Electronic Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information for

Dual-functional probes towards *in vivo* studies of brain connectivity and plasticity

Ilgar Mamedov, Jörn Engelmann, Oxana Eschenko, Michael Beyerlein and Nikos K. Logothetis

Included:

General Information

All chemicals were purchased from commercial sources and were used without further purification. All solvents were dried and stored under nitrogen. A solution of Gadolinium (III) ions of known concentration, was prepared by dissolving an accurately weighed quantity of the chloride salt into an appropriate volume of doubly distilled water, standardized by a complexometric titration with the disodium salt of EDTA.[1]

^{1}H NMR, and ^{13}C{^{1}H} NMR spectra were recorded on a Bruker DRX300 spectrometer at room temperature. Mass spectra (ESI-LRMS in positive and negative ion mode) were performed on an ion trap SL 1100 system (Agilent, Germany). FT-ICR-MS were performed on a Bruker FT-ICR Apex II spectrometer. Diafiltration was carried out on a Pall Minimate^{TM} Tangential Flow Filtration System using a Pall Minimate TFF Capsule with an Omega 3K PES ultrafiltration membrane.

Synthetic procedures

5-(1,4-dibenzyl-6-nitro-1,4-diazepan-6-yl)pentanoic acid (2). 6-nitrocaprionic acid **1** (1.00 g, 6.2 mmol) was dissolved in a toluene/ethanol mixture (1/1, 25 mL). Dibenzylethylenediamine (1.50 g, 6.2 mmol) was added and the mixture was heated to 60°C. Paraformaldehyde **(**1.00 g, 33.0 mmol) was added in small portions over 30 min with the resulting suspension heated to reflux temperature for 18 h. After cooling to room temperature, the solvent was evaporated *in vacuo* and the residue purified using a short path silica gel chromatography column eluted with 20% ethyl acetate in dichloromethane. The fractions containing the pure product were collected and evaporated *in vacuo* affording **2** as colorless oil (2.1 g, 80% yield). ^{1}H NMR ($CDCl_3$): δ 0.61-0.72 (m, 2H, CH_2), 1.14-1.24 (m, 2H, CH_2), 1.43-1.49 (m, 2H, CH_2), 2.00-2.05 (m, 2H, CH_2), 2.50-2.58 (m, 4H, CH_2), 2.86 (d. J=14.2 Hz, 2H, CH_2), 3.39 – 3.50 (br. m, 4H, CH_2), 3.62 – 3.67 (m, 2H, CH_2), 7.20 (br. m, 10H, C_6H_5), 10.38(s, 1H, COOH).^{13}C {^{1}H} NMR ($CDCl_3$): δ 22.2, 24.3, 33.7, 36.4, 58.8, 61.5, 63.9, 94.7 (CH_2), 127.3, 128.3, 129.2, 138.9 (C_6H_5), 179.3 (*C*OOH). ESI-MS: for $C_{24}H_{32}N_3O_4$ calcd. 426.2387 $[M+H]^+$, found: 426.2399

5-(6-amino-1,4-diazepan-6-yl)pentanoic acid (**3)**. In a round-bottomed 500 mL flask compound **2** (2.00 g, 4.7 mmol) is dissolved in methanol (50 mL) under N_2 atmosphere; Pd/C 10% (0.5 g). Ammonium formate (5.8 g, 94.1 mmol) was introduced to the methanolic solution and the mixture was slowly brought to reflux temperature. After 3h the mixture was cooled, filtered on a Celite® pad and evaporated *in vacuo* to give **3** as yellow viscous oil (0.85 g, 82%). ^{1}H NMR (MeOD): δ 1.44 (br. m, 2H, CH_2), 1.62 (br. m, 4H, CH_2), 2.27 (m, 2H, CH_2), 2.92-3.34 (m, 8H, CH_2) ^{13}C {^{1}H} NMR (MeOD): δ 23.8, 28.1, 38.0, 38.8, 50.3, 57.1, 59.2 (CH_2), 182.5 (*C*OOH). ESI-MS: for $C_{10}H_{22}N_3O_2$ calcd. 216.1706 $[M+H]^+$, found: 216.1780

