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# Synthesis and characterization of a novel Gd-based contrast agent for magnetic resonance imaging of myelination

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#### **Abstract**

Myelin is a membrane system that fosters nervous impulse conduction in the vertebrate nervous system. Myelin sheath disruption is a common characteristic of several neurodegenerative diseases such as multiple sclerosis (MS) and various leukodystrophies. To date, the diagnosis of MS is obtained using a set of criteria in which MRI observations play a central role. However, because of the lack of specificity for myelin integrity, the use of MRI as the primary diagnostic tool has not yet been accepted. In order to improve MR specificity, we began developing MR probes targeted towards myelin. In this work we describe a new myelin-targeted MR contrast agent, Gd-DODAS, based on a stilbene binding moiety and demonstrate its ability to specifically bind to myelin in vitro and in vivo. We also present evidence that Gd-DODAS generates MR contrast in vivo in T<sub>1</sub>-weighed images and in T<sub>1</sub> maps that correlates to the myelin content.

## **Keywords**

myelin; magnetic resonance imaging; multiple sclerosis; gadolinium; contrast agents; neuroimaging

# INTRODUCTION

Myelination is one of the most fundamental biological processes in the vertebrate nervous system. The presence of myelin sheaths wrapped around axons provides the necessary electrical insulation for efficient nervous impulse conduction. Disruption of the compacted myelin sheath of axons is associated with a number of debilitating diseases such as multiple sclerosis (MS) and various leukodystrophies. MS is the most common demyelinating disease and it is characterized by demyelination in the central nervous system (CNS). MS affects an estimated 400,000 people in the US and 2 million people worldwide. The etiology of MS is complex and has both genetic and environmental factors contributing to the risk of disease. Risk factors include Epstein–Barr virus, vitamin D deficiency, and smoking. MS is an inflammatory demyelinating disease that is considered by many to have an autoimmune origin. In the early phase of the disease, periods of active demyelination are followed by periodic and deteriorating remyelination. This phase is known as relapsing-

Supporting Information. Fluorimetric characterization of compound 10 and synthesis and characterization of compound 11. This material is available free of charge via the Internet at http://pubs.acs.org.

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remitting phase (RRMS). The RRMS phase is followed by a phase of continuous disease progression known as secondary progressive MS (SPMS). In some cases, during the transition from RRMS to SPMS, the continuous clinical deterioration is marked by active MS attacks (relapsing progressive MS; RPMS). Finally, about 10-20% of MS sufferers do not experience RRMS but present a continuous disease progression from the onset (primary progressive MS; PPMS). In RRMS and in progressive MS, active tissue injury is associated with both demyelination and inflammation. The mechanism of myelin disruption has been extensively studied and a wealth of experimental data has been reported. Accordingly, various pathways underlying the process of demyelination have been proposed based on mitochondrial injury caused by oxidative stress or based on autoimmune response to autoantigens.

Over the past decade, magnetic resonance imaging (MRI) has played an important role in the diagnosis and disease management of MS. 12 Diagnosis of MS is normally guided by the revised McDonald criteria as recommended by the International Panel on the Diagnosis of Multiple Sclerosis. 13 In 2001, MRI was first incorporated into the criteria as an imaging tool to facilitate diagnosis and subtyping of MS. Since then, the Macdonald criteria have been frequently revised, but they always included MRI as an indispensable tool to simplify diagnosis and provide a more accurate identification of the disease. Furthermore, tremendous efforts are being made to promote myelin repair in the brain as a new therapeutic strategy to restore neurological functions in the treatment of MS. 14 Development of myelin repair therapies must be accompanied by an imaging tool that can specifically and longitudinally monitor myelination. While MRI is very effective in detecting brain lesions, conventional MR techniques do not provide specific information on myelin pathology in the course of disease progression. <sup>15,16</sup> For example, lesions detected by T<sub>2</sub>-weighed images are not directly related to demyelination or remyelination: they could also originate from an increased fraction of mobile water protons in regions where myelin is replaced with gliosis or from edema where myelin sheaths may remain intact. MS lesions can also be detected in T<sub>1</sub>-weighed images, but they are less sensitive than T<sub>2</sub>-weighed images and, likewise, do not correlate with myelin pathology of underlying tissues. Clinical contrast agents such as Gd-DTPA (DTPA = diethylenetriamine pentaacetic acid) are widely used for contrast-enhanced MR studies in MS patients. The use of these contrast agents is possible due to the fact that the blood-brain-barrier (BBB) is often disrupted in MS patients. Thus, Gd-DTPA enhancement increases the reliability and sensitivity of detecting active lesions. However, none of the clinical contrast agents exhibit any affinity and specificity for myelin; instead, lesion enhancement by these agents is mainly indicative of disruption of the BBB. No information can be extracted from these images regarding myelin integrity in detected lesions. Recently gadofluorine, a fluorinated T<sub>1</sub> MR agent, has been reported. Gadofluorine binds to extracellular matrix proteins with high affinity and was found to detect brain lesions with high sensitivity in MR images. 17,18 Disruption of the BBB or blood to nerve barrier (BNB) allows the extravasation of gadofluorine and binding to extracellular matrix protein provides lesion enhancement. However, gadofluorine enhancement does not reflect any changes in myelin integrity.

In order to improve the specificity of MR enhancement, we set out to develop myelin-specific contrast agents with optimal MR properties. Such myelin-targeting MR probes are crucial to establish the use of MRI for efficacy evaluation of novel myelin repair therapies currently under development. Over the past decade, our laboratory has developed a series of myelin-targeting molecular probes for multimodality imaging based on positron emission tomography (PET), MRI, and near infrared (NIR). 19-25 Structure-activity relationship (SAR) studies of a library of compounds suggest a common structural motif that is important for binding to myelin (Figure 1A). Based on this knowledge, we explored the incorporation of some of these probes into the design of gadolinium-based contrast agents for MR studies of

myelin pathology. We first developed Gadolinium(III) [4,10-Bis-carboxymethyl-7-({3-[3-(4-dimethylaminophenyl)-2-oxo-2H-chromen-7-yloxy]-propylcarbamoyl}methyl)-1,4,7,10-tetraazacyclododec-1-yl]-acetate), a myelin-targeting MR agent termed MIC (Figure 1), which is based on a coumarin binding moiety, and demonstrated that MIC binds to myelin sheaths in vitro and in vivo and could be used to characterize myelin distribution based on  $T_{1w}$  MR imaging in murine models.  $^{26,27}$  This demonstrated the validity of this strategy and that it is possible to integrate previously developed myelin-imaging agents into the design of MR contrast agents without negative impact on the myelin-binding properties. In order to increase the structural diversity of the pool of myelin targeted agents, we designed and synthesized a second type of myelin-targeted MR contrast agent bearing an aminostilbene moiety, Gd-DODAS (compound 10, Figure 1B). The synthesis of 10, along with biological evaluation of binding properties and MR relaxometric properties in vivo, are described in comparison with the previously developed MIC.

