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***The ionic charge of Copper-64 complexes conjugated
to an engineered antibody effects biodistribution***

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**The Ionic Charge of Copper-64 Complexes Conjugated to
an Engineered Antibody Effects Biodistribution**

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Running Title: Effect of chelator charge on antibody biodistribution

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ABSTRACT

The development of biomolecules as imaging probes requires radiolabeling methods that do not significantly influence their biodistribution. Sarcophagine (Sar) chelators form extremely stable complexes with copper, and are therefore a promising option for labeling proteins with ^{64}Cu . However, initial studies using the first-generation sarcophagine bifunctional chelator SarAr to label the engineered antibody fragment ch14.18- $\Delta\text{C}_{\text{H}2}$ (MW 120 kDa) with ^{64}Cu showed high tracer retention in the kidneys (>38 % injected dose per gram (ID/g) 48 h post-injection), presumably because the high local positive charge on the Cu^{II} -SarAr moiety resulted in increased binding of the labeled protein to the negatively charged basal cells of the glomerulus. To test this hypothesis, ch14.18- $\Delta\text{C}_{\text{H}2}$ was conjugated with a series of Sar derivatives of decreasing positive charge and three commonly used macrocyclic polyaza polycarboxylate (PAC) BFCs. The immunoconjugates were labeled with ^{64}Cu , injected into mice, and PET/CT images were obtained at 24 and 48 h post injection (p.i.). At 48 h p.i., an *ex vivo* biodistribution was carried out. In addition, to demonstrate the potential of metastasis detection using ^{64}Cu -labeled ch14.18- $\Delta\text{C}_{\text{H}2}$, a preclinical imaging study of intrahepatic neuroblastoma tumors was carried out. Reducing the positive charge on the Sar chelators decreased kidney uptake of Cu -labeled ch14.18- $\Delta\text{C}_{\text{H}2}$ by more than 6-fold, from >45% ID/g to <6% ID/g, while the uptake in most other tissues, including liver, was relatively unchanged. However, despite this dramatic decrease, the renal uptake of the PAC BFCs was generally lower than that of the Sar derivatives as was the liver uptake. Uptake of ^{64}Cu -labeled ch14.18- $\Delta\text{C}_{\text{H}2}$ in metastases was detected using PET.

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Universität Tübingen; the original protein and production cell line were developed and made available for use by Stephen Gillies, PhD, Provenance Biopharmaceuticals (Billerica MA). This work was supported by National Institutes of Health Grants 5K08CA093554 (to SDV); the Children's Hospital Radiology Foundation; by the Comunidad de Madrid (through the Madrid-MIT M+Visión Fellowship, to JLJD); the Australian Research Council (to PSD), and a Victoria Fellowship (to BMP). Copper-64 was produced at Washington University School of Medicine (St. Louis, MO, USA) under the support of National Cancer Institute Grant R24CA86307.

Keywords: bifunctional chelator, ΔC_H2 , sarcophagine, radioimmunoimaging, NOTA, ^{64}Cu .

INTRODUCTION

Radiolabeled monoclonal antibodies are useful imaging agents because of their high specificity for the target antigen. When radiolabeled with a positron emitting radionuclide, they can provide quantitative, high-resolution data about the localization of a target antigen within the body. Antibody labeling with radioactive metals, such as ^{64}Cu ($t_{1/2} = 12.7$ h, β^+ 17.9%, 653 keV), requires the use of a bifunctional chelator (BFC) to attach the radioactive metal to the protein, ideally without adversely affecting the pharmacokinetics of the antibody. The resulting metal-BFC complex must be stable *in vivo* for the imaging data to accurately reflect the distribution of the antibody. While DOTA (1,4,7,10-tetraazacyclododecane tetraacetic acid) has often been used as a BFC, despite the fact that the kinetic instability of its copper complex (1) can lead to loss of the ^{64}Cu from the radioimmunoconjugate (RIC), there is considerable interest in developing BFCs that form ^{64}Cu complexes that are kinetically stable *in vivo*. One example is the sarcophagine-based BFCs, which have shown considerable promise in this application because of the very high stability of their copper complexes (2).