5-(6-(bis(2-(tert-butoxy)-2-oxoethyl)amino)-1,4-bis(2-(tert-butoxy)-2-oxoethyl)-1,4-diazepan-6-yl)pentanoic acid (4). In a round-bottomed 50 mL flask compound **3** (0.85 g, 3.95 mmol) is dissolved in acetonitrile (10 mL) under N_2 atmosphere. K_2CO_3 (2.7 g, 19.7 mmol), is added to the solution,

followed by slow addition of *t*-butyl bromoacetate (3.9 g, 19.7 mmol). Reaction mixture is stirred at 60°C overnight. The inorganic salts were removed by filtration and the solvent was evaporated *in vacuo*. The residue is charged on a silica gel column chromatography, eluted with DCM/ethyl acetate (8/2), obtaining pure **4** (1.80 g, 68%). ^{1}H NMR ($CDCl_3$): δ 1.15(br. m, 4H, CH_2), 1.29 – 1.30 (m, 36H, $C(CH_3)_3$), 2.47 – 2.63 (m, 8H, CH_2), 3.08 – 3.16 (m, 6H, CH_2), 3.48 – 3.60 (m, 6H, CH2), ^{13}C {^{1}H} NMR ($CDCl_3$): δ , 22.2, 25.3 (CH_2), 27.7, 27.8 ($C(CH_3)_3$),33.6, 44.1, 51.0, 53.2, 56.9, 61.5, 63.0 (CH_2), 80.4, 80.6 ($C(CH_3)_3$), 162.1 (COOt-Bu), 170.5 (COOH). ESI-MS: for $C_{34}H_{62}N_3O_{10}$ calcd. 672.44297 $[M+H]^+$, found: 672.4399.

2,2'-(6-(bis(carboxymethyl)amino)-6-(4-carboxybutyl)-1,4-diazepane-1,4-diyl)diacetic acid (L). 1g of **4** (2.2 mmol) was dissolved in formic acid (20 mL) and the mixture had been stirred at 60°C for 24 h. The solvent was removed under reduced pressure and viscous residue was taken up in a minimum amount of methanol and added drop wise to the cold diethyl ether. The resulting precipitate was isolated by filtration and re-suspended in 3 mL of water. A large excess of acetone (100mL) was added and the cloudy solutions were stored at -20°C for 16 h. Colorless crystalline powders were isolated by filtration, washed with acetone and dried in *vacuo*. ^{1}H NMR (D_2O): δ 1.18-1.30 (br. m, 2H, CH_2), 1.33-1.55 (br. M, 4H, CH_2), 2.23 (br. s, 2H, CH_2), 3.26-3.49 (br m, 4H, CH_2), 3.56-3.97 (br. M, 10H, CH_2), 4.05(br. s,4H, CH_2). ^{13}C NMR (D_2O): δ 22.0, 23.9, 24.4, 33.2, 52.1, 53.1, 58.7, 59.3, 63.5, (CH_2), 175.5, 178.3, 178.4(*C*OOH). FT-ICR-MS: for $C_{18}H_{28}N_3O_{10}$ calcd. 446.1178 $[M-H]^+$, found: 446.1187.

GdL. Gd^{3+} complex of **L** was formed by mixing the stock solution of $GdCl_3$ with the ligand in a molar ratio of 1:1. The solution was heated to 60°C for 12 h while the pH was maintained at ~7. The complex was finally treated with Chelex 100, filtered and lyophilized. The xylenol orange test was performed to ensure that there are no free metal ions in the solution. The formation of the complexes was confirmed by ESI-MS spectrometry in negative and positive modes. The appropriate molecular ion peak with the characteristic isotope pattern of the complex was present in the analyzed spectra. (LR)ESI-MS: for $C_{18}H_{25}GdN_3O_{10}$ calcd. 601.1 $[M-H]^-$, found: 601.0.

