

Dendrimer Brain Uptake and Targeted Therapy for Brain Injury in a Large Animal Model of Hypothermic Circulatory Arrest

Manoj K. Mishra,^{†,1} Claude A. Beaty,^{†,2} Wojciech G. Lesniak,¹ Siva P. Kambhampati,¹ Fan Zhang,¹ Mary A. Wilson,^{3,4,5} Mary E. Blue,^{3,4,5} Juan C. Troncoso,^{3,6} Sujatha Kannan,^{5,7} Michael V. Johnston,^{3,5,8} William A. Baumgartner,^{*,2} and Rangaramanujam M. Kannan^{*,1}

¹Center for Nanomedicine/Wilmer Eye Institute, Department of Ophthalmology, Johns Hopkins School of Medicine, Baltimore, MD 21287.

²Division of Cardiac Surgery, The Johns Hopkins Medical Institutions, Baltimore, MD 21287

³Department of Neurology, The Johns Hopkins Medical Institutions, Baltimore, MD21287

⁴Department of Neuroscience, The Johns Hopkins Medical Institutions, Baltimore, MD 21287

⁵Hugo W. Moser Research Institute at Kennedy Krieger, Inc., Baltimore, MD 21205

⁶Division of Neuropathology, The Johns Hopkins Medical Institutions, Baltimore, MD 21287

⁷Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins Medical Institutions, Baltimore, MD21287

⁸Department of Pediatrics, The Johns Hopkins Medical Institutions, Baltimore, MD 21287

[†] equal first authors

^{*} equal senior authors

Corresponding authors: Rangaramanujam M. Kannan, Center for Nanomedicine, Wilmer Eye Institute, Johns Hopkins Medicine, krangar1@jhmi.edu; William A. Baumgartner, Division of Cardiac Surgery, Johns Hopkins Medicine, wbaumgar@jhmi.edu.

Materials and Methods

Materials and reagents

Hydroxy functionalized ethylenediamine core generation four polyamidoamine (PAMAM) dendrimer (G4-OH; diagnostic grade; 64 hydroxyl end-groups) was purchased from Dendritech Inc. (Midland, MI, USA). N-Acetylcysteine (NAC), *N,N'*-Dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), piperidine, *N,N'*-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), 6-(Fmoc-amino)caproic acid, anhydrous dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-(Fmoc-amino)butyric acid (Fmoc-GABA-OH) and (Benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate (PyBOP) (Bachem Americas, Inc., Torrance, CA, USA), *N*-Succinimidyl-3-(2-pyridyldithio)- propionate (SPDP) (Toronto Chemicals Company, Toronto, CA), Valproic acid (VPA) (Acros Organics, Morris Plains, NJ, USA), Thiol-PEG3-alcohol (PEG-SH) (BroadPharm., San Diego, CA, USA) were purchased. Fluorescein isothiocyanate (FITC, Alfa Aesar) and Cy5-mono-NHS ester (Amersham Biosciences-GE Healthcare) were purchased. ACS grade DMF, DMSO, dichloromethane (DCM), diethylether, hexane, ethyl acetate, HPLC grade water, acetonitrile, and methanol were obtained from Fisher Scientific and used as received. Dialysis membrane (MW cut-off 1000 Da) was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA).

TSK gel ODS-80 Ts column (250 X 4.6 mm, i.d., 5 μm) and guard column were purchased from Tosoh Bioscience LLC. Size exclusion Ultrahydrogel 250 column (7.8 X 300 mm) and Ultrahydrogel DP guard column (6 X 40 mm) were purchased from Waters Corporation. Water used in all experiments was purified by a Branstead Nanopure Diamond Lab water purification system with resistivity 18.2 $\text{M}\Omega\text{ cm}^{-1}$.

Characterization of D-FITC and D-Cy5 conjugates

High Performance Liquid Chromatography (HPLC): FITC, Cy5, dendrimer, intermediate products, D-FITC, D-Cy5 conjugates and extracts from specimens were analyzed using a Waters 1525 binary HPLC separation module, equipped with a Waters In-Line degasser AF, a 717 plus auto sampler (kept at 4 °C), a Waters 2998 PDA detector, a Water 2475 multi wavelength fluorescence detector, controlled by Waters Empower software. Gradient separations were run using a TSK-Gel ODS-80 Ts column and a guard column with 0.1% v/v TFA in H₂O:ACN starting ratio 90:10 v/v increasing linearly to 10:90 v/v in 30 min at flow rate 1 mL/min. Total run time was 1 h. Triplicated injections for each analyzed sample were performed. Elution was simultaneously monitored by PDA detector (collecting UV-Vis spectra from 190-800 nm that allows for obtaining chromatograms at the desired wavelength in this range) as well as fluorescence detector, having 2 channels set for detection of FITC with excitation at 495 and emission at 520 nm as well as Cy5 with excitation at 645 nm and emission at 662 nm. To obtain calibration curves and determine the limits of detection (LOD) for D-FITC and D-Cy5, different amounts of the conjugates (ranging from 1 ng to 100 µg) were injected on the column and used as external standards for their quantification in dog organs, blood, cerebrospinal fluid, serum and urine.

Size Exclusion Chromatography (SEC): Analysis of dendrimer, D-FITC, D-Cy5 and specimen samples were performed using same HPLC system (describe above) with Waters 120 Ultrahydrogel 250 (7.8 x 300 mm) and Ultrahydrogel DP (6 x 40 mm) guard column. Phosphate buffer (0.1 M, pH 7.4) with 0.025% sodium azide was used as mobile phase (flow rate 0.6 mL/min). Different amount of D-FITC and D-Cy5 were injected on the column ranging from 1 ng to 100 µg and calibration curves were established for the lower limits of detection (LOD).