Although PET imaging with ^{64}Cu -labeled antibodies offers high specificity for the characterization of tumors, it is limited by the slow clearance of the RICs from normal tissues, including the blood. Fortunately, the pharmacokinetics of antibodies can be improved through protein engineering (3), including reducing the size of construct. For example, removing the $\text{C}_\text{H}2$ domain from the whole IgG antibody (MW 150 kDa), reduces the molecular weight of the protein to approximately 120 kDa. This results in more rapid clearance from non-target tissues and thus a shorter delay between tracer injection and imaging and reduces the radiation exposure of non-target tissue. The tradeoff is that the smaller molecule weight also decreases absolute tumor uptake because the time during which the protein can accumulate in the tumor tissue is reduced

(4). The goal, therefore, is to find the optimal balance between tumor uptake and clearance from non-target tissues.

The ΔC_H2 immunoprotein format (5) (MW 120 kDa) was examined in several previous studies, including those aimed at developing imaging probes. For example, Slavin-Chiorini *et al.* and Chinn *et al.* reported shorter blood half-lives for the HuCC49. ΔC_H2 proteins compared with intact antibodies (6),(7). Mueller *et al.* (8) reported similar findings for the anti-GD2 antibody ch14.18- ΔC_H2 , suggesting that it was a promising format for further study as a radiolabeled imaging agent.

Neuroblastoma, the most common extracranial solid tumor in children, presents with large clinical heterogeneity. Early-stage disease (i.e., I or II) typically responds well to therapy, while advanced-stage disease (i.e., III and IV) is typically more resistant. As with many malignancies, improved detection and characterization of neuroblastoma is critical to designing patient-specific therapeutic regimes. In current practice, patients with neuroblastoma are typically imaged with various radiopharmaceuticals including [^{18}F]FDG, [^{123}I]mIBG, and $^{99\text{m}}\text{Tc}$ -MDP (9),(10). The evaluation of [^{18}F]FDG imaging in children following radiation therapy, chemotherapy and surgery is complicated by the effects of these treatments. Iodine-labeled mIBG is very specific, but the value of post-therapy scans is controversial because uptake can persist long after effective treatment, producing a false negative result (11),(12). Technetium-99m-MDP is considered useful in initial staging, especially for bone metastases, but not in later restaging studies or in assessing response-to-therapy, again because of the effects of therapy (9). Consequently, there remains a clinical need for a more specific imaging probe for the detection and monitoring of neuroblastoma.

GD2 is a disialoganglioside that is expressed at high levels on the surface of neuroblastoma tumor cells but is not found in normal tissue (13),(14) making it a promising target for antibody-based *ex vivo* cytochemical identification (15),(16), imaging (17),(18),(19) and therapy (20) of neuroblastoma. Clinical studies, including those by Reuland (16) and Yeh (21), have described the advantages of anti-GD2 radioimmunoimaging over ^{123}I -mIBG imaging including early information about response-to-therapy and disease progression. Intact radiolabeled anti-GD2 antibodies, however, are suboptimal as imaging agents because the clearance half-life from non-target tissue is up to three days.

In previous work, we described the use of SarAr for radiolabeling the anti-GD2 antibody ch14.18 with ^{64}Cu , and compared the biodistribution of ^{64}Cu -SarAr-ch14.18 to that of ch14.18 labeled with ^{64}Cu using other BFCs (22). However, the biological half-life of this radioimmunoconjugate is somewhat longer than desirable for an imaging agent. In contrast, ^{64}Cu -labeling of ch14.18- $\Delta\text{C}_{\text{H}2}$ using SarAr as the BFC could produce a NB imaging agent with high specificity, improved pharmacokinetics, and high *in vivo* retention of the ^{64}Cu label. Unfortunately, initial studies of ^{64}Cu -SarAr-ch14.18- $\Delta\text{C}_{\text{H}2}$ showed very high renal uptake that we hypothesized was due to the high net positive charge of the Cu-SarAr moiety. To test this hypothesis, we have prepared a series of Sar-based chelators with lower net positive charges than SarAr and compared the biodistribution of ch14.18- $\Delta\text{C}_{\text{H}2}$ labeled with ^{64}Cu using these BFCs with that of several commonly used polyaza polycarboxylate BFCs (DOTA, NOTA, and TETA). Based on the results of this screening study, we evaluated the most promising of the radioimmunoconjugates in a mouse model of metastatic neuroblastoma.

