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A cell-permeable gadolinium contrast agent for magnetic resonance imaging of copper in a Menkes disease model†

Emily L. Que^{#a}, Elizabeth J. New^{#a}, and Christopher J. Chang^{*,a,b}

^aDepartment of Chemistry, University of California, Berkeley, California 94720, USA.

^bHoward Hughes Medical Institute, University of California, Berkeley, California 94720, USA

[#] These authors contributed equally to this work.

Abstract

We present the synthesis and characterization of octaarginine-conjugated Copper-Gad-2 (Arg8CG2), a new copper-responsive magnetic resonance imaging (MRI) contrast agent that combines a Gd³⁺-DO3A scaffold with a thioether-rich receptor for copper recognition. The inclusion of a polyarginine appendage leads to a marked increase in cellular uptake compared to previously reported MRI-based copper sensors of the CG family. Arg8CG2 exhibits a 220% increase in relaxivity ($r_1 = 3.9$ to $12.5 \text{ mM}^{-1} \text{ s}^{-1}$) upon 1 : 1 binding with Cu⁺, with a highly selective response to Cu⁺ over other biologically relevant metal ions. Moreover, Arg8CG2 accumulates in cells at nine-fold greater concentrations than the parent CG2 lacking the polyarginine functionality and is retained well in the cell after washing. *In cellulo* relaxivity measurements and T_1 -weighted phantom images using a Menkes disease model cell line demonstrate the utility of Arg8CG2 to report on biological perturbations of exchangeable copper pools.

Introduction

Copper is an essential element for life, acting as a cofactor in a number of important enzymes that govern redox processes within the body.¹ On the other hand, genetic disorders such as Wilson's and Menkes diseases arise from mutations in copper handling proteins, and disruption of copper homeostasis is associated with a number of neurodegenerative pathologies including Alzheimer's, Parkinson's and prion diseases. A number of fluorescent sensors have been developed to enable visualization of biological copper pools, based on nucleic acids,^{2,3} proteins,^{4,5} and small-molecule fluorophores.^{6–13} Gaining a better understanding of how copper contributes to healthy and disease states, however, requires the development of methods for imaging copper distribution in the whole body rather than in cultured cells or tissue samples. To this end, X-ray fluorescence microscopy,^{14,15} positron

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*chrischang@berkeley.edu.

emission tomography (PET),^{16,17} and near infrared imaging¹⁸ have been used to study copper distribution in tissue and animal models of copper mishandling.

Our laboratory has been interested in the application of magnetic resonance imaging (MRI) for studying copper biology, as this technique is a powerful imaging modality in which whole body images of live specimens can be obtained at high resolution without the use of ionizing radiation.^{19,20} The observed contrast in MR images can be increased by the use of contrast agents containing paramagnetic ions. Gd^{3+} ($S = 7/2$) is most commonly employed for this purpose, owing to its large number of unpaired electrons and its ability to increase the proton relaxation rate of water molecules interacting with the metal center. A number of elegant responsive MR contrast agents have been developed,^{21,22} in which a relaxivity change can report on pH,^{23,24} gene expression,²⁵ enzyme activity,^{26–28} or metal ion concentration.^{22,29–32} Notably, a number of recent studies have reported MR-based probes that respond to Cu^{2+} .^{33–36} We are also interested in studying Cu^+ , which is the primary copper species in the reducing intracellular environment. We have previously developed a series of MR-based probes that exhibit increases in longitudinal relaxivity (r_1) in response to Cu^+ and/or Cu^{2+} .^{36–38} Of particular note is CG2, a Cu^+ -sensitive contrast agent that displays a 360% increase in r_1 upon addition of one equivalent of Cu^+ ($r_1 = 1.5$ to $6.9 \text{ mM}^{-1} \text{ s}^{-1}$; Scheme 1).^{22,38}