# **RESULTS**

## **Synthesis**

The myelin-targeting MR contrast agent 10 was prepared in seven steps from p-aminobenzyl-diethyl phosphonate 1. The amino group was initially protected with Boc and subsequently methylated with methyl iodide to generate intermediate 3 in 90% yield over two steps. Intermediate 5, carrying a terminal alkyne group, was prepared from Horner-Wadsworth-Emmons reaction of 3 and 4 in 83% yield. Copper(I)-catalyzed azide alkyne-cycloaddition between intermediate 5 and 3-azidopropylamine was used to prepare compound 6, which was subsequently coupled to tris-tBu protected DOTA to give the protected ligand 8 in 50% yield over two steps. Deprotection of the tBu groups in neat TFA and metallation with GdCl<sub>3</sub> afforded 10 in 50 % yield over the last two steps with a 19% overall yield.

## In vitro chemical staining

The absorption spectrum of compound **10** shows a single intense long wavelength band with a maximum of absorbance at 341 nm. The fluorescent spectrum of **10** in water is similar to other p-aminostilbenes with a fluorescence maximum at 443 nm and a Stokes shift of 6848 cm<sup>-1</sup>. <sup>28</sup> The emission spectrum does not show any vibrational structure.

Thanks to the fluorescent nature of 10, its myelin binding properties were first investigated by analyzing the fluorescent staining pattern on freshly frozen tissue sections of two-month-old Swiss-Webster R/J mouse brains. Axial sections of the whole mouse brain close to the bregma were used to examine the myelin-binding properties of 10 in both myelin-deficient gray and myelin rich white matter regions. Axial sections were used to examine staining in the cerebellum.

As shown in Figure 2, **10** preferentially stains myelin-rich white matter regions such as the corpus callosum (Figure 2A), the external capsule (Figure 2B), striatum (Figure 2B), the anterior commissure (Figure 2C) and the cerebellum (Figure 2D). Myelin distribution was co-validated by chemical staining with Black-Gold II, a commonly used myelin stain, on adjacent sections.<sup>29</sup> As displayed in Figure 2 E-H, chemical staining with Black-Gold II and with compound **10** showed an identical pattern, which suggests that compound **10** binds to myelin with high specificity.

Compound 10 was also able to detect demyelinated lesions in vitro in a rat model of focal demyelination. For this purpose, Sprague-Dawley rats were treated with lysolecithin (LPC), a neurotoxin that induces demyelination at the site of injection. As shown in Figure 3, the

LPC-induced lesions in the corpus callosum can be readily detected by fluorescent staining with 10 as characterized by decreased fluorescence intensity. The same demyelinated lesion was confirmed by Black-Gold II staining of myelin in an adjacent brain tissue section.

The binding specificity and sensitivity of **10** for myelin was then further evaluated using a hypermyelinated *Plp*-Akt-DD transgenic mouse model.<sup>32,33</sup> Chemical staining with compound **10** of hypermyelinated *Plp*-Akt-DD transgenic mice frozen brain sections reveals the hypertrophic corpus callosum characteristic of this model (Figure 4). The same staining pattern is also observed by Black-Gold II staining using adjacent brain tissue sections.

In order to prove that compound 10 is able to identify demyelinated lesions in an animal model of the MS disease, frozen brain sections of cuprizone mouse brains were chemically stained with compound 10. In this model of toxic demyelination, mice are fed with the copper chelator cuprizone, which induces oligodendrocyte cell death and consequently demyelination. Sections stained with compound 10 revealed large demyelinated lesions in the corpus callosum and the external capsule. The demyelinated nature of these lesions was confirmed by Black-Gold II staining (Figure 5).

We investigated the capability of compound 10 to bind myelin other than in the brain white matter. For this purpose spinal cord sections of wild type mice were chemically stained with compound 10. The staining pattern observed correctly reflects the distribution of white matter in the spinal cord as co-validated by Black-Gold II staining of adjacent sections (Figure 6 A, B). Furthermore, we demonstrated that compound 10, similarly to what was observed in the brain, is able to detect demyelinated lesions induced by LPC injection in the spinal cord (Figure 6 D, E).

## In situ chemical staining

In situ staining of compound 10 was studied following intraventricular infusion in wild-type Sprague-Dawley rats. The rats were euthanized and perfused with 4% paraformaldehyde to remove the blood. The brains were extracted and dissected to prepare frozen axial sections. Fluorescent microscopy shows that 10 selectively localizes in myelinated regions such as corpus callosum and striatum (Figure 7). Adjacent sections were then subjected to Black-Gold II staining for myelin. As shown in Figure 6, the pattern of compound 10 staining (blue) is consistent with the pattern of Black Gold II staining. These data suggest that 10 can selectively label myelin fibers in situ and potentially be used to monitor myelin changes in vivo.

# **Relaxometric Properties**

The relaxometric properties of **10** were measured at 0.47 T (40 °C), 1.41 T (40 °C), and 9.4 T (21 °C), three magnetic field strengths commonly used in clinical and preclinical studies. Compound **10** relaxivity is dependent upon concentration (Figure 8). At high concentrations a sharp increase in relaxivity is observed at the two lower magnetic fields. At 9.4T, the relaxivity at higher concentration is almost identical to the low concentration relaxivity. The high dilution relaxivities of **10** at the measured fields in comparison with previously reported MIC and other clinical contrast agents are reported in Table 1.

## In vivo MR imaging

Similar to other Gd-based contrast agents, 10 is a hydrophilic compound that is not permeable across the BBB. For this reason, we evaluated compound 10 brain biodistribution after intracerebroventricular infusion to bypass the BBB. Sprague-Dawley rats were anesthetized and 10 ( $10~\mu L$ , 70~nmol) was administered via stereotaxic injection to the lateral ventricles (LV). The animals were imaged twice using a spin-echo multiple TR

saturation recovery method 5 and 24 h post injection. As shown in Figure 9A, compound 10 induced a dramatic shortening of  $T_1$  localized in correspondence of the corpus callosum.  $T_1$  shortening was clearly visible at a dose of 70 nmol (ca. 3.2 mg/Kg) and persisted at least for 24 h post infusion (data not shown). In contrast, brain  $T_1$  maps of untreated rats show little contrast between highly myelinated white matter and less myelinated gray matter regions (Figure 9B).  $T_1$  maps of the rat brain infused with compound 11 (10  $\mu$ L, 70 nmol), acquired 5 h post infusion, also lacked contrast between the white matter and gray matter, suggesting that compound 11, which contains only part of the pharmacophore responsible for myelin binding, is not retained in highly myelinated regions of the brain and produces a uniform shortening of the longitudinal relaxation time throughout the brain (Figure 9C).

# DISCUSSION

The inability of conventional MR to probe myelination status in the CNS and PNS prompted us to prepare MR contrast agents that could selectively interact with myelin sheaths. The design of the proposed agent is based on the structure of myelin probes for PET imaging that were previously identified in our group. SAR studies on a library of compounds revealed a common structural motif that is involved in binding with myelin (Figure 1A). These previously identified myelin probes, while sharing a common structural motif, can be categorized into coumarin and stilbene derivatives. We have recently reported the preparation and the myelin binding and MR properties of MIC, a myelin targeted MR agent derived from coumarin. In order to establish if myelin-targeted probes based on a stilbene core are also amenable to be functionalized with an MR reporter we synthesized and characterized a novel myelin-targeting agent derived from an amino-stilbene moiety conjugated with a DOTA monoamide gadolinium (III) complex. Compound 10 was synthesized in seven steps with an overall yield of ca. 19%.