EXPERIMENTAL PROCEDURES

General

Chemicals and reagents were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Cell culture reagents were obtained from Mediatech (Herndon, VA) unless otherwise stated. Glassware was washed with 2 M nitric acid and rinsed with ultra-pure water (>15 M Ω resistivity) (US Filter/Siemens Water Technologies, Warrendale, PA) before use. All solutions were prepared using ultra-pure water. Metal contaminants in prepared solutions were decreased by passing the solutions through a Chelex-100 resin column (Bio-Rad Laboratories, Hercules, CA). Metal-naïve pipette tips were purchased from Rainin Instrument (Oakland, CA). Copper-64 was purchased from Washington University – St. Louis (St. Louis, MO) (23) and was supplied in 0.04 M HCl. Radioactivity in the tissue samples was assayed with a Packard Cobra II automated gamma counter (Meriden, CT). Small-animal PET images were obtained using a Siemens Focus 120 camera (Siemens Medical Solutions USA, Malvern PA) (BFC studies) or a Bruker Albira multimodality (PET/SPECT/CT) small-animal imaging system (Bruker Corporation; Woodbridge CT) (metastatic model study). Computed Tomography (CT) images were obtained using a Siemens MicroCAT II scanner (Siemens Medical Solutions USA, Inc., Malvern PA). The anti-GD2 engineered immunoprotein ch14.18- Δ C_H2 (8) was kindly provided by Rupert Handgretinger, MD (Universität Tübingen, Germany). All animal procedures were carried out under a protocol approved by the Boston Children's Hospital Institutional Animal Care and Use Committee.

Bifunctional Chelators

The following BFCs were investigated in this study: 1-*N*-(4-aminobenzyl)-3,6,10,13,16,19-hexaazabicyclo[6.6.6]eicosane-1,8-diamine, *SarAr*, 5-((8-amino-3,6,10,13,16,19-

hexaazabicyclo[6.6.6]icosan-1-yl)amino)-5-oxopentanoic acid, $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$; methyl 5-(8-amino-3,6,10,13,16,19-hexaaza-bicyclo[6.6.6]icosan-1-ylamino-5-oxopentanoate, $(\text{CH}_3)(\text{CO}_2\text{H})\text{sar}$; 5-oxo-5-(8-(2-sulfobenzamido)-3,6,10,13,16,19-hexaaza-bicyclo[6.6.6]icosan-1-ylamino)pentanoic acid, $(\text{SO}_3\text{H})(\text{CO}_2\text{H})\text{sar}$; S-2-(4-aminobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, *p*- $\text{NH}_2\text{-Bn-DOTA}$; 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid, *BAT-6*; and S-2-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid, *p*- NCS-Bn-NOTA . $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ and *BAT-6* were generously donated by Matthew Harris, PhD, (Clarity Pharmaceuticals; Sydney, Australia) and Claude F. Meares, PhD, (UC Davis, CA), respectively. The compounds *p*- $\text{NH}_2\text{-Bn-DOTA}$ and *p*- NCS-Bn-NOTA were purchased from Macrocyclics (Dallas, TX). *SarAr* (24) and $(\text{CH}_3)(\text{CO}_2\text{H})\text{sar}$ (25) were synthesized as described previously. All other reagents and solvents were obtained from standard commercial sources and were used as received. Nuclear magnetic resonance (NMR) spectra were acquired on a Varian FT-NMR 500 spectrometer and a Varian FT-NMR 400 spectrometer. Hydrogen-1 nuclear magnetic resonance ^1H NMR spectra were acquired at 500 or 400 MHz and ^{13}C NMR spectra were acquired at 125.7 MHz. All NMR spectra were recorded at 25 °C. ^1H chemical shifts were referenced to the residual solvent peak, and ^{13}C chemical shifts were referenced to acetone in D_2O ($\delta = 30.89$ and 215.94 ppm). ESIMS spectra were recorded on an Agilent 6510 esi-TOF LC/MS mass spectrometer (Agilent, Palo Alto, CA). Microanalyses for C, H, and N were carried out by Chemical & Microanalytical Services (CMAS) Pty., Ltd. (Belmont, Victoria, Australia).