Despite the promising *in vitro* behaviour of the many metal-responsive Gd-based contrast agents,^{22,39} their ability to image changes in metal concentration *in cellulo* remains insufficiently explored. Previous biological, MR-based metal ion sensing has largely focused on Zn physiology, including elegant studies by Sherry and co-workers on the use of a Gd-based zinc sensor for the *in vivo* detection of extracellular zinc released by pancreatic beta cells³¹ and Lippard, Jasanoff, and colleagues on the application of a cell-permeable Mn-based contrast agent to sense zinc levels *in vivo*.^{30,40} Encouraged by the large dynamic range of CG2, we sought to exploit its properties in the design of an MR contrast agent for the imaging of Cu^+ in living systems. The +1 oxidation state of copper dominates in intracellular spaces; we expected that CG2, like other typical Gd^{3+} -containing MR contrast agents such as Gd-DTPA and Gd-DOTA, would be largely confined to the extracellular space due to a poor ability to cross cellular membranes.⁴¹ We therefore considered various strategies to elicit enhanced cellular uptake, which include electroporation,⁴² encapsulation into liposomes^{43,44} and conjugation to various macromolecules such as peptides,^{45–48} dendrimers,⁴⁹ dextrans,⁵⁰ and TiO_2 ⁵¹ or gold⁵² nanoparticles, and settled on the use of a polyarginine tag as a promising method to this end.^{53–56} In this context, Weissleder and coworkers have exploited the TAT peptide sequence from the HIV virus to render nanoparticle-based MR contrast agents cell-permeable,^{57–59} and Meade and coworkers have reported modified Gd^{3+} -DTPA and Gd^{3+} -DOTA complexes containing octaarginine (Arg₈) tails.^{41,45,51} For the latter, cellular uptake of these complexes was confirmed by inductively coupled plasma mass spectrometry and X-ray fluorescence techniques. Based on these precedents, we reasoned that appending an Arg₈ tag onto our CG2 scaffold should readily deliver our Cu^+ -responsive agent to the interior of the cell. In this edge article, we now report the design and synthesis of Arg₈CG2, and our comparisons of the cellular behaviour of this complex to the parent CG2. We then detail our studies of a murine cell line bearing a

mutant form of the copper efflux protein, ATP7A, using Arg8CG2, demonstrating that this molecular imaging agent can distinguish between normal and aberrant labile copper pools in a disease model, and reporting the *in cellulo* application of a metal-responsive Gd-based MR contrast agent.

Results and discussion

Design and synthesis of a Gd contrast agent bearing a polyarginine tail

The synthesis of Arg8CG2 is detailed in Scheme 2. Bis-protected cyclen **1** was synthesized by a previously-reported literature procedure in quantitative yield.⁶⁰ Alkylation of **1** with *tert*-butyl bromoacetate furnished compound **2**. The benzyl carbamate protecting groups were removed by hydrogenation over Pd/C to yield **3** in nearly quantitative yield. The trisubstituted cyclen **5** was prepared by slow addition of electrophile **4**³⁸ to a heated mixture of **3** and K₂CO₃. By this method, compound **5** was produced in 42% yield, with the tetrasubstituted by-product also observed. Triester **6** was obtained by alkylation of **5** with ethyl bromoacetate at room temperature in THF. The mono-carboxylate **7** was formed by the selective deprotection of the ethyl ester in 0.1 M NaOH in a water–dioxane (1 : 3) mixture at 50 °C. Arg8-functionalized Wang resin was obtained using standard solid phase peptide synthesis techniques. Coupling of **7** to the resin-bound polyarginine using HATU and DIPEA in DMF, followed by cleavage from the resin using TFA, gave ligand **8** following HPLC purification. Metalation of **8** was achieved with a slight excess of Gd(OH)₃ in H₂O (pH 6), followed by purification *via* reversed-phase chromatography using a C₁₈ SepPak cartridge.