Fluorescence spectroscopy (FLS): Fluorescence spectra of FITC, Cy5, D-FITC and D-Cy5 conjugates were recorded in phosphate PBS (c = 0.1M, pH = 7.4), using a Shimadzu RF-5301 spectrofluorometer.

Quantification of D-FITC and D-Cy5 in the biological specimens

Biodistribution studies D-Cy5 and D-FITC conjugates were done based on fluorescence spectroscopy, HPLC and SEC. To obtain calibration curves and determine the limits of detection (LOD) for both conjugates, their emission spectra with excitation at 495 nm for D-FITC, and 645 nm for D-Cy5 were recorded at concentrations ranging from 1 ng/ mL to 100 µg/mL using appropriate sets of excitation and emission slit widths. High sensitivity for detection of both conjugates (in a mixture) reaching concentration of 0.1 ng/mL was observed. To test the efficiency of the extraction procedure, known amounts of D-FITC and D-Cy5 were injected into tissue samples (around 150 mg of brain, kidney, liver, heart, lungs, and pancreas) which were homogenized in PBS (c = 0.1 M), using 2 mL low DNA binding eppendorf tubes and TissueLyser LT, Giagen homogenizer. Obtained suspensions were extensively vortexed and sonicated. Then samples were centrifuged at 21130 g (15000) rpm for 10 min at 4 °C using an Eppendorf centrifuge 5424 R. Resulting supernatants were filtered using 0.2 µM filters and analyzed by FLS, HPLC and SEC. Around 95% recovery of both conjugates for all tested organ samples was observed. Recovery rates were tested using external calibration curves acquired for D-FITC and D-Cy5 at different concentration ranges depending on known amount of injected conjugate into the tissue samples. At all concentration ranges calibration curves exhibited excellent linearity with R=0.998. Each sample was analyzed in triplicate.

Preparation of tissue specimens for quantification

Approximately 100-150 mg of snap frozen samples and stored at $-80\text{ }^{\circ}\text{C}$ were placed in 2 mL DNA LoBind eppendorf tubes and thawed, which was followed by homogenization in 1 mL of PBS using TissueLyser LT, Giagen homogenizer. Resulting suspensions were extensively vortexed and sonicated. To keep the same amount of tissue in each sample, appropriate volumes of obtained suspensions, containing 100 mg of tissue were diluted with PBS up to 1 mL, thus the concentration of tissue was 100 mg/mL. Maintaining the same tissue concentration was crucial to subtracting the autofluorescence originating from the tissue acquired using samples from control non-treated dogs. The fluorescence values, after appropriate subtraction for autofluorescence subtraction were recorded for samples obtained from dogs treated with D-FITC and D-Cy5 conjugates. Then samples were centrifuged at 21130 g (15000) rpm for 10 min at $4\text{ }^{\circ}\text{C}$ using an Eppendorf centrifuge 5424 R. Resulting supernatants were filtered using $0.2\text{ }\mu\text{M}$ filters and analyzed by FLS, HPLC, and SEC. Accumulation of the conjugates tissue is expressed in microgram per gram of organ weight ($\mu\text{g/g}$). For the analysis of CSF urine and blood serum, $100\text{ }\mu\text{L}$ of specimen mixed with $900\text{ }\mu\text{L}$ of PBS ($c = 0.1\text{ M}$) and filtered using $0.2\text{ }\mu\text{M}$ filters. Concentration of the conjugates in CSF, urine and blood are expressed in microgram of the analyte per milliliter ($\mu\text{g/mL}$).

Characterization of the dendrimer conjugates (D-VPA and D-NAC)

Nuclear Magnetic Resonance (NMR) Spectroscopy: NMR spectra of final conjugates as well as intermediates were recorded on a Bruker spectrometer (500 MHz), using deuterated solvents. Proton chemical shifts are reported in ppm (δ) and tetramethylsilane (TMS) used for internal standard. Deuterated solvents of dimethylsulfoxide ($\text{DMSO-}d_6$) and chloroform (CDCl_3) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, Massachusetts).

High performance liquid Chromatography (HPLC): The dendrimer-valproic acid (D-VPA) and dendrimer-N-acetylcysteine (D-NAC) conjugates were analyzed by HPLC instrument (Waters Corporation, Milford, Massachusetts) equipped with binary pump, photodiode array (PDA) detector, and auto sampler interfaced with Empower software. The HPLC chromatograms were monitored at 210 (G4-OH) and 205 nm (D-NAC and NAC); and at 212 nm for D-VPA, VPA and PEG-VPA using PDA detector. The water/acetonitrile (0.1% w/w TFA) was freshly prepared, filtered, degassed, and used as mobile phase. Symmetry C₁₈ reverse phase column (Tosoh, Japan) with 5 µm particle size, 25 cm length, 4.6 mm internal diameter was used. A gradient flow was used with initial condition of 100:0 (H₂O/ACN) and gradually increasing the gradient condition of 90:10 (H₂O/CAN) till 10 min to 50:50 (H₂O/ACN) in 20 min and returning to 100:0 (H₂O/ACN) in 40 min with flow rate of 1 mL/min for D-NAC conjugate. For D-VPA, the gradient flow start with 90:10 to 10:90 (H₂O/ACN) for 30 min, and later back to 90:10 at 40 min and run up to 60 min with flow rate 1 mL/min.