TR-Dextran-GdL. 0.03g of **GdL** (0.049 mmol, 10 equiv.), 0.01g EDC (0.059 mmol, 12 equiv.) and 0.008g HOBt (0.059 mmol, 12 equiv.) were dissolved in 1mL of DMF. After stirring for 10 min at 0°C, a solution of 0.005g TR-Dextran 10.000 ($5.0x10^{-4}$ mmol, 1 equiv.) in 1mL DMF was added to the reaction mixture followed by addition of 0.005g NMM (0.049mmol, 10 equiv.). The reaction was left stirring at room temperature overnight. The solvent was evaporated under reduced pressure and the crude product was purified by diafiltration using Omega 3K PES ultrafiltration membrane with cut off 3000 Da.

Relaxometric Experiments

The T_1 and T_2 of the **TR-Dextran- GdL** were measured on a Bruker DRX300 spectrometer at room temperature operating at 7T. The r_1 and r_2 relaxivities were calculated as the slope of the functions shown in following equation: $1/T_{1,2(obs)} = 1/T_{1,2(d)} + r_{1,2}x[Gd^{3+}]$, where $T_{1,2(obs)}$ is the measured $T_{1,2}$, $T_{1,2(d)}$ is the diamagnetic contribution of the solvent and $[Gd^{3+}]$ is the concentration of Gd^{3+} in mmol/L (Fig.S1).

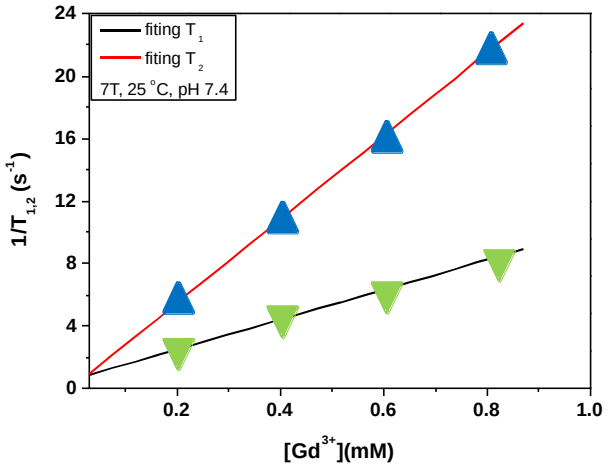

Fig.S1. Determination of r_1 and r_2 relativities at 7T. r^2 = 0.98 for T_2 and 0.97 for T_1

Cell Culture and Fluorescence microscopy

N18 mouse neuroblastoma cells (kind gift of Prof. Bernd Hamprecht, University of Tübingen, Germany) were cultured as a monolayer at 37°C with 5% CO_2 in antibiotic free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 4 mM L-glutamine, (all purchased from Biochrom AG, Germany). Cells were passaged by trypsinization with trypsin/EDTA 0.05/0.02% (w/v) in phosphate-buffered saline (PBS; Biochrom AG, Germany) for 5 minutes every second to third day.

To achieve more neuronal metabolic and morphologic features in the neuroblastoma cells, serum deprivation was used to differentiate the cells prior to the subsequent experiments. [2] In the course of the slow stepwise reduction of FBS content in the culture medium to 1.25% the growth rate slowed down and cells started to show a neuronal morphology with a network of neurite-like cellular processes which were completely absent at 10% FBS.

Differentiated N18 neuroblastoma cells were grown in 96-well microplates in Dulbecco's Modified Eagle's Medium supplemented with 1.25% FBS and 4 mM L-glutamine (all purchased from Biochrom AG, Germany). After reaching 70–80% confluence, cells were treated with different concentrations of a tracer **TR-Dextran-GdL** molecule in complete culture medium for 18 h under routine culture conditions. Subsequently, the supernatant was removed and the nuclear stain Hoechst 33342 (Hoe) in complete culture medium (100 µL/well) was added to cells in order to estimate their number per well (correlating with the DNA content). After 30 min of incubation, cells were repeatedly washed with HBSS. Cell-associated **TR-Dextran-GdL** (Ex 530 nm/ Em 590 nm) and the cell number based on Hoe fluorescence (Ex 346 nm/Em 460 nm) were measured in the multiplate reader (Fluostar Optima, BMG Labtech, Germany).