Spectroscopic properties and response to Cu⁺

The relaxivity properties of Arg8CG2 were characterized in phosphate buffered saline (PBS; pH 7.4) at 37 °C and Gd³⁺ concentrations were determined by ICP-OES. In the absence of Cu⁺, the relaxivity of Arg8CG2 is 3.9 mM⁻¹ s⁻¹ (Fig. S1†). This r_1 is significantly higher than that of the parent complex CG2 ($r_1 = 1.5 \text{ mM}^{-1} \text{ s}^{-1}$), which is likely to reflect an increased rotational correlation time (τ_R) due to the large octaarginine group, and increased second-sphere hydration due to the presence of additional hydrogen-bonding groups from the arginine residues. Upon addition of Cu⁺, the r_1 of Arg8CG2 increases to 12.5 mM⁻¹ s⁻¹, a 220% increase in relaxivity ($r_1 = 8.6 \text{ mM}^{-1} \text{ s}^{-1}$; Fig. 1a). Although the observed turn-on response is a smaller percent increase in r_1 than for our original CG2 platform (360%), this value is competent for *in vivo* applications. We note that the presence of the polyarginine tail does not appear to greatly affect the ability of our platform to respond to Cu⁺ ions.

Since our polyarginine-functionalized Gd³⁺ complex responds to Cu⁺, we sought to characterize the binding interaction between Arg8CG2 and Cu⁺. A Job plot analysis confirms a 1 : 1 Arg8CG2/Cu⁺ binding stoichiometry (Fig. S2†). The binding affinity of our complex for Cu⁺ was determined by monitoring changes in the absorbance spectrum of the Arg8CG2–Cu⁺ complex in the presence of varying amounts of thiourea, which is known to be a competitive ligand for Cu⁺.⁶¹ Analysis of the resulting data gave an apparent K_d of $8.3 \times 10^{-15} \text{ M}$ (Fig. 1b), signifying even tighter binding than the parent CG2 complex ($K_d = 2.6 \times 10^{-13} \text{ M}$). Moreover, the presence of the polycationic tail on the Gd core does not affect

the metal ion selectivity of the CG2-based scaffold (Fig. 2). Arg8CG2 selectivity responds to Cu^+ over a range of biologically relevant metal ions including 10 mM Na^+ , 2 mM K^+ , Mg^{2+} , and Ca^{2+} , 0.2 mM Fe^{2+} , Fe^{3+} , and Cu^{2+} , and Zn^{2+} at both 0.2 mM and 2 mM concentrations. Arg8CG2 does have some response to Cu^{2+} , but we do not anticipate this behavior to be a drawback for the *in cellulo* use of Arg8CG2, since the reducing environment of the cell favors Cu^+ over Cu^{2+} . Phantom MR images of Arg8CG2 in PBS showed that the probe displays different levels of contrast in samples with and without added Cu^+ at clinically-relevant field strengths (Fig. S3†).

Arg8CG2 exhibits greater cellular uptake and *in cellulo* relaxivity than CG2

We anticipated that the polyarginine tail would afford Arg8CG2 much greater cellular uptake compared to the parent complex, CG2. This behavior was tested using HEK 293T as a model cell line. Cells were treated for one hour with either of the complexes at a range of concentrations, washed with PBS, lysed and digested prior to the determination of Gd content by ICP-MS. For each sample, protein content was also determined using a BCA assay and Gd uptake per cell was determined by calibration of the BCA assay to total cell number. These results show a ten-fold greater uptake of Arg8CG2 than CG2 at a dosing concentration of 500 μM (Fig. 3), confirming that the octaarginine tail contributes to better transport across the cell membrane. Notably, the calculated number of complexes per cell is within the range of values observed by Allen *et al.* for their series of polyarginine-containing complexes.⁴¹ An analysis of the uptake of Arg8CG2 after 1 h and 15 h indicates greater cellular uptake after the shorter time period (Fig. S4†), which may be due to cellular clearance mechanisms. This observation is consistent with the findings of a broad-scale study of cell-penetrating peptides, which reported that maximal uptake was achieved between 30 min and 2 h.⁶² On the basis of these results, all subsequent experiments were performed with an incubation time of 1 h and a dosing concentration of 500 μM .