Synthesis of $[\text{Cu}(\text{NH}_3)(\text{CO}_2\text{CH}_3)](\text{CF}_3\text{SO}_3)_3 \cdot 0.5(\text{C}_2\text{H}_6\text{O})$

To a solution of $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ (26) (0.20 g, 0.5 mmol) in distilled water (50 mL) was added copper acetate hydrate (0.10 g, 0.5 mmol) and the solution was stirred at room temperature for 2 h. The solution was applied to a column of Dowex 50WX2 cation exchange resin (H^+ form, 10

x 5 cm). The column was washed with H₂O (500 mL) and 1 M HCl (200 mL) and eluted with 3 M HCl (300 mL). The solution was taken to dryness under reduced pressure leaving a purple residue. To the residue was added methanol (30 mL), followed by the dropwise addition of trifluoromethanesulfonic acid (~2 mL). The resulting blue solution was heated at reflux for 2 h. The solution was allowed to cool before the volatiles were removed under reduced pressure. The residue was cooled in an ice bath and diethyl ether was added to precipitate a purple solid, which was collected by filtration and washed with copious amounts of diethyl ether. The purple solid was recrystallised from acetonitrile/diethyl ether followed by acetone/diethyl ether, collected by filtration and dried to give [Cu(NH₃)(CO₂CH₃)sar](CF₃SO₃)₃·0.5(C₂H₆O) as a light blue powder (0.24 g, 24 mmol, 48%). (Found: C, 29.75; H, 5.15; N, 11.50; calcd for C₂₄H₄₆CuF₉N₈O_{12.5}S₃: C, 29.49; H, 4.74; N, 11.46). ESIMS: (+ve ion) [M – H]²⁺ m/z 100% 252.63 (experimental), 252.63 (calcd).

Synthesis of Cu(SO₃)(CO₂)sar)·2.5(H₂O):

To a solution of [Cu(NH₃)(CO₂CH₃)sar](CF₃SO₃)₃·0.5(C₂H₆O) (0.09 g, 0.09 mmol) in anhydrous *N,N*-dimethylacetamide (2 mL) was added 2-sulfobenzoic anhydride (0.04 g, 0.2 mmol) in anhydrous *N,N*-dimethylacetamide (1 mL) and *N,N*-diisopropylethylamine (70 μL, 0.4 mmol) in anhydrous *N,N*-dimethylacetamide (1 mL). The solution was heated at 50 °C for 2 h. The reaction was monitored on a microcolumn of SP Sephadex C25 cation exchange resin (Na⁺ form) eluted with 0.05 M sodium citrate. When a single, fast-moving, band was detected the reaction mixture was cooled and water (50 mL) was added. The mixture was applied to a column of SP Sephadex C25 (Na⁺ form, 6 x 3 cm), and after washing with water, the complex was eluted with 0.05 M sodium citrate. The major band was applied to a Dowex 50W-X2 cation exchange column (H⁺ form, 10 x 5 cm). After the column was washed with water (500 mL) and 1 M HCl (500 mL), the complex was eluted with 3 M HCl, and the eluent was evaporated to

dryness under reduced pressure, leaving $[\text{Cu}(\text{SO}_3\text{H})(\text{CO}_2\text{H})\text{sar}]\text{Cl}_2 \cdot x\text{HCl}$ a purple residue (0.08 g). To the residue was added Dowex 1X8 anion exchange resin (acetate form). The resulting slurry was left standing with occasional swirling for 20 min before being filtered through a sintered glass frit. Elution with water removed a blue solution that was concentrated to ~1 mL under reduced pressure. Methanol was slowly added until the solution was turbid. After standing at room temperature, fine blue needles were obtained that were collected by filtration. The crystals were washed with methanol and diethyl ether and then dried to give $\text{Cu}(\text{SO}_3)(\text{CO}_2)\text{sar} \cdot 2.5(\text{H}_2\text{O})$ (0.04 g, 0.05 mmol, 55%). Elemental analysis: found: C, 43.26; H, 6.66; N, 15.53; calcd for $\text{C}_{26}\text{H}_{47}\text{CuN}_8\text{O}_{9.5}\text{S}$: C, 43.41; H, 6.59; N, 15.58. ESIMS: (+ve ion) $[\text{M} + \text{H}^+]^+$ m/z 100% 674.23 (experimental), 674.23 (calcd). $[\text{M} + 2\text{H}^+]^{2+}$ m/z 100% 337.62 (experimental), 337.62 (calcd). Crystals suitable for x-ray crystallography were grown by vapor diffusion of acetone into a solution of the complex in water.