Preparation of the intermediates and final conjugates

Preparation of dendrimer-Cy5 conjugate (D-Cy5)

The preparation of dendrimer-Cy5 conjugate is a three steps process and we have established the preparation of the bifunctional dendrimer previously which is the main intermediate [ref 54]. In the final step, Cy5-mono-NHS ester, a near IR dye was reacted with the bifunctional dendrimer in sodium bicarbonate buffer to get Cy5-labelled dendrimer (D-Cy5). The final conjugate was purified by dialysis followed by GPC fractionation. The resulting D-Cy5 conjugate was characterized by proton NMR, HPLC/GPC, MALDI TOF mass, and fluorescence spectroscopy. In NMR spectrum appearance of methylene protons peaks of

Cy5 (1.2-2.1 ppm) and aromatic protons (6.3-8.4) along with dendrimer and linker protons confirm the formation of the conjugate. The HPLC trace showed a peak at 16.23 min for D-Cy5 (monitored at 645 nm), different from that of the starting dendrimer and free Cy5, suggesting a pure and stable conjugate. The fluorescence spectrum showed peaks at 645 nm (for excitation) and 665 nm (for emission) which follow a similar pattern to Cy5 also suggesting the conjugation of Cy5 to the dendrimer and did not alter the characteristic nature of the dye. The resulting amide bond between dendrimer and Cy5 dye is stable and did not release any Cy5 in PBS over a week. Only one mole equivalent of Cy5 was used in the reaction and the percentage payload of the Cy5 to the dendrimer is ~ 4-5%.

*Preparation of bifunctional dendrimer [(OH)₄₂-D-(GABA-NH₂)₂₂, **2**]*

Fmoc-GABA-OH (7.25 gm, 22.30 mmol) was dissolved in DMF (200 mL) in a 1 liter two neck round bottom flask under nitrogen environment. PyBOP (13.90 gm, 26.71 mmol) dissolved in DMF (100 mL) and DIEA (3.68 mL) were added, and the reaction mixture was allowed to stir for 30 min under ice bath. Finally, PAMAM G4-OH (3.25 gm, 0.232 mmol) dissolved in anhydrous DMF (100 mL) was added to the reaction mixture at in 30 min. The reaction mixture was stirred for 48 h and the solvent was evaporated at 25 °C under vacuum. The crude product was redissolved in DMF (200 mL) and subjected to dialysis in DMF (dialysis membrane MW cut off = 1 kDa) for 36 h by changing the solvent at least three times. The obtained solvent was evaporated under reduced pressure at room temperature, and the final product was subjected to high vacuum overnight, which produced an off-white fluffy semi-solid Fmoc-functionalized dendrimer conjugate (4.5 gm). ¹H NMR (DMSO-*d*₆) δ 1.72 (m, CH₂ protons, linker), 2.27-3.40 (m, CH₂ protons of G4-OH and COCH₂ protons of linker), 4.00 (bs, CH₂OC=O protons, G4-OH), 4.18 (bs, CH proton of Fmoc group), 4.29 (bs, OCH₂

protons of Fmoc group), 7.30-8.10 (m, carbamate protons, aromatic protons of Fmoc and internal amide protons of G4-OH). The whole batch of Fmoc-functionalized dendrimer was dissolved in anhydrous DMF (100 mL) and piperidine/DMF (2:8; 100 mL) was added to it under nitrogen. The reaction mixture was stirred for 1 hr and the mixture of solvents was evaporated under vacuum at 28-30 °C. The crude product was co-evaporated using DMF (50 mL) under high vacuum and subjected to dialysis (membrane MWCO = 1000 Da) for 24 h. The solvent was evaporated and the product was triturated with diethylether to take out traces of DMF. Finally, the product was kept in high vacuum overnight to get bifunctional dendrimer (3.23 gm). The bifunctional dendrimer has hydroxy groups on the surface with some free reactive amine groups. ¹H NMR (DMSO-*d*₆) δ 1.63-1.74 (m, CH₂ protons, linker), 2.06-2.09 (t, CH₂ protons, linker), 2.21 (bs, CH₂ protons of G4-OH), 2.30 (m, COCH₂ protons of linker), 2.39-2.43 (m, CH₂ protons, G4-OH), 2.65 (bs, CH₂ protons, G4-OH), 3.11 (bs, CH₂ protons, G4-OH), 3.28 (bs, CH₂NH protons, linker), 3.40 (bs, CH₂ protons, G4-OH), 4.02 (s, CH₂OC=O protons, G4-OH), 7.86-8.13 (m, NHCO protons of G4-OH).

Synthesis of dendrimer-N-acetylcysteine conjugate (D-NAC, 5)

To a stirred solution of bifunctional dendrimer, **2** (3.23 gm, 0.202 mmol) in anhydrous DMF (180 mL), DIEA (1.95 mL) was added and the reaction mixture was cooled to 0 °C. SPDP (2.17 gm, 6.98 mmol) dissolved in DMF (50 mL) was added to the reaction mixture and allowed to stir for 8 h. Finally N-acetylcysteine (2.31 gm, 14.16 mmol) dissolved in DMSO (50 mL) was added, and the resulting mixture was stirred overnight at room temperature. All volatiles were evaporated at 25 °C under high vacuum and dialyzed in DMF using membrane (MW CO 1 kDa) for 28 h. The obtained DMF was evaporated at 25 °C under high vacuum and again dialyzed in water by dissolving in PBS buffer 7.4 (100 mL) for 8 h. Finally the water