Having established the superior cellular uptake of Arg8CG2, we then sought to investigate whether this difference translated into a change in *in cellulo* relaxivity. We pretreated HEK cells with CuCl_2 or bathocuproine disulfonate (BCS), a membrane-impermeable copper chelator, which is commonly used to globally deplete intracellular copper. After washing, we then treated the cells with 500 μM CG2 or Arg8CG2 for 1 h. The cells were again washed, trypsinized, pelleted, resuspended in PBS, and immediately placed in a relaxometer tube, in which the T_1 was measured. Cu-treated cells incubated with CG2 exhibited a relaxivity that was significantly higher than control cells ($p < 0.05$, Fig. 4), confirming that the probe is able to detect increases in the cellular copper pool. On the other hand, BCS-treated cells, in which there is a global depletion of labile copper, exhibited a significantly lower relaxivity, showing that CG2 is sensitive to basal levels of copper and can detect depletions in this pool. The relaxivity values obtained for this pool, however, were not much greater than those measured for cells alone, in the absence of probe. Cells treated with Arg8CG2, on the other hand, exhibited markedly higher relaxivities than those cells with no probe, and while they showed the same trends as CG2 for Cu- and BCS-treated cells, these differences were much more pronounced. The collective results in the model HEK 293T cell line indicate that, compared to CG2, Arg8CG2 exhibits *in cellulo* relaxivities that are much more suitable for the study of perturbations in labile cellular Cu levels.

Arg₈CG2 can report on labile copper pools in a disease model

Since Arg₈CG2 can report on basal labile copper levels, and on increases and decreases in this pool, we sought to apply this probe to study the effects of mutations in the *Atp7a* gene on intracellular copper availability. This gene encodes the copper-transporting ATPase 1, ATP7A, which is the major copper efflux protein, and is therefore essential in the maintenance of cellular Cu homeostasis.⁶³ In humans, mutations in the X-linked *Atp7a* gene are responsible for Menkes syndrome. On a cellular level, mutations in *Atp7a* give rise to elevated copper levels, exacerbated in cases of Cu supplementation.^{12,64} To investigate whether our probe could detect differences between healthy and disease states, we used the Menkes model WG1005 fibroblast line, which bears mutations in *Atp7a*, in comparison to control fibroblasts, MCH58, which express wild-type *Atp7a*.¹²

We initially sought to evaluate the toxicity of our two Gd complexes in the fibroblast cell lines, as well as in HEK 293T cells, by using the WST-1 assay (Table 1). No appreciable toxicity for CG2 was observed in any cell line at concentrations up to 2 mM over a 24 h period. Arg₈CG2, on the other hand, decreased cellular viability over 24 h in the three cell lines, particularly HEK 293T and WG1005. This observation is consistent with reports of the toxicity of octaarginine-bearing compounds.⁶² The results of the 4 h incubations, however, demonstrate much better tolerance for the complex, and indicate that our dosing conditions of 500 μ M for 1 h are unlikely to elicit significant cell death.

Uptake studies show a 9-fold greater accumulation of Arg₈CG2 than CG2 in both cell lines, with similar intracellular Gd concentrations measured to those observed for HEK cells (Fig. S5[†]). Egress studies were also performed in these cell lines; following 1 h incubation with complex, the medium was removed, and replaced with fresh medium for 30 min, after which time intracellular Gd concentrations were measured. These results show that Arg₈CG2 is retained very well, with a greater than 80% retention in both cell lines, while only 20% of CG2 is retained in the cell. These data provide further support for the use of a polyarginine tag for an *in cellulo* probe. Since all *in cellulo* relaxivity and phantom imaging studies reported here were performed within 30 min of cell collection, we can be confident that the localization of Arg₈CG2 is primarily intracellular.

The $1/T_1$ values of the two cell lines upon treatment with CG2 and Arg₈CG2 are shown in Fig. 5. As observed for the HEK 293T cells, there is a significant increase in $1/T_1$ for cells treated with Cu and then Arg₈CG2, and a significant decrease for cells treated with BCS, compared to control and copper-treated cells. Notably, there is a significantly higher relaxivity, corresponding to a higher copper concentration, for the WG1005 cell line than for MCH58 cells. This observation is consistent with a mutation in *Atp7a* perturbing the ability of the cell to excrete copper. The same trend can also be observed for CG2-treated cells, although only the control cells exhibit a statistically significant difference in relaxivity, highlighting the superiority of Arg₈CG2 for the *in cellulo* assessment of labile copper levels.