Synthesis of $(\text{SO}_3\text{H})(\text{CO}_2\text{H})\text{sar} \cdot x\text{HCl} \cdot x\text{H}_2\text{O}$

The pH of an aqueous solution (20 mL) of $[\text{Cu}(\text{SO}_3\text{H})(\text{CO}_2\text{H})\text{sar}]\text{Cl}_2 \cdot x\text{HCl}$ (0.12 g, 0.16 mmol, based on $x = 0$) was increased to pH ~ 9 by addition of NaOH solution (0.2 M). The resulting blue solution was slowly added to a flask sparged with N_2 containing NaBH_4 (0.10 g) and Pd/C (0.50 g) in distilled water (20 mL) causing effervescence. The reaction mixture was stirred at room temperature overnight, and the resulting suspension was filtered through a 0.45 μm filter. The colorless filtrate was acidified with 1 M HCl (100 mL) and applied to a Dowex 50W $\times$ 2 cation exchange column (H^+ form, 8 x 3 cm). After the column was washed with 1 M HCl solution (500 mL), the compound was eluted with 4 M HCl (175 mL). The eluate was evaporated to dryness under reduced pressure at 40 $^\circ\text{C}$ leaving the $(\text{SO}_3\text{H})(\text{CO}_2\text{H})\text{sar} \cdot x\text{HCl} \cdot x\text{H}_2\text{O}$ as a colorless residue (0.09 g, 0.15 mmol, 94% approx. yield based on $x = 0$). ESIMS: (+ve ion) $[\text{M} + \text{H}^+]^+$ m/z

100% 613.31 (experimental), 613.31 (calcd). ^1H NMR (D_2O): δ = 1.90, m, 2H, CH_2 ; 2.38, t, ^3J = 7.4 Hz, 2H, CH_2 ; 2.45, t, ^3J = 7.2 Hz, 2H, CH_2 ; 3.1-3.9, broad, 24H, cage CH_2 ; 7.48-7.52, m, 1H, ArH; 7.64-7.69, m, 2H, ArH; 7.91-7.94, m, 1H, ArH. ^{13}C NMR (D_2O): δ = 20.8, 33.4, 35.4, glutarate CH_2 ; 46.9, 48.0, 51.4, cage CH_2 ; 56.3, 57.4, quat. C of cage cap; 127.7, 128.4, 131.4, 132.4, 133.4, 139.7, ArC; 172.7, 177.8, 178.5, CO.

Crystallography

Intensity data for $\text{Cu}(\text{SO}_3)(\text{CO}_2)\text{sar} \cdot 9(\text{H}_2\text{O})$ was collected with an Oxford Diffraction SuperNova CCD diffractometer using Cu-K α radiation (graphite crystal monochromator λ = 1.54184 Å). The temperature during data collection was maintained at 130.0(1) K using an Oxford cooling device. Data were reduced and corrected for absorption (27). The structures were solved by direct methods and difference Fourier synthesis (28). Thermal ellipsoid plots were generated using ORTEP-3 (29) integrated within the WINGX (30) suite of programs.

Conjugation of the BFCs to ch14.18- $\Delta\text{C}_\text{H}2$

The ch14.18- $\Delta\text{C}_\text{H}2$ antibody was buffer-exchanged and concentrated using centrifugal filter units (30 kDa MWCO; Centricon, Millipore, Billerica MA). SarAr, $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$, $(\text{CH}_3)(\text{CO}_2\text{H})\text{sar}$, $(\text{SO}_3\text{H})(\text{CO}_2\text{H})\text{sar}$ and $p\text{-NH}_2\text{-Bn-DOTA}$ were conjugated to the antibody using the zero length linker 1-ethyl-3-(3-(dimethylamino) propyl) carbodiimide hydrochloride (EDC; Pierce, Rockford, IL). The method for $p\text{-NH}_2\text{-Bn-DOTA}$ is presented as an example. To 20 mg $p\text{-NH}_2\text{-Bn-DOTA}$ in 50 μL Me_2SO (400 mg/mL) was added 3 volume equivalents of 0.1 M sodium acetate buffer (pH 5.0) to give a final BFC concentration of 100 mg/mL. The pH was brought to 5.0 by addition of 1 N NaOH. The chelator was added to the antibody at a molar ratio of 250:1, followed by sodium acetate buffer (to bring final protein concentration to 5 mg/mL), and then 500 molar equivalents of EDC, prepared immediately before use in ultrapure water at