was lyophilized to get dendrimer-NAC conjugate, D-NAC (3.54 gm). ^1H NMR ($\text{DMSO-}d_6$) δ 1.64-1.67 (m, CH_2 protons, linker), 1.85 (s, CH_3 protons, NAC), 2.08 (bs, CH_2 protons, linker), 2.23 (bs, CH_2 protons of G4-OH), 2.31 (m, COCH_2 protons of linker), 2.47 (s, CH_2 protons of G4-OH), 2.68 (bs, CH_2 protons, G4-OH), 2.84-2.92 (m, SCH_2 protons of NAC and CH_2 protons of SPDP) 3.11-3.12 (d, CH_2 protons, G4-OH), 3.28 (bs, CH_2NH protons, linker), 3.39-3.41 (t, CH_2 protons, G4-OH), 4.01 (s, $\text{CH}_2\text{OC=O}$ protons, G4-OH), 4.35-4.48 (m, CH protons, NAC), 7.85-8.09 (m, NHCO protons of G4-OH, amide protons of NAC).

Supplementary figures

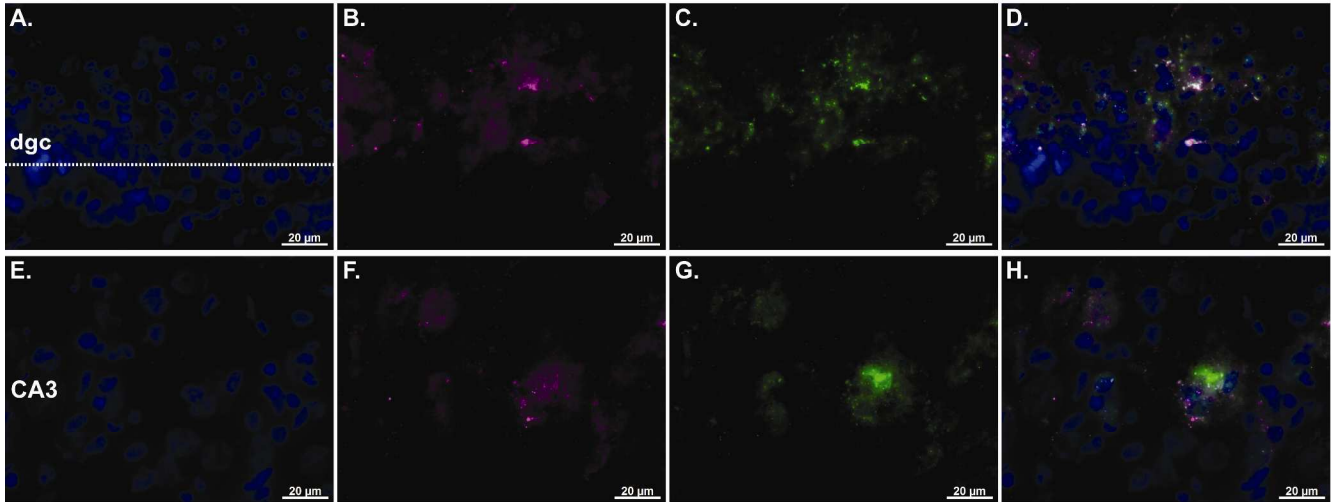


Fig. S1. Similar distribution of dendrimer injected intravenously or into the cisterna magna. 24h after 2hHCA, D-Cy5 was injected into the cisterna magna and D-FITC was injected IV. Dendrimer distribution was examined in the hippocampus 48h later. In the dentate gyrus (**A-D**), DAPI-stained nuclei in the outer two-thirds of the dentate granule cell layer (dgc, above the dashed line) have a fragmented morphology characteristic of apoptotic neurons (**A**). D-Cy5 administered ICM (**B**) and D-FITC administered IV (**C**) are comparably distributed in the injured part of this layer. In CA3 (**E-H**), clusters of necrotic neurons are typically observed after HCA, and D-Cy5 and D-FITC have a comparable distribution in scattered clusters of cells.