Finally, we collected phantom images of the two cell lines treated in the same manner as for the relaxivity studies (Fig. 6). Cells treated with CG2 or Arg₈CG2 exhibit higher contrast than cells alone, with copper-treated cells displaying the highest signal. It is also possible to observe a difference in the contrast between the two cell lines for the copper- and Arg₈CG2-

treated samples. The phantom images also highlight the enhanced contrast of cells treated with Arg₈CG2 compared to CG2-treated cells. These results indicate the potential of this probe for use in MRI studies in higher living model systems.

Conclusions

In summary, we have described the synthesis, relaxivity behavior and cellular characterization of Arg₈CG2, a Cu⁺-responsive MRI contrast agent that exhibits improved cellular uptake. This work demonstrates the utility of the octaarginine group to promote cellular uptake and retention, and the great advantage that such behavior lends to *in cellulo* experiments. Using this new probe, we have been able to detect differences in copper accumulation between cells bearing a mutant copper transporter and wildtype cells. The clear differences in relaxivity between *Atp7a* mutant and wildtype cells as detected by Arg₈CG2, and the resulting variation in contrast in phantom imaging experiments, points to the applicability of this probe for *in vivo* imaging in disease states. We have identified a number of animal models of copper mishandling diseases, to which we can apply Arg₈CG2, including the toxic milk⁶⁵ and *Atp7b* knockout mice,⁶⁶ which are models for Wilson's disease, and which exhibit markedly different copper distributions from wildtype mice.^{17,18} We are currently pursuing the use of Arg₈CG2 in a variety of whole animal imaging studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

1. Que EL, Domaille DW, Chang CJ. Chem. Rev. 2008; 108:1517–1549. [PubMed: 18426241]
2. Liu J, Lu Y. J. Am. Chem. Soc. 2007; 129:9838–9839. [PubMed: 17645334]
3. Liu J, Lu Y. Chem. Commun. 2007:4872–4874.
4. Wegner SV, Arslan H, Sunbul M, Yin J, He C. J. Am. Chem. Soc. 2010; 132:2567–2569. [PubMed: 20131768]
5. Wegner SV, Sun F, Hernandez N, He C. Chem. Commun. 2011; 47:2571–2573.
6. Yang L, McRae R, Henary MM, Patel R, Lai B, Vogt S, Fahrni CJ. Proc. Natl. Acad. Sci. U. S. A. 2005; 102:11179–11184. [PubMed: 16061820]
7. Miller EW, Zeng L, Domaille DW, Chang CJ. Nat. Protoc. 2006; 1:824–827. [PubMed: 17406313]
8. Zeng L, Miller EW, Pralle A, Isacoff EY, Chang CJ. J. Am. Chem. Soc. 2006; 128:10–11. [PubMed: 16390096]
9. Domaille DW, Zeng L, Chang CJ. J. Am. Chem. Soc. 2010; 132:1194–1195. [PubMed: 20052977]
10. Taki M, Iyoshi S, Ojida A, Hamachi I, Yamamoto Y. J. Am. Chem. Soc. 2010; 132:5938–5939. [PubMed: 20377254]