Gd(III) ions, thanks to their high magnetic moment and long electron relaxation time, are able to efficiently shorten the longitudinal or transversal relaxation rate ( $R_1$  and  $R_2$ ) of the solvent water protons. The effectiveness with which a paramagnetic agent is able to shorten the relaxation rate of solvent water protons is given by a parameter called relaxivity ( $r_i$  i=1,2) which represents the increase in relaxation rate of a water solution occurring after the addition of the agent, normalized to a concentration of 1 mM (Equation 1).

$$R_i = \frac{1}{T_i} = r_i \cdot [GD] + R_i^{\text{Solv}}$$
  $i = 1, 2$  (Equation 1)

Here  $T_i$  is the longitudinal or transversal relaxation time of the solution,  $r_i$  is the relaxivity and  $R_i^{solv}$  is the relaxation rate of the solvent. Relaxivity is not a constant but depends on a number of external parameters, such as the applied magnetic field strength and temperature, as well as molecular parameters including the number of coordinated water molecule(s) (q), the mean residence time  $(\tau_M)$  of the coordinated water molecule(s), and the reorientational correlation time  $(\tau_R)$ . The relaxivity of small Gd complexes such as compound 10 is strongly influenced by the reorientational correlation time especially at magnetic fields lower than 1.5T.

At 0.47 and 1.41 T (40 °C), the low concentration relaxivity of **10** is nearly identical to the relaxivity of MIC and about 1.5-1.7 times higher than that of commercial MR contrast agents such as GdDOTA or GdDTPA. Unlike MIC, compound **10** tends to aggregate in solution at concentrations above ca. 0.4 mM which is apparent from the increase in relaxivity observed at high concentration. The higher relaxivity observed under these conditions is a reflection of the increased rotational correlation time of the aggregated

complex. At 9.4T, the increase in relaxivity is less pronounced since the influence of the rotational correlation time upon the relaxivity becomes less important.<sup>36</sup> An increase in relaxivity can also be expected upon binding of compound **10** to myelin, provided that the metal center retains access to bulk water and that the bound water exchange rate is not significantly reduced.

Compound 10 is fluorescent and therefore it is possible to investigate its myelin-binding properties using fluorescent microscopy. For this purpose the ability of 10 to selectively bind myelin was investigated by chemical staining of wild type mouse brain sections. As shown in Figure 2, the fluorescence originating from 10 is more intense in highly myelinated white matter brain regions such as the corpus callosum, the external capsule, the caudate putamen, the anterior commissure, and in the cerebellar arbor vitae. The co-localization of compound 10 with the myelin distribution in the brain was confirmed by chemical staining with Black-Gold II, a commonly used myelin stain. The ability of 10 to detect and identify areas of decreased or increased myelination was tested by in vitro histochemistry of murine animal models of abnormal myelination. Chemical staining with compound 10 of brain sections of an LPC rat model of demyelination (Figure 3), demonstrates that compound 10 can be used to identify focal demyelinated lesions. Decreased myelination was also easily detected in the corpus callosum and external capsule of a cuprizone mouse model of demyelinating disease (Figure 5). Furthermore, histochemical staining of AKT mouse brain with compound 10 showed an increased fluorescence in highly myelinated white matter which correlates well to the enhanced myelination that is characteristic of this kind of animal model (Figure 4).

Myelin is a complex blend of proteins and lipids and its composition changes not only between the CNS and PNS, but also within the CNS. In order to test if compound 10 retains its binding ability to myelin fibers outside the brain, we stained axial sections of a wild type spinal cord with compound 10 (Figure 6). The staining pattern observed for compound 10 reflects the myelin distribution within the spinal cord. Moreover, compound 10 could detect LPC-induced demyelinated lesion in the spinal cord of a wild type mouse similarly to what was observed for brain lesions.

Once established that compound 10 is able to selectively bind myelin sheaths in vitro, its ability to bind myelin sheaths in vivo was tested by measuring its distribution in the brain of live animals by magnetic resonance imaging relaxometry. Compound 10 is a hydrophilic, highly water soluble gadolinium complex with a molecular weight (904 Da) that exceeds 500 Da, a value that is usually recognized as the upper limit for BBB permeability by passive diffusion. Thus, like other clinical contrast agents, compound 10 is not permeable across the BBB. It is important to note that in MS the BBB is normally disrupted in correspondence of new or reactivated lesions. Therefore, compound 10 can be expected to cross the compromised BBB similarly to GdDTPA. In the present study, we examined the distribution of 10 in the brain of wild type rats with an intact BBB. In order to bypass the BBB, compound 10 was administered by intracerebroventricular infusion. T<sub>1</sub> maps generated 5 h post-infusion show that compound 10 accumulates preferentially in the highly myelinated genus of the corpus callosum. The diffusion from the site of injection is slow and the contrast pattern in T<sub>1</sub> maps acquired 24 h post-injection remains unchanged (data not shown). The contrast pattern generated by compound 10 was compared with the pattern generated by compound 11. Compound 11 is characterized by the same coordination cage as compound 10 modified with a hydrophobic moiety. T<sub>1</sub> maps obtained 5h post-infusion show that compound 11 does not accumulate in highly myelinated region and produces a uniform decrease of the longitudinal relaxation time throughout the entire brain (Figure 9C).

## CONCLUSION

There is an urgent and unmet need to improve the sensitivity of MR techniques towards myelin changes in the nervous system. Such improvements will facilitate the early diagnosis of neurodegenerative diseases such as MS, provide a powerful tool to monitor response to current and new therapies, and assist in drug development.

A number of myelin-targeting probes for PET and optical imaging have been previously developed in our group. Optical imaging is an effective tool for preclinical drug development; however, it lacks deep tissue penetration, while PET, despite having a superb sensitivity, is characterized by a low resolution and lack of anatomical information. MRI, on the other hand, can provide deep tissue penetration, high resolution and an excellent soft tissue contrast. For this reason, we decided to modify our previously developed probes for MR imaging. We recently reported MIC, a Gd complex containing a coumarin binding moiety, as the first myelin-targeting MR contrast agent. In this work we describe the synthesis, relaxometric characterization and myelin binding properties of compound 10, the first MR myelin-targeting agent based on a stilbene binding moiety. Compound 10 binding to myelin was demonstrated both in vitro and in situ by fluorescent microscopy and in vivo by T<sub>1w</sub> MR imaging. The fact that both myelin-targeted coumarin and stilbene probes can be successfully modified and functionalized with MR reporter while retaining their targeting specificity, suggests that it is possible to create structurally diverse libraries of myelintargeted MR probes by modifying the lanthanide-chelating unit. Like all the other hydrophilic MR probes, BBB permeability of 10 remains a challenge. In this work we exploited intracerebroventricular infusion to bypass the BBB. A number of methodologies have been reported in the literature to shuttle hydrophilic compounds across the BBB, we are currently exploring the application of some of these techniques to deliver these agents. The utility of these probes however is not entirely dependent on their ability to cross the intact BBB. In neurodegenerative diseases such as MS, the BBB is transiently open in active lesions and this is already exploited clinically for GdDTPA-enhanced MR imaging. For this reason, we believe that the development of myelin-targeting MR probes can prove to be an extremely useful tool for neuroimaging.