Microscopy on the same cells was performed without fixation using a Zeiss Axiovert 200 M microscope (Germany) with an LD Plan NeoFluor 40X objective. The imaging conditions were kept constant for the observation of different samples. Cellular localization and distribution of **TR-Dextran-GdL** was determined by irradiating at 545 nm and observing at 610 nm, and nuclear labeling by Hoechst was imaged at 460 nm. Additionally, cell morphology was observed by capturing

phase contrast images with differential interference contrast (DIC) microscopy of the same areas used for fluorescence detection. High resolution microscopy was done in single channel Ibidi slides (Ibidi GmbH, Germany) by inoculation of N18 cells (3 x 10^5 cells/mL). After reaching 70-80% confluency, cells were incubated with 50 µM of tracer molecule. After repeated cell washings with Hank's buffered saline solution (HBSS, Biochrom AG, Germany), fluorescence microscopy was performed on a Zeiss Axiovert 200M (Carl Zeiss AG, Germany) with a Plan-Apochromat 63×/1.4 oil objective. The structured light illumination device OptiGrid (Improvision/Perkin-Elmer, UK) was used to acquire fluorescence images of confocal quality. Image acquisition and processing were done with the Volocity Acquisition and Visualization software (Improvision/Perkin–Elmer, UK).

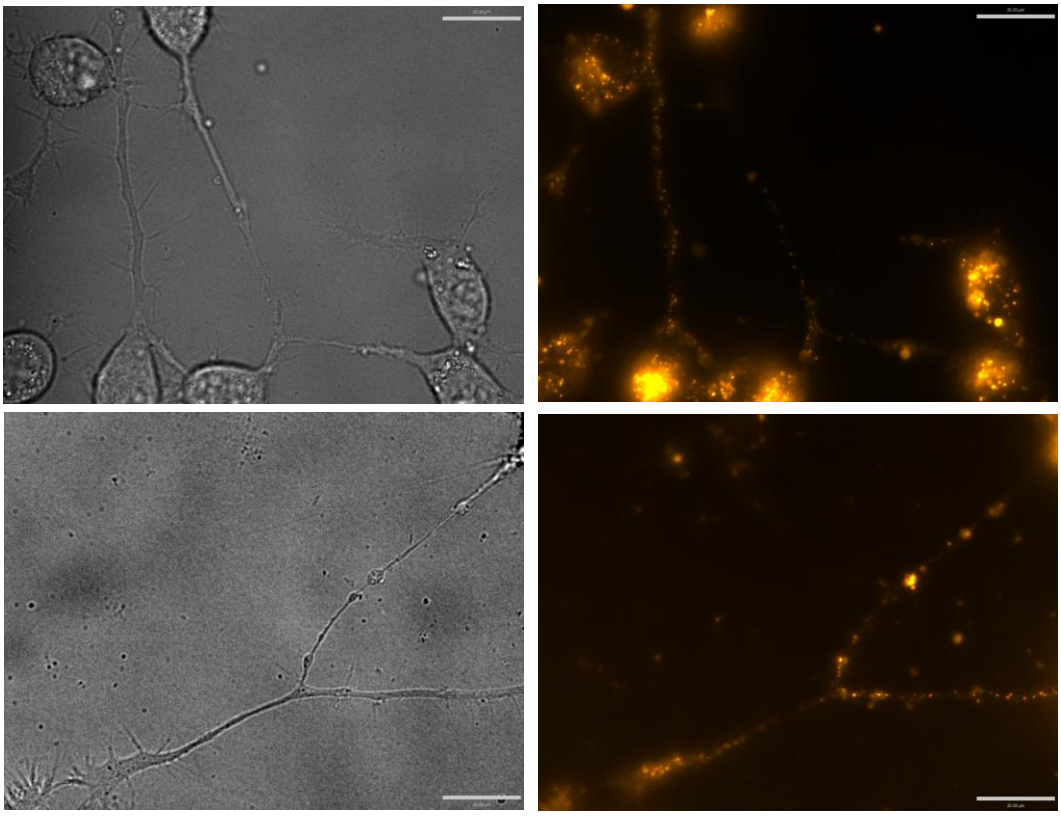

Fig.S2. Cellular uptake of **TR-Dextran-GdL** and localization after labelling of mouse N18 neuroblastoma cells in serum containing complete medium for 18 h at 37°C. Labelling of the cell bodies and processes (upper row) and terminals (bottom row). Fluorescence (right) and corresponding phase contrast (left) images are shown. Bars represent 20 µm.

MR Measurement in Cells

For MR imaging, serum deprived N18 cells (1.25% FBS) were cultured in 175 cm^2 tissue culture flasks and labeled with 25 or 50 µM (Gd concentration) of **TR-Dextran-Gd** in culture medium for