11. Dodani SC, Domaille DW, Nam CI, Miller EW, Finney LA, Vogt S, Chang CJ. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:5980–5985. [PubMed: 21444780]
12. Dodani SC, Leary SC, Cobine PA, Winge DR, Chang CJ. *J. Am. Chem. Soc.* 2011; 133:8606–8616. [PubMed: 21563821]
13. Lim CS, Han JH, Kim CW, Kang MY, Kang DW, Cho BR. *Chem. Commun.* 2011; 47:7146–7148.
14. Chwiej J, Trace J. *Elem. Med. Biol.* 2010; 24:78–88.
15. Ide-Ektessabi A, Rabionet M. *Anal. Sci.* 2005; 21:885–892. [PubMed: 16038516]
16. Fodero-Tavoletti MT, Villemagne VL, Paterson BM, White AR, Li Q-X, Camakaris J, O’Keefe GJ, Cappai R, Barnham KJ, Donnelly PS. *J. Alzheimer’s Dis.* 2010; 20:49–55. [PubMed: 20164590]
17. Peng F, Lutsenko S, Sun X, Muzik O. *Mol. Imaging Biol.* 2012; 14:70–78. [PubMed: 21327972]
18. Hirayama T, Van de Bittner GC, Gray L, Lutsenko S, Chang CJ. *Proc. Natl. Acad. Sci. U. S. A.* 2012; 109:2228–2233. [PubMed: 22308360]
19. Caravan P, Ellison JJ, McMurry TJ, Lauffer RB. *Chem. Rev.* 1999; 99:2293–2352. [PubMed: 11749483]
20. Werner EJ, Datta A, Jocher CJ, Raymond KN. *Angew. Chem., Int. Ed.* 2008; 47:8568–8580.
21. Meade TJ, Taylor AK, Bull SR. *Curr. Opin. Neurobiol.* 2003; 13:597–602. [PubMed: 14630224]
22. Que EL, Chang CJ. *Chem. Soc. Rev.* 2010; 39:51–60. [PubMed: 20023836]
23. Aime S, Castelli D, Delli, Terreno E. *Angew. Chem.* 2002; 114:4510–4512.
24. Kálmán FK, Woods M, Caravan P, Jurek P, Spiller M, Tircsó G, Király R, Brücher E, Sherry AD. *Inorg. Chem.* 2007; 46:5260–5270. [PubMed: 17539632]
25. Chang Y-T, Cheng C-M, Su Y-Z, Lee W-T, Hsu J-S, Liu G-C, Cheng T-L, Wang Y-M. *Bioconjugate Chem.* 2007; 18:1716–1727.
26. Chauvin T, Durand P, Bernier M, Meudal H, Doan B-T, Noury F, Badet B, Beloeil J-C, Tóth É. *Angew. Chem., Int. Ed.* 2008; 47:4370–4372.
27. Li Y, Sheth VR, Liu G, Pagel MD. *Contrast Media Mol. Imaging.* 2011; 6:219–228. [PubMed: 21861282]
28. Urbanczyk-Pearson LM, Meade TJ. *Nat. Protoc.* 2008; 3:341–350. [PubMed: 18323804]
29. Hanaoka K, Kikuchi K, Urano Y, Nagano T. *J. Chem. Soc., Perkin Trans.* 2001; 2:1840–1843.
30. Lee T, Zhang X.-a. Dhar S, Faas H, Lippard SJ, Jasanoff A. *Chem. Biol.* 2010; 17:665–673. [PubMed: 20609416]
31. Lubag AJM, De Leon-Rodriguez LM, Burgess SC, Sherry AD. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:18400–18405. [PubMed: 22025712]
32. Mishra A, Logothetis NK, Parker D. *Chem.–Eur. J.* 2011; 17:1529–1537. [PubMed: 21268155]
33. Kasala D, Lin T-S, Chen C-Y, Liu G-C, Kao C-L, Cheng T-L, Wang Y-M. *Dalton Trans.* 2011; 40:5018–5025. [PubMed: 21451883]
34. Li W-S, Luo J, Chen Z-N. *Dalton Trans.* 2011; 40:484–488. [PubMed: 21113542]
35. Patel D, Kell A, Simard B, Xiang B, Lin HY, Tian G. *Biomaterials.* 2011; 32:1167–1176. [PubMed: 21035183]
36. Que EL, Chang CJ. *J. Am. Chem. Soc.* 2006; 128:15942–15943. [PubMed: 17165700]
37. Que EL, Gianolio E, Baker SL, Aime S, Chang CJ. *Dalton Trans.* 2010; 39:469–476. [PubMed: 20023983]
38. Que EL, Gianolio E, Baker SL, Wong AP, Aime S, Chang CJ. *J. Am. Chem. Soc.* 2009; 131:8527–8536. [PubMed: 19489557]
39. Bonnet CS, Toth E. *Future Med. Chem.* 2010; 2:367–384. [PubMed: 21426172]
40. Zhang, X.-a.; Lovejoy, KS.; Jasanoff, A.; Lippard, SJ. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:10780–10785. [PubMed: 17578918]
41. Allen MJ, MacRenaris KW, Venkatasubramanian PN, Meade TJ. *Chem. Biol.* 2004; 11:301–307. [PubMed: 15123259]
42. Terreno E, Crich SG, Belfiore S, Biancone L, Cabella C, Esposito G, Manazza AD, Aime S. *Magn. Reson. Med.* 2006; 55:491–497. [PubMed: 16450336]