#### **EXPERIMENTAL SECTION**

# **Materials and Methods**

All chemicals, unless otherwise stated, were purchased from commercial sources and used without further purification. L- $\alpha$ -Lysophosphatidylcholine from egg yolk was purchased from Sigma-Aldrich. All NMR spectra were acquired on an Inova 400 NMR system (Varian) equipped with a 5 mm broadband probe. Analytical HPLCs were performed on an Agilent 1100 Series system equipped with a dual channel UV/VIS detector using a Phenomenex 5 $\mu$  C18(2) 100A (250×4.56 mm, 5 $\mu$ m) column (4.6 × 250 mm); eluent A: H<sub>2</sub>O/0.1% TFA, B: MeOH/0.1% TFA, Elution: 10% B 3m, 10% B to 100% B in 15 min; flow rate 1 mL/min). The purity of tested compounds as determined by analytical HPLC were >95%. ESI-MS were acquired on a Finnigan LCQ Deca. pH was measured using a PHM210 Standard pH meter (Radiometer Analytical) connected to a symphony pH glass electrode (VWR). UV absorption was measured on a Cary 50 Bio spectrophotometer using a standard 1×1 cm quartz cuvette. Fluorescence was measured with a Cary Eclipse spectrophotofluorimeter using a standard 1×1 cm quartz cuvette.

*tert*-butyl (4-((diethoxyphosphoryl)methyl)phenyl)carbamate (2)—To a 100 mL round bottom flask fitted with a magnetic stir bar were added diethyl 4-aminobenzylphosphonate (1, 2.500 g, 10.28 mmol), di-*tert*-butyl dicarbonate (2.240 g, 10.27 mmol), THF (15 mL), and water (6.0 mL). The reaction was stirred at room temperature

overnight. THF was removed in vacuo and the resulting residue was diluted with water and extracted with EtOAC three times. The organic layers were combined and washed twice with water and once with brine. The organic layer was dried over  $MgSO_4$ , filtered, and concentrated in vacuum. The resulting white solid (3.393 g, 96%) was used without further purification.

 $^{1}\text{H-NMR (CDCl}_{3}, 400 \text{ MHz}) & \text{£ } 1.22 \text{ (6H, td, }^{3}\text{J}_{\text{H,H}} = 6.8 \text{ Hz, }^{4}\text{J}_{\text{H,P}} = 0.4 \text{ Hz, CH}_{2}\text{C}_{\text{H}_{3}}), 1.49 \\ \text{(9H, s, CC}_{\text{H}_{3}}), 3.07 \text{ (2H, d, }^{2}\text{J}_{\text{H,P}} = 21.2 \text{ Hz, PC}_{\text{H}_{2}}), 3.91-4.04 \text{ (4H, m, OC}_{\text{H}_{2}}), 6.87 \text{ (1H, br s, N}_{\text{H}}), 7.17 \text{ (2H, m, Ar}_{\text{H}}), 7.30 \text{ (2H, br d, J} = 8.4 \text{ Hz, Ar}_{\text{H}}); $^{13}\text{C-NMR (CDCl}_{3}, 100 \text{ MHz}) \\ & \text{£ } 16.3 \text{ (CH}_{2}\text{CH}_{3}), 28.8 \text{ (CCH}_{3}), 32.9 \text{ (d, }^{1}\text{J}_{\text{C,P}} = 138 \text{ Hz, PCH}_{2}), 62.0 \text{ (OCH}_{2}), 80.3 \text{ (CMe}_{3}), \\ & 118.4 \text{ ($C_{\text{aromatic}})}, 125.5 \text{ ($C_{\text{aromatic}})}, 130.1 \text{ ($C_{\text{aromatic}})}, 137.4 \text{ ($C_{\text{aromatic}})}, 152.8 \text{ ($C_{\text{aromatic}})}; \\ & \text{ESI-MS ($m/z$): measured } 366.03 \text{ [M+Na]}^{+}; \text{ expected } 366.14 \\ \end{aligned}$ 

tert-butyl (4-((diethoxyphosphoryl)methyl)phenyl)(methyl)carbamate (3)—To an oven dried 100 mL round bottom flask purged with argon and fitted with a magnetic stir bar was added sodium hydride (0.35 g, 8.74 mmol, 60% dispersion in mineral oil). The sodium hydride was washed three times with hexanes (6 mL) and the solvent was discarded. Compound 2 (2.0g, 5.82 mmol) was added under argon and the mixture was suspended in dry THF (18 mL). The reaction was cooled to 0 °C and methyl iodide (0.75 mL, 11.7 mmol, 2.28 g/mL) was added dropwise. The reaction was allowed to warm up to room temperature and stirred under argon overnight. The reaction was quenched with water and the THF was removed under vacuum. The residue was dissolved in EtOAc and water and the aqueous layer was extracted three times with EtOAc. The organic layers were combined and washed twice with water and once with brine. The organic layer was dried over MgSO<sub>4</sub>, filtered, and concentrated to give pure 3 as a yellow oil (1.96 g, 94%).

<sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz) δ:1.23 (6H, t, J = 7.2 Hz, CH<sub>2</sub>C $H_3$ ), 1.42 (9H, s, CC $H_3$ ), 3.11 (2H, d,  ${}^3J_{H,P}$  = 21.6 Hz, PC $H_2$ ), 3.22 (3H, s, NC $H_3$ ), 3.94-4.07 (4H, m, OC $H_2$ ), 7.16 (2H, br d, J = 8.0 Hz, ArH), 7.24 (2H, m, ArH); <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz) δ: 16.3 (CH<sub>2</sub>C $H_3$ ), 28.1 (CCH<sub>3</sub>), 33.0 (d,  ${}^2J_{C,P}$  = 138 Hz, PCH<sub>2</sub>), 37.1 (NCH<sub>3</sub>), 61.9 (OCH<sub>2</sub>), 80.1 (CMe<sub>3</sub>), 125.4 (Caromatic), 128.4 (Caromatic), 129.7 (Caromatic), 142.4 (Caromatic), 154.5 (Caromatic); ESI-MS (m/z): measured 380.06 [M+Na]<sup>+</sup>; expected 380.16

**(E)-tert-butyl methyl(4-(4-(prop-2-yn-1-yloxy)styryl)phenyl)carbamate (5)**—A mixture of compound **3** (3, 1.12 g, 3.14 mmol) and sodium hydride (0.25 g, 6.29 mmol) in dry DMF (5mL) was stirred under Ar for 1h. A solution of 4-(prop-2-yn-1-yloxy)benzaldehyde (4, 0.40 g, 2.51 mmol) in dry DMF (2 mL) was added and the mixture stirred for an additional 12 h. The reaction mixture was diluted with EtOAc (50 mL) and extracted with  $K_2CO_{3(aq)}$  10% (2X30 mL). The organic layer was dried over sodium sulfate, filtered and evaporated. The residue was purified by column chromatography over silica gel eluting with DCM. The fractions containing the product were joined and evaporated under reduced pressure to give 5 (0.76 g, 83%) as a white solid.

