*tert*butoxycarbonyl)-1,4,7,10-tetraazacyclododecane (200 mg, 0.270 mmol) in TFA (7 ml) and CH_2Cl_2 (5 ml) was stirred at room temperature under an argon atmosphere overnight. The solvents were removed under reduced pressure and the residue was dissolved in CH_2Cl_2 (50 ml) and the solvent was removed. This procedure was repeated three times. The residue was dissolved in aqueous KOH solution (1M) and the product was extracted into CH_2Cl_2 (3x20ml). The combined organic extracts were dried over K_2CO_3 and the solvent was removed to yield the product as a red glass (100 mg, 0.228 mmol, 85 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.59 (4H, m, ring CH_2), 2.71 (8H, s, ring CH_2), 2.82 (4H, m, ring CH_2), 3.89 (2H, s, CH_2), 3.95 (3H, s, OCH_3), 7.62 (1H, d, *J* 8.5, H^9), 7.69 (1 H, d, *J* 8, H^3), 8.38 (1H, dd, *J* 8.5, 2, H^8), 8.67 (1H, d, *J* 8, H^4), 8.95 (1H, d, *J* 2, H^6). ^{13}C NMR (75 MHz, $CDCl_3$): δ 45.4, 46.5, 47.4, 52.3 (C ring), 52.8 (OCH_3), 61.2 (CH_2AZA), 115.5 ($C^{4'}$), 119.1 (C^9), 121.6 ($C^{6'}$), 122.0 (C^3), 127.0 (C^7), 129.4 (C^6), 136.2 (C^8), 137.7 (C^4), 158.4 ($C^{9'}$), 159.8 ($C^{1'}$), 160.0 (C^2), 165.9 ($COOCH_3$), 177.1 (C^5). m/z (ES^+) 440.3 (MH^+). HRMS (ES^+) 440.2288 ($C_{23}H_{30}O_4N_5$ requires: 440.2292) (MH^+).

1-(2-Methyl-7-methoxycarbonyl-1-azaxanthone)-4,7,10-tris[(S)-1-(1-phenyl)ethylcarbamoylmethyl]-1,4,7,10-tetraazacyclododecane, L^1

A solution of 1-(2-methyl-7-methoxycarbonyl-1-azaxanthone)-1,4,7,10-tetraaacyclododecane (190 mg, 0.43 mmol), (*S*)-N-chloroacetyl-1-phenylethylamine

(285 mg, 1.44 mmol), K_2CO_3 (178 mg,) and KI in acetonitrile (10 ml) was heated to reflux overnight. The reaction mixture was filtered and the salts collected were washed with CH_2Cl_2. The solvents were removed under reduced pressure and the residue was purified by chromatography on alumina (CH_2Cl_2 to 2 % MeOH) to yield the product as a pale brown glass (300 mg, 0.32 mmol, 74%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (9 H, m, CH_3), 2.3-3.5 (22H, m, ring and arm CH_2), 3.79 (2H, s, CH_2), 3.95 (3H, s, OCH_3), 4.7-4.95 (3H, m, CH), 7.0-7.4 (15, m, Ph), 7.58 (1H, d, *J* 8.5, H^9), 7.65 (1 H, d, *J* 8, H^3), 8.36 (1H, dd, *J* 8.5, 2, H^8), 8.46 (1H, d, *J* 8, H^4), 8.93 (1H, d, *J* 2, H^6). m/z (ES^+) 923.4 (MH^+), 945.4 (MNa^+). HRMS (ES^+) 923.4818 ($C_{53}H_{63}O_7N_8$ requires: 923.4814) (MH^+).

[TbL^{1a}]Cl$_3$,

A solution of 1-(2-methyl-7-methoxycarbonyl-1-azaxanthone)-4,7,10-*tris*[(*S*)-1-(1-phenyl)ethylcarbamoylmethyl]-1,4,7,10-tetraazacyclododecane (100 mg, 0.108 mmol) and $Tb(OTf)_3$ (64 mg, 0.106 mmol) in acetonitrile (6ml) was stirred at 60 ^{o}C overnight. The solvent was removed under reduced pressure and the residue redissolved in the minimum volume of acetonitrile. The solution was dropped onto diethyl ether (15 ml) and the precipitate was collected by centrifugation. This procedure was repeated three times. The residue was dissolved in MeOH/H_2O mixture and the triflate counter-ions were exchanged for chloride using a strongly basic anion exchange resin. The solvent was removed to yield the product as an off-white solid (70 mg). m/z (MALDI-TOF^+) 1079.4 (M-$2H^+$). HPLC: t_R 8.0 min (Method A, Appendix). τ_{Tb} (H_2O) 1.53 ms.