43. Hak S, Sanders HMHF, Agrawal P, Langereis S, Grüll H, Keizer HM, Arena F, Terreno E, Strijkers GJ, Nicolay K. *Eur. J. Pharm. Biopharm.* 2009; 72:397–404. [PubMed: 18940253]
44. Kabalka GW, Davis MA, Moss TH, Buonocore E, Hubner K, Holmberg E, Maruyama K, Huang L. *Magn. Reson. Med.* 1991; 19:406–415. [PubMed: 1881329]
45. Allen MJ, Meade TJ. *JBIC, J. Biol. Inorg. Chem.* 2003; 8:746–750.
46. Bhorade R, Weissleder R, Nakakoshi T, Moore A, Tung C-H. *Bioconjugate Chem.* 2000; 11:301–305.
47. Curtet C, Maton F, Havet T. *Invest. Radiol.* 1998; 33:752–761. [PubMed: 9788138]
48. Heckl S, Debus J, Jenne J, Pipkorn R, Waldeck W, Spring H, Rastert R, von der Lieth CW, Braun K. *Cancer Res.* 2002; 62:7018–7024. [PubMed: 12460922]
49. Wiener EC, Konda S, Shadron A, Brechbiel M, Gansow O. *Invest. Radiol.* 1997; 32:748–754. [PubMed: 9406015]
50. Casali C, Janier M, Canet E. *Acad. Radiol.* 1998; 5:214–218.
51. Endres PJ, Paunesku T, Vogt S, Meade TJ, Woloschak GE. *J. Am. Chem. Soc.* 2007; 129:15760–15761. [PubMed: 18047347]
52. Song Y, Xu X, MacRenaris KW, Zhang X-Q, Mirkin CA, Meade TJ. *Angew. Chem., Int. Ed.* 2009; 48:9143–9147.
53. Deshayes S, Morris MC, Divita G, Heitz F. *Cell. Mol. Life Sci.* 2005; 62:1839–1849. [PubMed: 15968462]
54. Matsui H, Tomizawa K, Lu YF, Matsushita M. *Curr. Protein Pept. Sci.* 2003; 4:151–157. [PubMed: 12678854]
55. Rothbard JB, Jessop TC, Wender PA. *Adv. Drug Delivery Rev.* 2005; 57:495–504.
56. Wender PA, Galliher WC, Goun EA, Jones LR, Pillow TH. *Adv. Drug Delivery Rev.* 2007; 60:452–472.
57. Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, Weissleder R. *Nat. Biotechnol.* 2000; 18:410–414. [PubMed: 10748521]
58. Wunderbaldinger P, Josephson L, Weissleder R. *Bioconjugate Chem.* 2002; 13:264–268.
59. Zhao M, Kircher MF, Josephson L, Weissleder R. *Bioconjugate Chem.* 2002; 13:840–844.
60. De Leon-Rodriguez LM, Kovacs Z, Esqueda-Oliva AC, Miranda-Olvera AD. *Tetrahedron Lett.* 2006; 47:6937–6940.
61. Smith, RM.; Martell, AE. *Critical Stability Constants*. Plenum Press; New York: 1989.
62. Jones SW, Christison R, Bundell K, Joyce CJ, Brockbank SMV, Newham P, Lindsay MA. *Br. J. Pharmacol.* 2005; 145:1093–1102. [PubMed: 15937518]
63. Kaler SG. *Nat. Rev. Neurol.* 2011; 7:15–29. [PubMed: 21221114]
64. Bellingham SA, Lahiri DK, Maloney B, La Fontaine S, Multhaup G, Camakaris J. *J. Biol. Chem.* 2004; 279:20378–20396. [PubMed: 14985339]
65. Theophilos MB, Cox DW, Mercer JF. *Hum. Mol. Genet.* 1996; 5:1619–1624. [PubMed: 8894697]
66. Huster D, Finegold MJ, Morgan CT, Burkhead JL, Nixon R, Vanderwerf SM, Gilliam CT, Lutsenko S. *Am. J. Pathol.* 2006; 168:423–434. [PubMed: 16436657]

Fig. 1.