 $^{1}$ H-NMR (CDCl<sub>3</sub>, 400 MHz) &1.46 (9H, s, OC*H*<sub>3</sub>), 2.54 (1H, t, J=2.4 Hz, CC*H*), 3.27 (3H, s, NC*H*<sub>3</sub>), 4.70 (2H, d, J=2.4 Hz, OC*H*<sub>2</sub>), 6.93-7.05 (4H, m, C*H*<sub>alkene</sub> and Ar*H*), 7.22 (2H, d, J=8.4 Hz, Ar*H*), 7.42-7.47 (4H, m, Ar*H*).  $^{13}$ C-NMR (CDCl<sub>3</sub>, 100 MHz) & 28.29 (O*C*H<sub>3</sub>), 37.15 (N*C*H<sub>3</sub>), 55.76 (O*C*H<sub>2</sub>), 75.60 (*C*H <sub>alkyne</sub>), 78.38 (*C* <sub>alkyne</sub>), 80.32 (O*C*Me<sub>3</sub>), 115.03 (*C*H <sub>aromatic</sub>), 125.40 (*C*H <sub>aromatic</sub>), 126.29 (*C*H <sub>aromatic</sub> + *C* <sub>aromatic</sub>), 127.56 (*C*H <sub>aromatic</sub>), 127.70 (*C*H <sub>alkene</sub>), 130.90 (*C*H <sub>alkene</sub>), 134.47 (*C* <sub>aromatic</sub>), 142.77 (*C* <sub>aromatic</sub>), 154.61 (*C* <sub>aromatic</sub>), 157.06 (*C*O). ESI-MS (*m/z*): measured 364.33 [M+H]<sup>+</sup>; expected 364.19

(E)-tert-butyl (4-(4-((1-(3-aminopropyl)-1H-1,2,3-triazol-4-yl)methoxy)styryl)methylcarbamate (6)—A suspension of 5 (0.62 g, 1.71

mmol), 3-azidopropan-1-amine (0.26 g, 2.57 mmol), Hunig's base (0.55 g, 4.28 mmol) and copper(I) iodide (0.049 g, 0.26 mmol) in dichloromethane (25 mL) was stirred under argon. After 3h a fresh aliquot of 3-azidopropan-1-amine (0.21 g, 2.12 mmol) was added in a dichloromethane solution (2 mL). The reaction was stirred under Ar at room temperature for 12 h. The solvent was removed under reduced pressure and the residue was dissolved in ethyl acetate (25 mL) and extracted with NH<sub>4</sub>OH<sub>aq</sub> 0.1 M (2 × 25 mL). The aqueous extracts were combined and washed with dichloromethane (2 × 25 mL). The dichloromethane solutions were combined and extracted with NH<sub>4</sub>OH<sub>aq</sub> 0.1 M (4 × 50 mL) and brine (1 × 50 mL). The organic layer was dried over sodium sulfate and filtered. The solvent was removed under reduced pressure and the residue was purified by column chromatography over silica gel eluting with a gradient of methanol in dichloromethane from 0% to 5%. The fractions containing the product were combined and evaporated to give 6 (0.43 g, 54%) as a white solid.

<sup>1</sup>H NMR (400 MHz, CDCl3) &: 1.45 (9H, s, OC*H*<sub>3</sub>), 2.02 (2H, q, J=6.8 Hz, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.72 (2H, b, N*H*<sub>2</sub>), 3.25 (3H, s, NC*H*<sub>3</sub>), 4.47 (2H, t, J=6.9 Hz, C*H*<sub>2</sub>N), 5.21 (2H, s, OC*H*<sub>2</sub>), 6.90-7.05 (4H, m, Ar*H* and C*H*<sub>alkene</sub>), 7.20 (2H, m, Ar*H*), 7.42 (4H, m, Ar*H*), 7.63 (1H, s, NC*H*). <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz) &: 28.28 (O*C*H<sub>3</sub>), 33.30 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 37.15 (N*C*H<sub>3</sub>), 38.48 (H<sub>2</sub>N*C*H<sub>2</sub>), 47.73 (N*C*H<sub>2</sub>), 62.03 (O*C*H<sub>2</sub>), 80.32 (*C*Me<sub>3</sub>), 114.92 (*C*H <sub>aromatic</sub>), 122.77 (*C* <sub>aromatic</sub> triazol), 125.39 (*C*H <sub>aromatic</sub>), 126.11 (*C* <sub>aromatic</sub>), 126.26 (*C*H <sub>aromatic</sub>), 127.62 (*C*H <sub>aromatic</sub>), 127.71 (*C*H <sub>alkene</sub>), 130.55 (*C*H <sub>alkene</sub>), 134.48 (*C* <sub>aromatic</sub>), 142.74 (*C* <sub>aromatic</sub>), 143.97 (*C* <sub>aromatic</sub> triazol), 154.61 (*C* <sub>aromatic</sub>), 157.78 (*C*O). ESI-MS (*m*/*z*): measured 464.07 [M+H]<sup>+</sup>; expected 464.27

(E)-tri-tert-butyl 2,2',2"-(10-(2-((3-(4-((4-((4-((tert-butoxycarbonyl) (methyl)amino)styryl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)propyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (8)—A solution of 7 (0.48 g, 0.83 mmol), 6 (0.35 g, 0.76 mmol), HBTU (0.30 g, 0.79 mmol), HOBT (0.12 g, 0.79 mmol) and DIPEA (0.15 g, 1.13 mmol) in dimethylformamide (7 mL) was stirred at rt for 12 h. The solution was diluted with EtOAc (25 mL) and extracted with water (3 × 25 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel eluting with a gradient of methanol in dichloromethane from 0% to 5%. The fractions containing pure product 8 were evaporated. The fractions containing the product contaminated with aromatic byproducts were combined and evaporated. The residue was dissolved in dichloromethane (30 mL) and extracted with CH<sub>3</sub>COONa<sub>aq</sub> 0.1 M (6 × 50 mL) and brine (1 × 50 mL). The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced pressure to give compound 8 as an amber glassy solid. The combined yield of product 8 was 92 % (0.71 g).

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 1.28 1.51 (36H, m, b, CC*H*<sub>3</sub>), 1.67-3.64 (28H, b, C*H*<sub>2</sub> macrocycle, NC*H*<sub>2</sub>), 3.23 (3H, s, NC*H*<sub>3</sub>), 4.38 (2H, m, C*H*<sub>2</sub>N), 5.17 (2H, s, OC*H*<sub>2</sub>), 6.87-7.02 (4H, m, Ar*H* and C*H*<sub>alkene</sub>), 7.17 (2H, d, J= 8.4 Hz, Ar*H*), 7.40 (4H, d, J= 8.6 Hz, Ar*H*), 7.88 (1H, s, C*H* triazol); <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz) δ: 27.72 (OC(*C*H<sub>3</sub>)<sub>3</sub>, b), 28.18 (OC(*C*H<sub>3</sub>)<sub>3</sub>), 29.89 (CH<sub>2</sub>*C*H<sub>2</sub>CH<sub>2</sub>), 36.02 (H<sub>2</sub>N*C*H<sub>2</sub>), 37.06 (N*C*H<sub>3</sub>), 47.61 (N*C*H<sub>2</sub>), 47.1-53.5 (NCH<sub>2</sub> ring, broad), 55.2-55.7 (NCH<sub>2</sub> acetic, broad), 61.42 (O*C*H<sub>2</sub>), 80.20 (*C*Me<sub>3</sub>), 81.5-82.05 (macrocycle *C*Me<sub>3</sub>, broad), 114.90 (*C*H <sub>aromatic</sub>), 123.91 (*C*H <sub>aromatic</sub>), 125.27 (*C*H <sub>aromatic</sub>), 125.74 (*C* <sub>aromatic</sub>), 126.14 (*C*H <sub>aromatic</sub>), 127.49 (*C*H <sub>aromatic</sub>), 127.76 (*C*H <sub>alkene</sub>), 130.16 (*C*H <sub>alkene</sub>), 134.45 (*C* <sub>aromatic</sub>), 142.55 (*C* <sub>aromatic</sub>), 143.14 (*C* <sub>aromatic</sub> triazol), 154.53 (*C* <sub>aromatic</sub>), 157.87 (N*C*O), 171.9-173.9 (*C*O <sub>macrocycle</sub>).