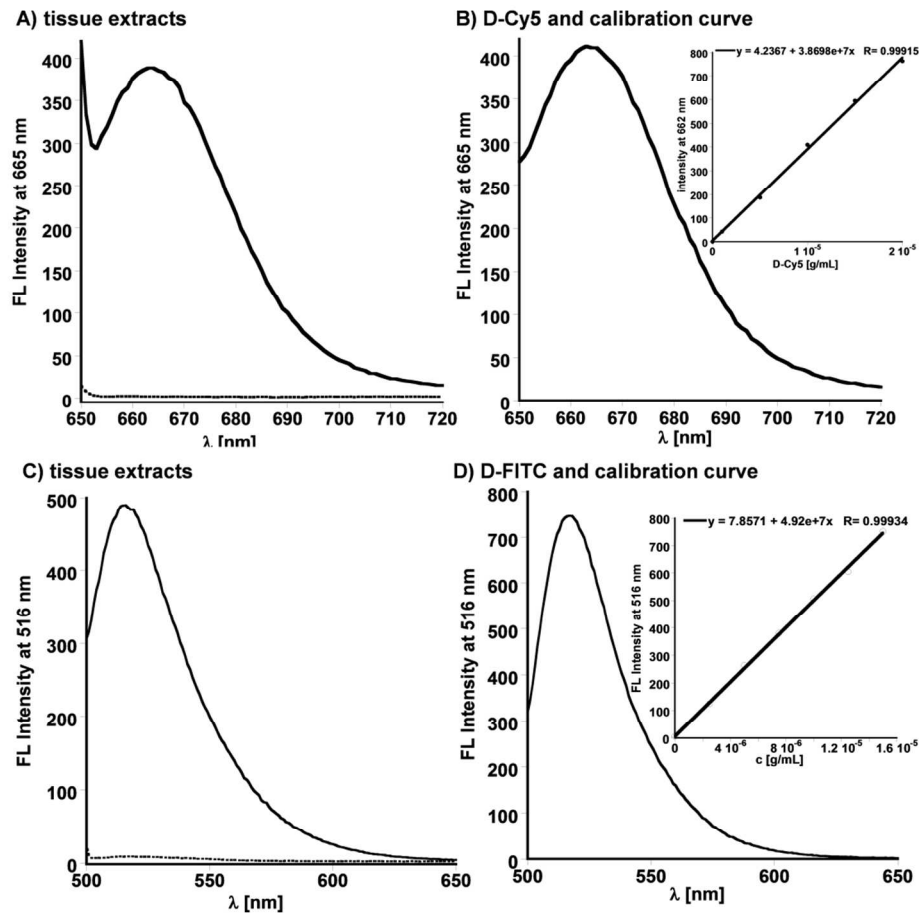


Fig. S2. A) Fluorescence emission spectra of extracts obtained from kidney of dog treated with D-Cy5 (solid line), and from non-treated control dog (dotted line). The dogs were treated 1hr IV infusion and euthanized 6 hrs post administration. An increase in fluorescence intensity indicated presence of fluorophore in the sample. **B)** The fluorescence spectrum of D-Cy5 conjugate (10 $\mu\text{g/mL}$) was used for the quantification of D-Cy5 in the kidneys (insert - calibration curve). **C)** Fluorescence emission spectra of extracts obtained from kidney of dog treated with D-FITC (high intensity spectrum, 48 hrs post administration) and from non-treated dog; **D)** The fluorescence spectrum of D-FITC conjugate (15 $\mu\text{g/mL}$) was used for the quantification of D-FITC (insert - calibration curve). All spectra were recorded in a Shimadzu RF-5301 spectrofluorometer using excitation at 645 nm (slit width 3) and emission ranging from 650 nm to 720 nm (slit width 5) in case of D-Cy5 and excitation at 495 nm and emission ranging from 500 to 650 nm in case of D-FITC quantification.

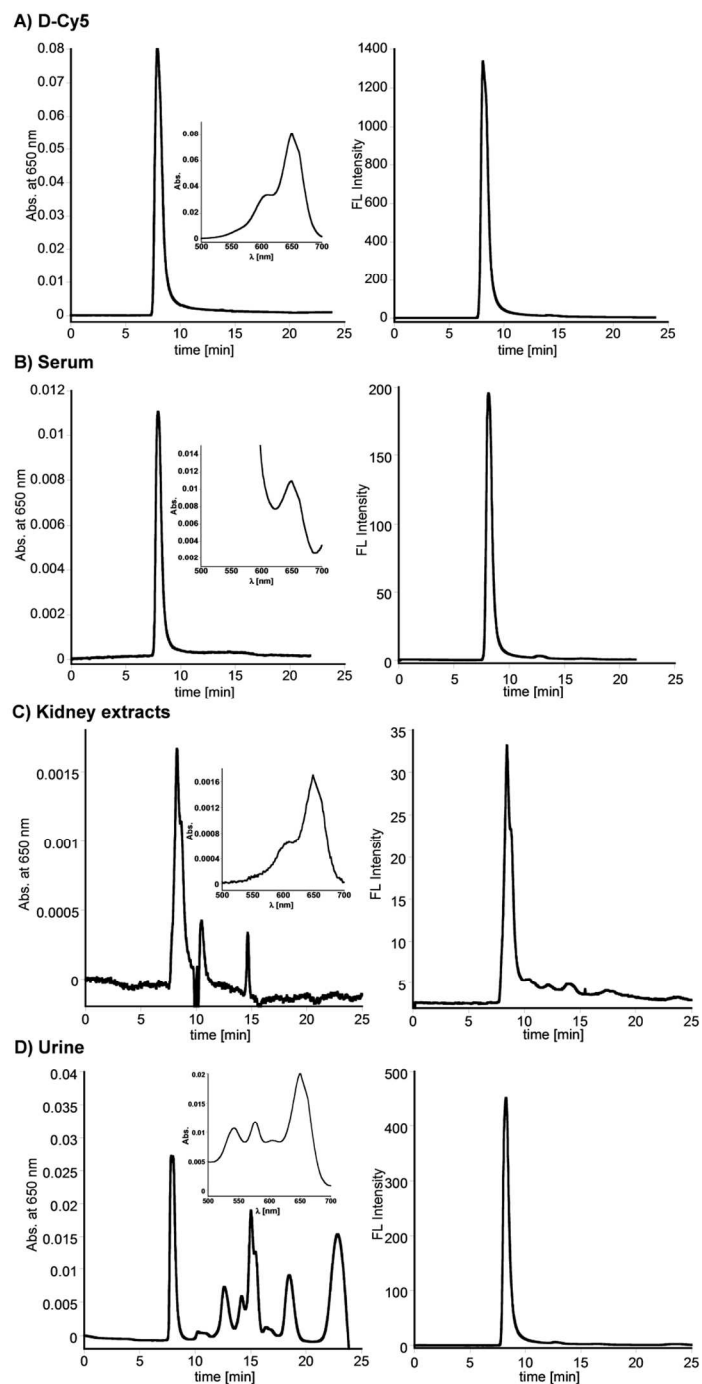


Fig. S3. Size exclusion chromatograms of D-Cy5 (absorbance at 650 nm), and inserts - UV-Vis spectra recorded under the peak of analyte (left panel) and fluorescence spectra with excitation: 645 nm; and emission: 665 nm (right panel) of: **A)** D-Cy5 conjugate, **(B)** serum, **(C)** kidney extracts, and **(D)** urine of the dogs treated with D-Cy5 and sacrificed 6 hr post infusion. The same retention time

and spectral pattern under the peak of D-Cy5 indicate the presence of the conjugate in each analyzed specimen. The results clearly demonstrate that there is no free Cy5 in the samples (retention time 16-18 mins) and the D-Cy5 conjugate is intact (covalent attachment of Cy5 to the dendrimer).