Interaction of Arg8CG2 with Cu^+ . (a) Plot of r_1 relaxivity *versus* added Cu^+ for a 0.2 mM solution of Arg8CG2 in PBS (pH 7.4), at 37 °C at a proton Larmor frequency of 60 MHz. (b) Normalized absorbance response of 0.2 mM Arg8CG2 to buffered Cu^+ solutions for determination of the apparent K_d value. Spectra were acquired in PBS (pH 7.4) at 25 °C. The black trace represents the best fit ($K_d = 8.3 \times 10^{-15} \text{ M}$, $R^2 = 0.986$).

Fig. 2.

Relaxivity responses of ArgCG2 to various metal ions. White bars represent the addition of the appropriate metal ion (10 mM for Na⁺; 2 mM for K⁺, Mg²⁺, and Ca²⁺; 0.2 mM for Fe²⁺, Fe³⁺ and Cu²⁺) to 0.2 mM ArgCG2. Response to Zn²⁺ was measured both at 0.2 mM Zn²⁺ (Zn²⁺ 1 ×) and 2 mM Zn²⁺ (Zn²⁺ 10 ×). Black bars represent the subsequent addition of 0.2 mM Cu⁺ to the contrast agent solution. Relaxivity measurements were acquired at 37 °C in PBS (pH 7.4) at a proton Larmor frequency of 60 MHz.

Fig. 3. Cellular uptake of complexes in HEK 293T cells. (a) CG2 (black) and ArggCG2 (gray) in HEK 293T cells following 1 h incubation at various dosing concentrations. After incubation, cells were rinsed with PBS, lysed with RIPA buffer, dissolved in nitric acid and analyzed by ICP-MS. Error bars represent one standard deviation ($n = 4$).

Fig. 4.

$1/T_1$ values of HEK 293T cells treated with 500 mM CG2 or Arg8CG2 following incubation with vehicle control (black), copper (light gray, 100 μ M, 48 h) or BCS (dark gray, 200 μ M, 48 h). Relaxivity measurements were acquired at 37 °C in PBS (pH 7.4) at a proton Larmor frequency of 60 MHz. Error bars represent one standard deviation ($n = 3$) and statistical analyses were performed with a two-tailed Student's t -test relative to the control. * $P < 0.05$, ** $P < 0.01$.

Fig. 5.

$1/T_1$ values of MCH58 (black) and WG1005 (gray) cells treated with 500 μ M CG2 (left) or Arg₈CG2 (right) following incubation with vehicle control, copper (100 μ M, 48 h) or BCS (200 μ M, 48 h). Relaxivity measurements were acquired at 37 °C in PBS (pH 7.4) at a proton Larmor frequency of 60 MHz. Error bars represent one standard deviation ($n = 3$) and statistical analysis were performed with a two-tailed Student's t -test relative to the control. * $P < 0.05$, ** $P < 0.01$.

Fig. 6. T_1 -weighted phantom images of MCH58 and WG1005 cells treated with CG2 or Arg₈CG2 (500 μ M, 1 h). Images were acquired at 25 $^{\circ}$ C at 1.5 T ($\sim$ 64 MHz proton Larmor frequency).

Scheme 1.
Copper-Gad contrast agent, CG2.

Scheme 2.
Synthesis of Arg₈CG₂.

Table 1Cellular toxicities (IC₅₀) for complexes^a

	CG2 IC₅₀/μM		Arg₈CG2 IC₅₀/μM	
	4 h	24 h	4 h	24 h
HEK	>2000	>2000	>2000	295 (23)
MCH58	>2000	>2000	1864 (19)	1153 (58)
WG1005	>2000	>2000	1517 (41)	499 (18)

^aValues represent the mean of triplicate readings of at least 3 independent measurements, with standard deviations in parentheses.