(E)-2,2',2"-(10-(2-((3-(4-((4-((4-(methylamino)styryl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)propyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-

**triyl)triacetic acid (9)**—A solution of **8** (0.16 g, 0.16 mmol) in trifluoroacetic acid (2 mL) was stirred at room temperature for 24 h. Trifluoroacetic acid (TFA) was evaporated under reduced pressure, the residue was dissolved in the minimum amount of water and loaded on a Hypersep C18 SPE cartridge (1g bed). The cartridge was eluted with a gradient of water/TFA (99.9 : 0.1, eluant A) and acetonitrile/TFA (99.9 : 0.1; eluant B). The cartridge was eluted extensively with solvent A, and then with solvent A/Solvent B 9/1 (15 mL). The product was recovered eluting with solvent A/solvent B 8/2. The fraction containing the product was evaporated to give **9**·×TFA (0.085 g) as an amber glass solid.

<sup>1</sup>H NMR (400 MHz, pH ca. 3 ,D<sub>2</sub>O, external ref. *t*BuOH) & 1.96 (2H, q, J=6.6 Hz CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.70-4.44 (26H, broad-m, CH<sub>2</sub> macrocycle , NCH<sub>2</sub>), 2.91 (3H, s, NCH<sub>3</sub>), 4.32 (2H, t, J=6.5 Hz, CH<sub>2</sub>N), 4.95 (2H, s, OCH<sub>2</sub>), 6.57 (1H, d, J=16.1 Hz, CH<sub>alkene</sub>), 6.68-6.78 (3H, m, Ar*H*, CH<sub>alkene</sub>), 7.15 (2H, d, J= 8.7 Hz, Ar*H*), 7.23 (4H, s, Ar*H*) 7.94 (1H, s, C*H* triazol); <sup>13</sup>C NMR (600 MHz, 50 C, pH ca. 3 , D2O, external ref. *t*BuOH) & 30.68 (CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 38.34 (NCH<sub>2</sub>), 38.78 (NCH<sub>2</sub>), 40.93 (NCH<sub>2</sub>), 50.03 (NCH<sub>2</sub> ring, broad), 51.04 (NCH<sub>2</sub> ring, broad), 52.5 (NCH<sub>2</sub> ring, broad), 55.34 (NCH<sub>2</sub> acetic, broad), 56.05 (NCH<sub>2</sub> acetic, broad), 56.66 (NCH<sub>2</sub> acetic, broad), 63.03 (OCH<sub>2</sub>), 117.40 (CH aromatic), 118.24 (q, J= 292 Hz, CF<sub>3</sub>) 124.00 (CH aromatic), 126.94 (C aromatic), 127.01 (CH aromatic), 129.57 (CH alkene), 130.02 (CH alkene), 131.63 (CH aromatic), 132.19 (CH aromatic), 136.78 (C aromatic), 140.6 (C aromatic), 144.98 (C aromatic triazol), 159.4 (C aromatic), 164.29 (q, J= 35.3 Hz, CF<sub>3</sub>COOH), 173.84 (very broad, CO); ESI-MS (m/z): measured 750.32 [M + H]<sup>+</sup>; expected: 750.39

Gadolinium (E)-2,2',2"-(10-(2-((3-(4-((4-(4-(methylamino)styryl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)propyl)amino)-2oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acetate (10)—The pH of a solution of 9·×TFA (0.019 g) in deionized water (10 mL) was adjusted to 5 with NaOH<sub>aq</sub> 1M. A solution of GdCl3 (6.09 mg, 0.023 mmol) in water (4.0 mL) was added in four aliquots maintaining the pH between 5.5 and 6 with NaOH<sub>aq</sub> 1M. The solution was stirred at room temperature for 5h and subsequently the temperature was raised to 60 C and the reaction continued for 12 h. The solution was allowed to cool to room temperature and the pH was adjusted to 9.5 with  $NaOH_{aq}$  1M. The solution was then filtered through a 0.2um syringe filter and immediately loaded onto a Hypersep C18 SPE cartridge (1g bed). The cartridge was eluted with a gradient of water and acetonitrile from water/acetonitrile 100/0 to water/acetonitrile 60/40. The fractions containing the product were concentrated under reduced pressure to remove the organic solvent and then lyophilized to give 10 (0.017g, 50% over two steps) as a yellow solid. ESI-MS (m/z): measured 905.2  $[M + H]^+$ ; expected: 905.29. A correct isotopic pattern was observed. Elemental analysis calcd (%) for C<sub>37</sub>H<sub>48</sub>N<sub>9</sub>O<sub>8</sub>Gd 3 H<sub>2</sub>O: C 46.68, H 5.64, N 13.24, found: C 46.76, H 5.54, N 12.93.

# Animals

All animal experiments were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee of Case Western Reserve University (Protocol 2010-0006, 2010-0007). Two-month-old Swiss-Webster R/J mice were purchased from Harlan Laboratories, Indianapolis, IN. Two-month-old C57BL/6 mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Sprague Dawley rats were purchased from Harlan Laboratories, Haslett, MI. The *Plp*-Akt-DD transgenic mice were generated as described previously and used at 2 months of age. <sup>32,33</sup> Briefly, the transgenic mice expressing constitutively active Akt (HAAkt308D473D, Akt-DD) driven by the *Plp* promoter were generated and used as a hypermyelinated animal model. The Akt cDNA was inserted into the AscI/PacI sites of the modified *Plp* promoter cassette, and hypermyelination was induced after the *Plp* promoter/Akt-DD insert was injected into SJL/SWR F1 mice. Positive founders

were identified by PCR amplification of tail DNA using IntronSV40F (5'-GCAGTGGACCACGGTCAT-3') and Akt lower (5'-CTGGCAACTAGAAGGCACAG-3') primer pairs. Analyses were done from wild-type littermate mice in all developmental experiments and, when possible, with older animals.

#### **Induction of LPC lesions**

One Sprague Dawley rat was anesthetized by IP injection of 225  $\mu L$  of rodent cocktail [9 parts ketamine (100mg/ml) + 9 parts xylazine (20mg/ml) + 3 parts acepromazine (10 mg/ml) +79 parts sterile saline]. The skull was shaved and a 3-cm longitudinal incision was made on the scalp. A burr hole was created using a spherical dental burr over the site of the intended injection. 6  $\mu L$  of a 1% LPC solution was injected at a rate 0.25  $\mu L$ /min. The injection coordinates were AP=0.0 mm, ML= 2.0 mm, DV=3.2 mm, corresponding to the corpus callosum. After the injection the incision was sutured and the animal allowed to recover while being warmed with a heating pad. The lesions were allowed to develop for 7 days and subsequently the animal brain was used to prepare frozen section as described below.