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[EuL^{1a}]Cl$_3$

The europium complex was prepared in an analogous manner. The product was obtained as an off-white solid (190 mg). m/z (MALDI-TOF^+) 1073.3 (M-$2H^+$). HPLC: t_R 8.0 min (Method A, Appendix). τ_{Eu} (H_2O) 0.58 ms.

[GdL^{1a}]Cl$_3$

The gadolinium complex was prepared in an analogous manner. The product was obtained as an off white solid (90 mg). m/z (MALDI-TOF^+) 1078.5 (M-$2H^+$). HRMS (MALDI) 1078.3825 ($C_{53}H_{60}{}^{158}GdN_8O_7$ requires 1078.3833) (M-$2H^+$), HPLC: t_R 8.0 min (Method A, Appendix).

$[TbL^{1b}]Cl_3$,

A solution of $[TbL^{1a}]Cl_3$ (85 mg) in the mixture of H_2O (4 ml) and MeOH (4 ml) was stirred at room temperature for 24 h, maintaining the pH at 10 by the addition of aqueous KOH solution (1M). The reaction progress was monitored by HPLC (Method A, Appendix). The pH was adjusted to 7 with HCl solution (1M). The solution was syringe filtered and the solvent was removed. The residue was purified by reverse phase HPLC (Method B, Appendix). The solvent was removed to yield the product as a white powder (30 mg). m/z (MALDI-TOF$^+$) 1065.4 (M-2H$^+$). HPLC: t_R 7.7 min (Method A, Appendix). $\tau_{□b}$ (H_2O) 1.62 ms.

$[TbL^{1c}]Cl_3$

EDC (120 mg, 0.404 mmol) and N-hydroxysuccinimde (80 mg, 0.695 mmol) were added to the stirred solution of $[TbL^{1b}]$(150 mg, worked up reaction mixture without HPLC purification) in dry DMSO (10 ml). The reaction mixture was stirred overnight at room temperature. The reaction progress was monitored by HPLC (Method A, Appendix). The product was precipitated by dropping the DMSO solution onto dry diethyl ether (30 ml). The residue was triturated with acetonitrile and was re-dissolved in H_2O. The solvent was removed by freeze-drying to yield the product as a white solid (100 mg). m/z (ES$^+$) 639.5 (M+NHS-H^{2+}), 1277.1 (M+NHS-2H$^+$). HRMS (ES$^+$): found 639.210 (M+NHS-H^{2+}); $C_{60}H_{67}N_{10}O_{12}{}^{159}Tb$ requires 639.209, found 1277.415 (M+NHS-2H$^+$); $C_{60}H_{66}N_{10}O_{12}{}^{159}Tb$ requires 1277.411. HPLC: t_R 8.1 min (Method A, Appendix).

$[TbL^{5}]Cl_3$

Dodecylamine (3.7mg mg, 20□mol) was added to a stirred solution of $[TbL^{1c}]Cl_3$ (10 mg) in dry DMF (1ml**).**The reaction mixture was stirred at room temperature under an argon atmosphere overnight and the reaction progress was monitored by HPLC. The reaction mixture was dropped onto diethyl ether (2 ml) and the product was collected by centrifugation. The residue was re-dissolved in H_2O and the solvent was removed by freeze-drying to yield the product as a white solid. The product was obtained as a white solid (7 mg). m/z (MALDI-TOF$^+$) 1232.5 (M-2H$^+$). HRMS (ES$^+$) 1232.5725 ($C_{64}H_{83}N_9O_6Tb$ requires: 1232.5714) (M-2H$^+$); 1278.5800 ($C_{65}H_{85}N_9O_8Tb$ requires:

1278.5769) (M+HCOOH-2H$^+$); 639.7926 ($C_{65}H_{86}N_9O_8Tb$ requires: 639.7921) (M+HCOOH-H^{2+}). HPLC: t_R 10.3 min (Method A, Appendix). τ_{Tb} (H_2O) 1.71 ms.

[TbL^4]Cl

The tetraguanidinium vector (10 mg, 6.4 μmol) was added to a stirred solution of [TbL^{1c}]Cl_3 (10 mg) in dry DMF (1ml). The reaction mixture was stirred at room temperature under an argon atmosphere overnight and the reaction progress was monitored by HPLC. The reaction mixture was dropped onto diethyl ether (10 ml) and the product was collected by centrifugation. The residue was purified by HPLC (Method C, Appendix). The product was obtained as a white powder (3 mg). m/z (MALDI-TOF$^+$) 2134.7 (M-2H$^+$). HPLC: t_R 8.3 min (Method A, Appendix). τ_{Tb} (H_2O) 1.63 ms.