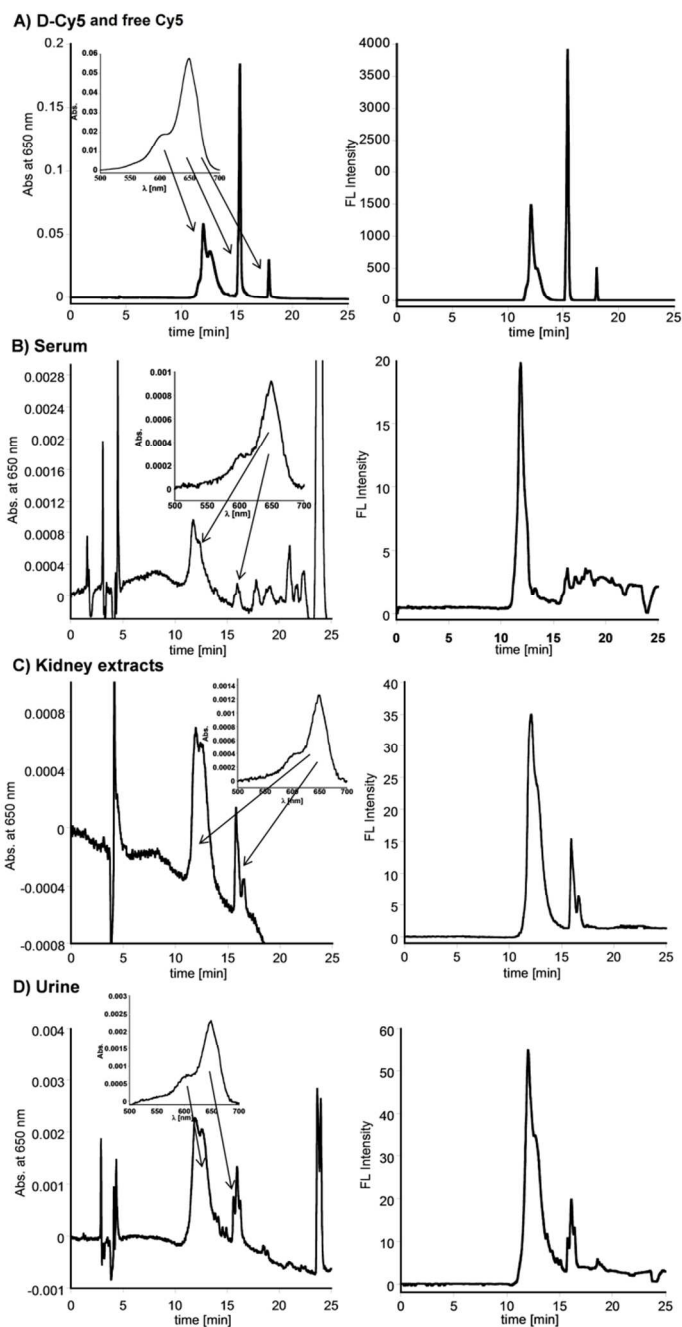


Fig. S4. HPLC chromatograms obtained for D-Cy5 and Cy5 based on absorbance at 650 nm, inserts UV-Vis spectra acquired under the peak of the analytes (left panel) and fluorescence spectra

(excitation: 645 and emission: 665 nm) (right panel) of: **(A)** physical mixture of D-Cy5 (broad peak between 10 and 15 min) and Cy5 (sharp peaks at 16 and 18 min; **(B)** serum, **(C)** kidney extracts and **(D)** urine of the dog treated with D-Cy5, which sacrificed 6 h post infusion. Mainly D-Cy5 was present in all samples and only trace amount of free Cy5 could be detected.