# Preparation of frozen sections

Mice were deeply anesthetized with isoflurane and perfused via the ascending aorta with PBS followed by 4% paraformaldehyde in PBS. Brains were removed and incubated for 24 hours in 4% paraformaldehyde at 4 °C and then the tissues were incubated in 30% sucrose at 4 °C until submerged. Frozen sections were used for fluorescence microscopy. For preparation of fresh frozen sections, the cryoprotected tissues were first frozen in OCT on dry ice before axial sectioning (20  $\mu m$ ) with a cryostat at –20 °C. Tissue sections from the midline of the brain containing the whole corpus callosum were selected for staining. Stained sections were covered with fluorescence mounting medium (Vectashield, Vector Laboratories) and stored at 4 °C for future analysis.

#### **Chemical Staining**

Freshly frozen sections  $20~\mu m$  in thickness were incubated in 0.1% Triton-100 in  $1\times PBS$  for 10~min and then incubated in a solution of  $10~(100~\mu M)$  in  $H_2O$  for 30~min at room temperature. The fresh frozen sections were then washed three times for 5~min each with PBS before cover-slipping with fluorescence mounting medium. Images of the stained mouse brain sections were acquired on a Nikon TE2000 inverted microscope (UV-2E/C Ex: 340-380, DM: 400, BA: 435-485) or with a Leica DM5000B microscope equipped with an HCX PL FLUOTAR 1.25x/0.04 objective and using the A4 filter (360/40~nm band pass excitation, 400~nm dichromatic mirror, 470/40~nm band pass suppression).

## Intracerebroventricular injection of Gd complexes

Sprague Dawley rats were anesthetized by IP injection of 200-250  $\mu$ L of rodent cocktail (9 parts ketamine (100mg/ml) + 9 parts xylazine (20mg/ml) + 3 parts acepromazine (10 mg/ml) +79 parts sterile saline) and were fixed to the rat head-restraining stereotaxic surgical table. The skull was shaved and a 3-cm longitudinal incision was made on the scalp. A burr hole was created using a spherical dental burr over the site of the intended injection. A water solution of **10** or compound **11** (7 mM, 10  $\mu$ L, 70 nmol) was injected into the brain through a 10  $\mu$ L syringe equipped with a 28 gauge needle. After injection the incision was sutured and the animals allowed to recover consciousness while warmed with a heating pad. The injections were performed on both sides of the brain in the lateral ventricles with half of the dose injected on each side. Typical coordinates for the injection sites relative to the bregma are: AP=  $-0.5\pm0.1$  mm, ML =  $2.3\pm0.1$  mm, DV =  $3.5\pm0.1$  mm.

## In situ staining of myelin

The rats that were used for MR imaging were euthanized less than 48h post compound **10** intracerebroventricular infusion, perfused to remove the blood and the brains were extracted and sectioned to prepare frozen axial sections. Images of the stained mouse brain sections were acquired on a Nikon TE2000 inverted microscope (UV-2E/C Ex: 340-380, DM: 400, BA: 435-485).

## Relaxometry

The relaxivity of 10 was measured at 1.41 T (40 °C) and 0.47 (40 °C) on a Bruker minispec and at 9.4T (21 °C) on a Varian Inova 400 NMR system equipped with a 5 mm broadband probe. The longitudinal relaxation rates of five water solutions, with concentrations of compound 10 ranging from 0 to 0.7 mM, were measured using an inversion recovery pulse sequence with at least 10 inversion times. Attention was paid to assure that the relaxation delay between pulses was at least 5 times  $T_1$ . The absence of free Gd(III) was confirmed using a standard colorimetric test with xylenol orange.

# **MR Imaging**

All MR experiments were performed at 9.4 T (Bruker 'BIOSPEC', Bruker, Germany) using a 60 mm-diameter volume coil. Anesthesia was maintained by mask inhalation of isoflurane vaporized at concentrations of up to 4% in the induction phase, at 1.5% during the imaging experiments. Prior to measurement of the longitudinal relaxation time, axial images of the rat brain were acquired, using a multislice RARE pulse sequence (TE =11.3 ms, TR =5000ms, spatial resolution=0.195×0.195 mm/pixel, matrix size 256×256, 30 slices, slice thickness = 0.5 mm, 1 average), for the identification of the region of interest (ROI).  $T_1$  measurements were carried out using a spin-echo multiple TR saturation recovery method with at least 10 TRs (TE=6.99 ms, TR=385-12500 ms, spatial resolution=0.195×0.195 mm/pixel, Matrix size=256×256, 30 slices, slice thickness=0.5mm, 1 average).  $T_1$  maps were generated using the QuickVol II plug-in in ImageJ. The animals were imaged twice using a spin-echo multiple TR saturation recovery method five to seven hours post injection and then again 20 to 24 h post injection.

# **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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# **Abbreviations Used**

**DIPEA** N,N-Diisopropylethylamine

**DMF** dimethylformamide

DOTA tetraazacyclododecanetetraacetic acid

DTPA diethylenetriamine pentaacetic acid

(methylamino)styryl)phenoxy)methyl)-1H-1,2,3-triazol-1-

yl)propyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-

triyl)triacetic acetate

**HBTU** O-(Benzotriazol 1 yl) N,N,N',N'-tetramethyluronium hexafluorophosphate

**HOBT** 1-hydroxybenzotriazole

MBP myelin basic protein

MIC gadolinium(III) [4,10-bis-carboxymethyl-7-({3-[3-(4-

dimethylaminophenyl)-2-oxo-2H-chromen-7-yloxy]-

propylcarbamoyl}methyl)-1,4,7,10-tetraazacyclododec-1-yl]-acetate

**PET** positron emission tomography

TFA trifluoroacetic acid
THF tetrahydrofuran

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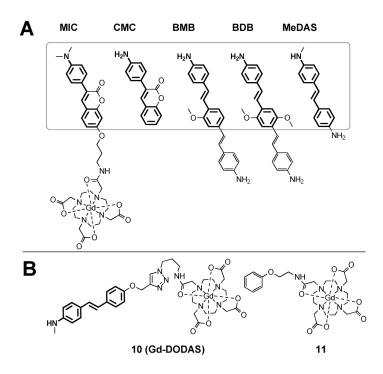


Figure 1.

(A) Chemical structure of myelin-targeting probes previously identified by our group (MIC = Gadolinium(III) [4,10-Bis-carboxymethyl-7-({3-[3-(4-dimethylaminophenyl)-2-oxo-2H-chromen-7 yloxy]-propylcarbamoyl}methyl)-1,4,7,10-tetraazacyclododec-1-yl]-acetate; CMC = 3-(4-aminophenyl)-2H-chromen-2-one; BMB = 4,4'-((1E,1'E)-(2-methoxy-1,4-phenylene)bis(ethene-2,1-diyl))dianiline; BDB = 4,4'-((1E,1'E)-(2,5-dimethoxy-1,4-phenylene)bis(ethene-2,1-diyl))dianiline; MeDAS = (E)-4-(4-aminostyryl)-N-methylaniline). In bold is highlighted a shared structural motif that is involved in binding with myelin. (B) Chemical structure of complexes that are discussed in this paper (Gd-DODAS: Gadolinium (E)-2,2',2"-(10-(2-((3-(4-((4-(4-(methylamino)styryl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)propyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acetate; Compound 11: Gadolinium 2,2',2"-(10-(2-oxo-2-((2-phenoxyethyl)amino)ethyl)-1,4,7,10-

tetraazacyclododecane-1,4,7-triyl)triacetate).