[TbL^3]

[TbL^{1c}] Cl_3(3 mg) was added to a stirred solution of Arg_7 (1 mg) in aqueous HEPES buffer (0.1 ml, 0.1 M, pH 7.4). The reaction mixture was stirred at room temperature for 24 h. The reaction progress was monitored by HPLC and further two additions of [TbL^{1c}] Cl_3 (3 mg) were made. The solvent was removed under reduced pressure and the residue was purified by HPLC, to give the product as a white solid (1 mg). m/z (MALDI-TOF$^+$) 2160.0 (M-2H$^+$). HPLC: t_R 6.1 min. τ_{Tb} (H_2O) 1.53 ms.

[TbL^2]

[TbL^{1c}] Cl_3 (5 mg) was added to the stirred solution of $LysArg_7$ (1 mg) in aqueous HEPES buffer (0.1 ml, 0.1 M, pH 7.4). The reaction mixture was stirred at room temperature for 24 h. The reaction progress was monitored by HPLC and further two additions of [TbL^{1c}] Cl_3 (5 mg) were made. The solvent was removed under reduced pressure and the residue was purified by HPLC to give the product as a white solid (1 mg). m/z (MALDI-TOF$^+$) 2286.2 (M-2H$^+$) HPLC: t_R 6.2 min. τ_{Tb} (H_2O) 1.53 ms.

[Tb.L^6]

[TbL^{1c}] Cl_3 (6.7 mg) was added to the stirred solution of human serum albumin (20 mg) in H_2O (3 ml). The reaction mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure and the residue was purified by

dialysis (Dialysis Tubing: BDH D102, bio-desalting dialysis tubing, cellulose, cut off 8000 g/mol) to give the product as a white solid (15 mg). τ_{H20} (1.63 ms).

Appendix 1: HPLC Conditions

Method A (Basic analytical gradient)

Solvent A: H_2O/0.1% HCOOH

Solvent B: ACN/0.1% HCOOH

Flow rate: 1 ml/min

Time (min)	Solvent A (%)	Solvent B (%)	Curvature
0	100	0	0
15	0	100	1
20	0	100	0
25	100	0	-3
27	100	0	0

Method B

Solvent A: H_2O/0.1% HCOOH

Solvent B: ACN/0.1% HCOOH

Flow rate: 5 ml/min

Time (min)	Solvent A (%)	Solvent B (%)	Curvature
0	100	0	0
11	50	100	1
13	0	100	-3
16	0	100	0
16	100	0	-3
22	100	0	0

Method C

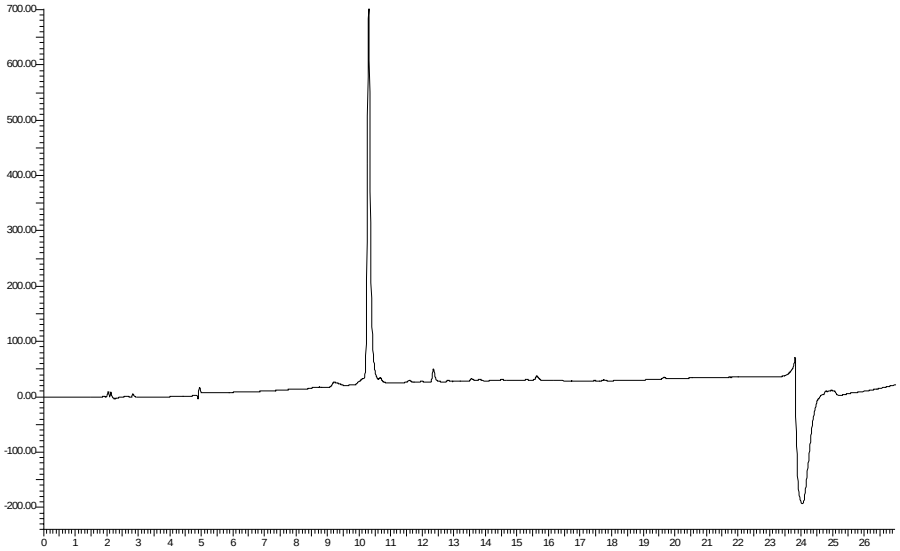
Solvent A: H_2O/0.1% HCOOH

Solvent B: ACN/0.1% HCOOH

Flow rate: 1 ml/min

Time (min)	Solvent A (%)	Solvent B (%)	Curvature
0	100	0	0
6	60	40	1
11	60	40	0
13	0	100	-3
15	0	100	0
18	100	0	-3
20	100	0	0

Chromatogram of dodecyl amide complex, [Tb.L^5]



Chromatogram of the oligoguanidinium conjugate, [Tb.L^4]