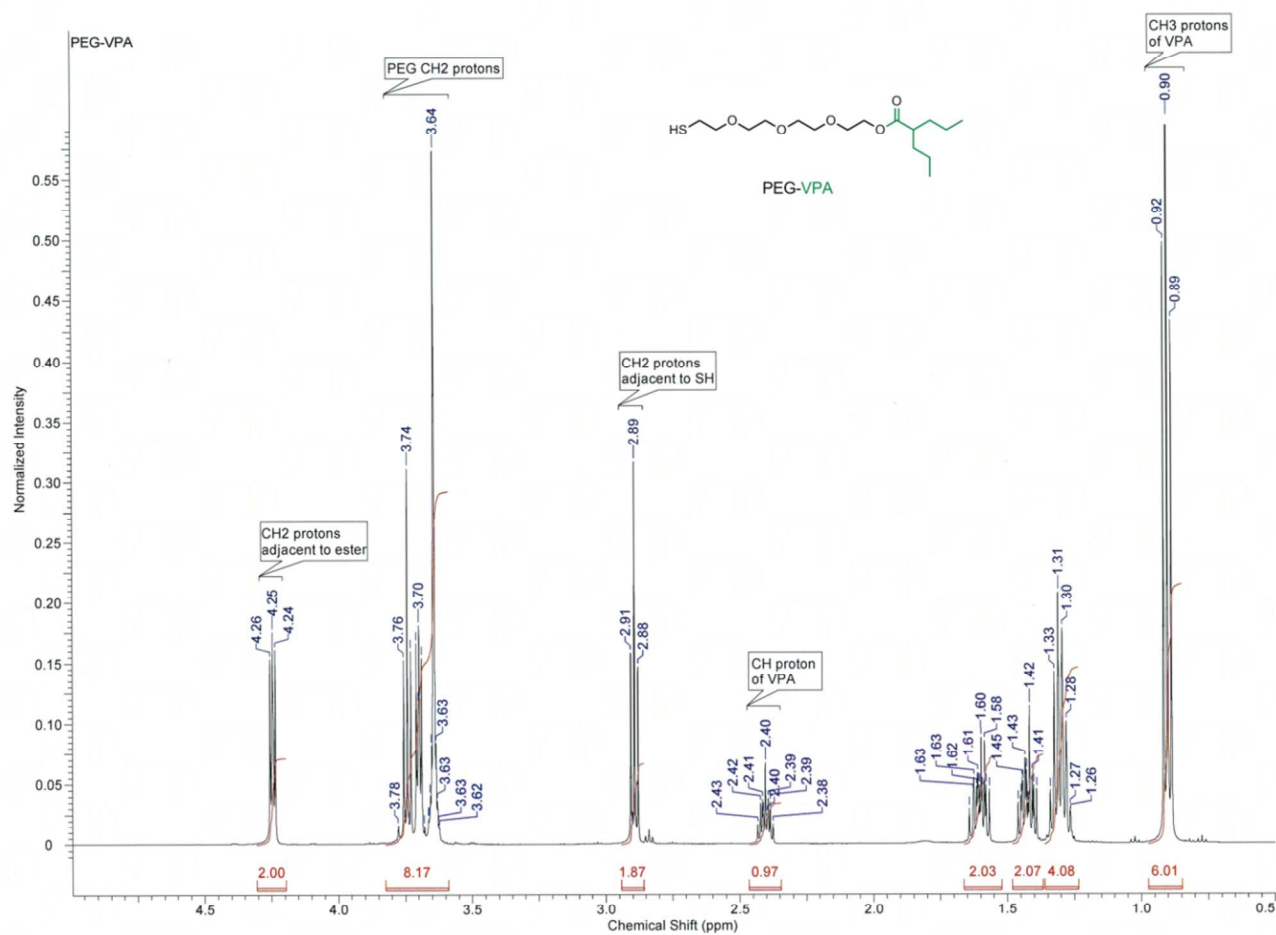


Fig. S5. Proton NMR spectrum of PEG-VPA in CDCl_3 . Presence of methyl protons of VPA and aliphatic protons of PEG in NMR chart confirms the formation of the product.