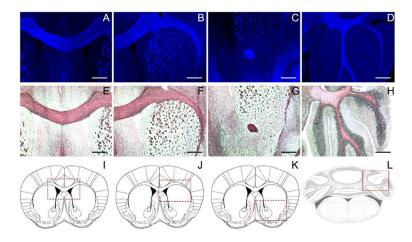


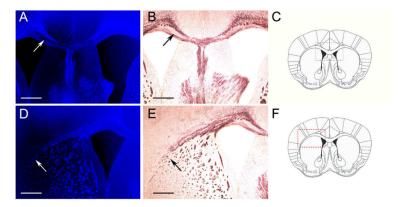
Figure 2. Chemical staining of representative frozen sections of wild type mouse brain showing that 10 selectively stains various myelinated white matter regions in the brain such as corpus callosum, striatum, anterior commissure, and cerebellum. (A-D, respectively). Gd-DODAS staining were correlated with Black-Gold II staining of adjacent sections (E-H). Atlas figures representing the stained regions (I-L).  $^{30}$  Scale bar =  $500\,\mu m$ .



Figure 3. (A) Chemical staining with 10 of a brain section of LPC-treated rat. (B). The correspondence of compound 10 fluorescence with myelin distribution within the brain was confirmed by black Black-Gold II staining. The demyelinated lesion is indicated by the arrow. Scale bars =  $500 \, \mu m$ . (C) Atlas figure showing the represented region.<sup>31</sup>



Figure 4. (A) Chemical staining with compound 10 of a brain section of a hypermyelinated *Plp*-Akt-DD D transgenic mouse. (B). The correspondence of compound 10 fluorescence with myelin distribution within the brain was confirmed by Black-Gold II staining. Scale bars =  $250 \ \mu m$ . (C) Atlas figure showing the represented region.<sup>30</sup>



**Figure 5.**Chemical staining with compound **10** of a brain section of a cuprizone mouse model showing demyelinated lesions (arrows) in the corpus callosum (A) and external capsule (B). Demyelination was confirmed by Black-Gold II staining (D, E). Atlas figures showing the represented regions (C,F).<sup>30</sup>

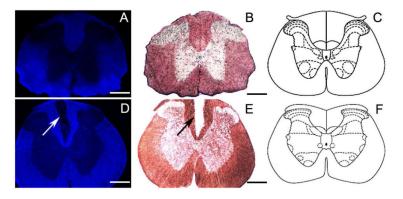
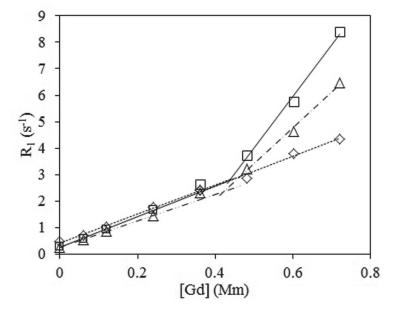


Figure 6.
Chemical staining with compound 10 of an axial spinal cord section of a wild type mouse (A) and Black-Gold II staining of an adjacent section (B). A large demyelinated lesion (arrow) can be detected in a spinal cord section of an LPC-induced mouse model of demyelination (D). The demyelinated lesion was confirmed by Black-Gold II staining of an adjacent section (E). Spinal cord diagrams showing the represented regions (C,F).



**Figure 7.**A) In-situ fluorescent microscopy of a brain tissue section following in vivo contrast enhanced MR imaging. (B) Bright Field microscopy Black-Gold II myelin staining of an adjacent section. Scale Bars =  $500 \, \mu m$ . (C) Atlas figure showing the represented region. <sup>31</sup>



**Figure 8.** Dependence of the relaxation rate of PBS buffered solutions (pH=7.4) of **10** upon Gd concentration at 0.47 T (40  $^{\circ}$ C; squares), 1.41 T (40  $^{\circ}$ C; triangles) and 9.4 T (21  $^{\circ}$ C; diamonds).

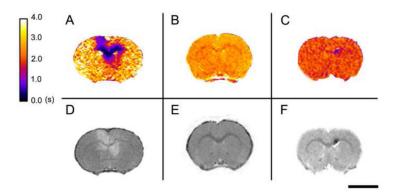


Figure 9. MR  $T_1$  maps (spin-echo multiple TR saturation recovery , TE=6.99 ms, TR=385-12500 ms, spatial resolution=0.195×0.195 mm/pixel, matrix size=256×256, 30 slices, slice thickness=0.5mm, 1 average) of a A) Sprague-Dawley rat brain 5h after compound 10 injection, B) Sprague-Dawley rat brain without infusion of contrast agent, C) Sprague-Dawley rat brain after injection of compound 11, and corresponding anatomical images (D-F).

Scheme 1. Synthesis of compound 10. i) Boc<sub>2</sub>O, THF, 96%; ii) MeI, NaH, THF, 94%; iii) NaH, DMF, 83%; iv) 3-azidopropan-1-amine, CuI, DIPEA, 54%; v) HBTU, HOBT, DIPEA, DMF, 92%; vi) TFA; vii) GdCl<sub>3</sub>, pH=5.5, 50% (over two steps).

Table 1

Longitudinal relaxivity of compound  ${\bf 10}$  compared to MIC and the clinically approved MR contrast agents GdDTPA and GdDOTA

|        | $\begin{matrix} 10 \\ r_1  (s^{-1}  mM^{-1}) \end{matrix}$ | $\begin{array}{c} MIC \\ r_1  (s^{-1}  mM^{-1}) \end{array}$ | $\begin{array}{c} GdDTPA \\ r_1 \ (s^{-1} \ mM^{-1}) \end{array}$ | $\begin{array}{c} GdDOTA \\ r_1  (s^{-1}  mM^{-1}) \end{array}$ |
|--------|--|--|---|---|
| 0.47 T | 5.8 ±0.2 <sup>a</sup>                                      | $5.8 \pm 0.2^{a}$  | 3.4 ±0.2 <sup>34,a</sup>  | $3.4 \pm 0.2^{34,a}$  |
| 1.41 T | 5.1 ±0.2 <sup>a</sup>                                      | 5.1 ± 0.1 <sup>a</sup>                                       | 3.3 ±0.2 <sup>34,C</sup>  | $2.9 \pm 0.2^{34,C}$  |
| 9.4 T  | 5.5 ±0.3 <sup>b</sup>                                      | $5.2 \pm 0.1^{b}$  | 3.9 <sup>35, d</sup>  | 4.1 <sup>35,</sup> d  |

*a*<sub>40 °C</sub>

*b*<sub>21 °C</sub>

<sup>c</sup>15 T 37 °C

<sup>d</sup>25 °C