18h. The cells were repeatedly washed with HBSS, trypsinized, centrifuged and re-suspended in 0.5 mL Eppendorf tubes at a cell density of 1×10^7/500µL in complete medium. Cells were allowed to settle before MR measurements. Tubes with medium only and cells without CA were used as controls. MR imaging of the cell pellets at room temperature (~21 °C) was performed in a 3T (123 MHz) human MR scanner (MAGNETOM Tim Trio, Siemens Healthcare, Germany), using a 12-channel RF Head coil and slice selective measurements from a slice with a thickness of 1 mm positioned through the cell pellet. T_1 was measured using an inversion-recovery sequence, with an adiabatic inversion pulse followed by a turbo-spin-echo readout. Between 10 and 15 images were taken, with the time between inversion and readout varying from 23 ms to 3000 ms. With a repetition time of 10 s, 15 echoes were acquired per scan and averaged six times. Diffusion sensitivity was reduced by minimizing the crusher gradients surrounding the refocusing pulse. All experiments scanned 256^2 voxels in a field-of-view of 110 mm in both directions resulting in a voxel volume of $0.43 \times 0.43 \times 1$ mm^3. Data analysis was performed by fitting to relaxation curves with self-written routines under MATLAB 7.1 R14 (The Mathworks Inc., United States). The series of T_1 relaxation data were fitted to the following equations:

T_1 series with varying $t = TI$: $S = S_0 (1 - \exp(-t / T_1)) + S_{(TI = 0)} \exp(-t / T_1)$.

Nonlinear least-squares fitting of three parameters S_0, $S_{(TI = 0)}$, and T_1 was done for manually selected regions-of-interest with the Trust-Region Reflective Newton algorithm implemented in MATLAB. The quality of the fit was controlled by visual inspection and by calculating the mean errors and residuals.

From the determined T_1 values in the cell pellet corresponding apparent cellular relaxation rates $R_{1,cell}$ were calculated and given as % of control rates (Fig S3).

Evaluation of the signal intensities in the T_1-weighted MR images (Fig. S3) were performed in ImageJ (http://rsb.info.nih.gov/ij) by defining an ellipsoid region of interest (ROI) inside one tube image and measuring the mean signal intensity and standard deviation of the included voxels. Further statistical analyses were performed in GraphPad Prism 5.03 (GraphPad Software, Inc., USA).