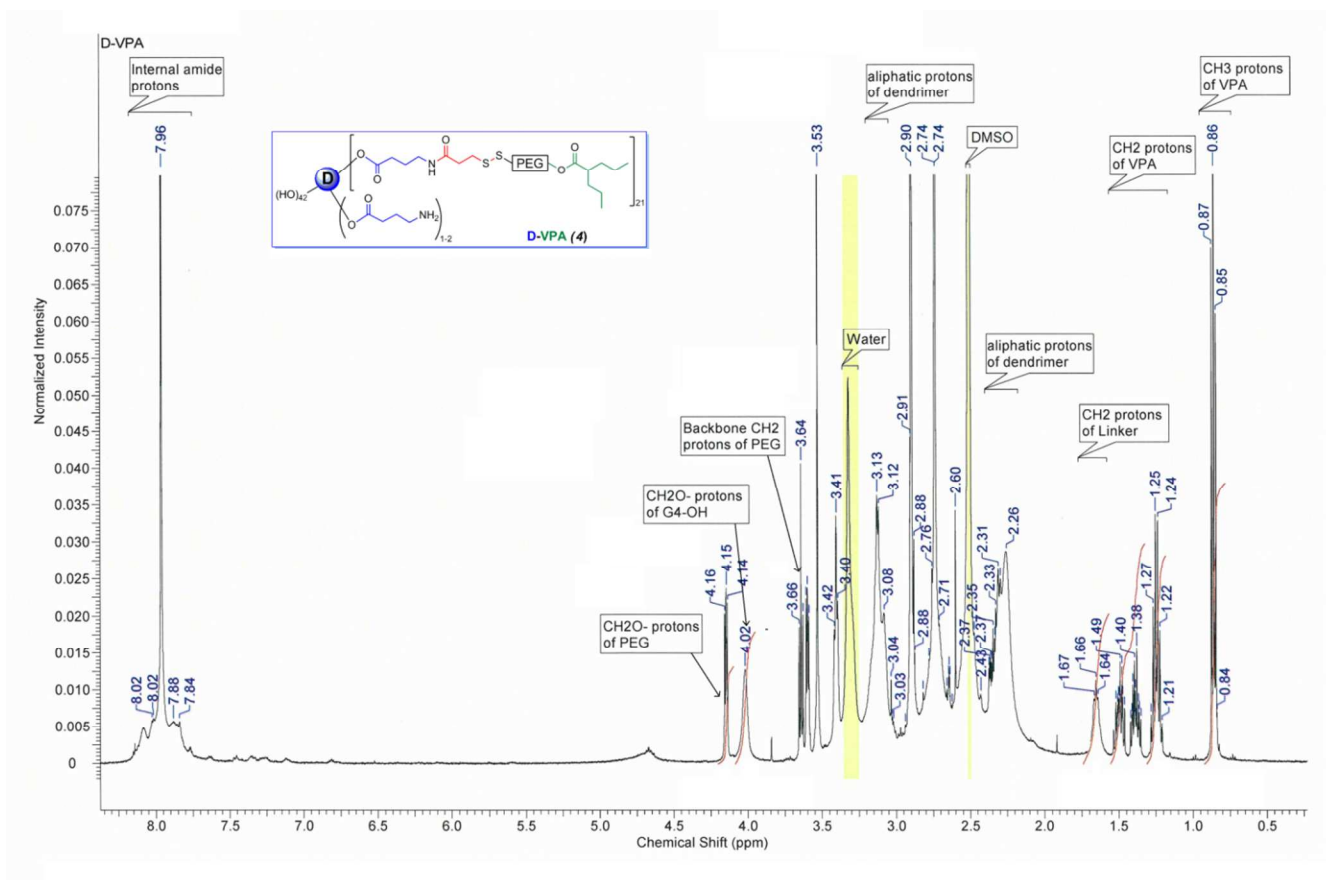


Fig. S6. Proton NMR spectrum of dendrimer-valproic acid (D-VPA, **4**) in DMSO- d_6 .

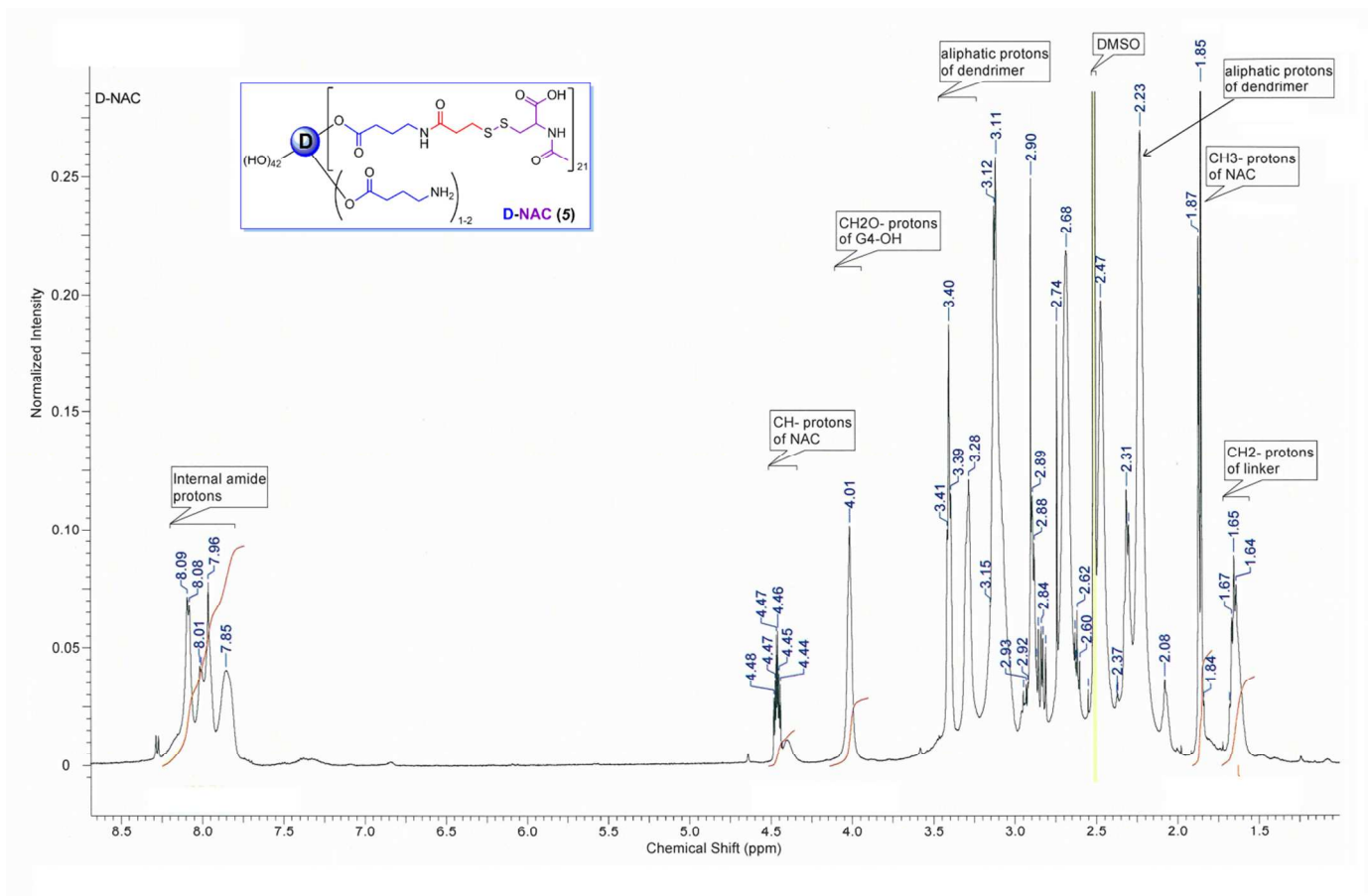


Fig. S7. Proton NMR spectrum of dendrimer-N-acetylcysteine (D-NAC, **5**) in DMSO-*d*₆.