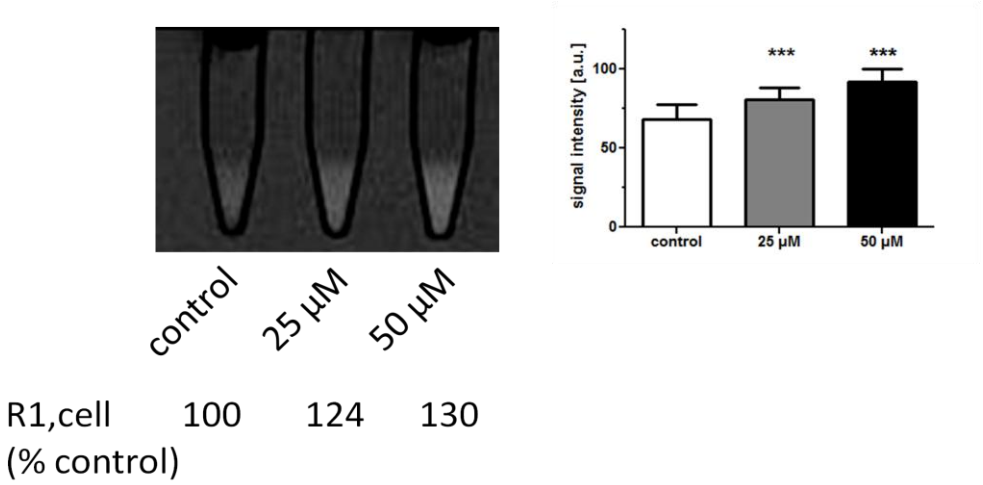

Fig.S3. T_1-weighted images and cellular relaxation rates of labeled cells at 3T (left) and evaluation of the corresponding signal intensities (right). Values represent mean±SD (n=159); ***, $p<0.001$ significantly different compared to the control.

Surgery and Anesthesia

Male Sprague-Dawley and Long-Evans rats (200-250 g) were used for *in vivo* studies. All experiments were approved by the local authorities (Regierungspraesidium) and were in full compliance with the guidelines of the European Community (EUVD 86/609/EEC) for the care and use of the laboratory animals. The animals were anesthetized with 2.0% isoflurane (Forene, Abbott, Wiesbaden, Germany) and placed in a stereotaxic frame (Kopf Instruments). The tracer was stereotaxically injected using a 0.5 µL Hamilton syringe at a rate of 0.5 nL/min under general anesthesia, using aseptic techniques. The needle was left in the brain for 15 min post injection and then retracted stepwise to avoid leakage of the contrast agent along the needle track. During the scan, the isoflurane anesthesia was reduced to 1.5-1.7%. Rats were immobilized on a nonmagnetic stereotaxic head holder. The rat body was placed on a heating pad to maintain a body temperature of 37 ^{o}C.

Each rat was imaged up to four times: 1, 96, 144, 216 and 336 h after tracer injection. Body temperature, heart rate, CO_2 and SpO_2 was monitored throughout the scanning session.

MRI experiment

The MRI (Magnetic Resonance Imaging) was done on a 7T (300 MHz) magnet with a 30-cm horizontal bore (Bruker BioSpec 70/30, Ettlingen , Germany) equipped with the 12cm inner diameter gradient insert (Bruker BGA-12S Ettlingen, Germany) . The MR system was controlled by a Bruker BioSpec console (ParaVision 5.1) running under the Linux operating system. The RF-Coil was a circular polarized rat head volume coil (Bruker, Ettlingen, Germany) with an inner diameter of 40cm. The coil was placed at a fixed position on the animal bed with the rat fixed stereotaxically. This ensured the same positioning of rat and coil within the magnet bore between separate measurements. We used a modified driven equilibrium Fourier transform (MDEFT) method with MDEFT preparation to obtain T_1-weighted anatomical images.[3] The scan parameters were: TR = 22ms, TE = 3ms, FA =20°, ID = 1000ms and four segments. The geometric parameters of the 3D scans were: matrix 240x170x126 and FOV 48x34x25.2mm resulting in a voxel size of 0.2x0.2x0.2mm. For each measurement 8 MDEFT images were acquired.

Literature

[1]Schwarzenbach, G. (1957), *Standardization of Lanthanides with EDTA in Complexometric Titrations*, Interscience: New York, USA, 77.
[2]Marchisio, P.C., Weber, K., and Osborn, M. (1979). Identification of multiple microtubule initiating sites in mouse neuroblastoma cells. Eur J Cell Biol 20, 45-50.
[3]Lee, J.-H., Garwood, M., Menon, R., Adriany, G., Andersen, P., Truwit, C.L., Ugurbil, K., (1995), High contrast and fast three-dimensional magnetic resonance imaging at high fields. Magn. Reson. Med. 34, 308–312.