a concentration of 50 mg/mL. After 30 min, unbound chelator was separated from the immunoconjugate by size-exclusion HPLC (BioSep SEC-S3000 column; Phenomenex, Torrance, CA) with an aqueous phase of 0.1 M sodium acetate, pH 5.0 (flow rate: 1 mL/min). The retention time of the immunoconjugate averaged 8.1 min, with the unbound chelator eluting at approximately 11.0 min. Purified immunoconjugate fractions were pooled, concentrated and then stored in aliquots at -80 °C.

BAT-6 was conjugated to the immunoprotein as previously described (31). Briefly, to 1 mg of protein (16.4 mg/mL) in 60.9 μ L of tetramethylammonium phosphate buffer (pH 8.0) was added 0.133 μ M BAT-6 in ultrapure water (9.25 μ L) and freshly prepared 2-iminothiolane (2IT, 0.01 mg) in 50 mM triethanolamine hydrochloride (13.1 μ L) at pH 8.7 such that final concentrations were: Ab, 0.1 mM; BAT-6, 2 mM; 2IT, 1 mM. The reaction was mixed by gentle pipetting, briefly centrifuged, and then placed in a 37 °C water bath for 30 min. Unbound chelator was removed by HPLC as before, and the immunoconjugate was concentrated into NH₄OAc buffer (0.1 M, pH 5.0) as previously described and stored in aliquots at -80 °C.

The conjugation of *p*-NCS-Bn-NOTA to ch14.18- Δ C_H2 was carried out as described by Vosjan *et al.* (32). Briefly, to 1 mg of ch14.18- Δ C_H2 (4 mg/mL) in sodium carbonate buffer (0.1 M, pH 9.1) was added 14.7 μ L of *p*-NCS-Bn-NOTA (0.855 mg/mL) divided into 3 aliquots separated by 5 min (final molar ratio of Ab to BFC = 1:3). The reaction was mixed by gentle pipetting following each addition then placed in a 37 °C water bath for 1 h. Unbound chelator was removed by HPLC as before, and the immunoconjugate was concentrated and buffer exchanged into NaOAc buffer (0.1 M, pH 5.0) as before and stored in aliquots at -80 °C.

Antigen Binding of Immunoconjugates

The bioactivity of the conjugated immunoproteins was assayed by enzyme-linked immunosorbent assay (ELISA). Lyophilized GD2 (Calbiochem, EMD Biosciences, Inc., La Jolla, CA) was dissolved in ethanol (20 µg/mL) and aliquoted into 96 well plates (100 µL per well). The plates were allowed to dry in a laminar flow hood overnight. Non-specific protein binding was blocked for 1 h with 200 µL of phosphate buffered saline (PBS) containing 0.05% Tween 20, 1% bovine serum albumin (BSA) (w/v), and 1% normal goat serum (v/v). Following removal of the blocking solution, dilutions of the immunoproteins in the blocking solution were applied to the blocked wells and incubated for 2 h. The wells were then washed with PBS (200 µL x 3), and 100 µL horseradish peroxidase-conjugated goat anti-mouse polyclonal IgG (40 ng/mL; Jackson Laboratories, Bar Harbor, ME) diluted in PBS containing 0.05% Tween 20 and 1% BSA was applied to the wells. After 1 h, the wells were washed, and 100 µL of TMB peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was applied to the wells, followed 20 min later by 100 µL of 1 M H₃PO₄. Absorbance (O.D.) was then read at 450 nm using an automated 96-well plate reader.

Isotope Dilution Assay

The ratio of available chelating molecules to protein molecules was measured as previously described (33) using the method reported by Meares *et al.* (34) except that ⁶⁴Cu was used instead of ⁵⁷Co. Briefly, a small amount of ⁶⁴Cu was added to a solution of known concentration of non-radioactive ("cold") copper from a commercially available standard (Sigma Aldrich, St. Louis, MO) in acetate buffer (0.1 M, pH 5.0). An aliquot of immunoprotein conjugated with chelator was added and incubated for 1 h. The degree of labeling was then analyzed using thin-layer chromatography (ITLC SG, Biodex, Shirley, NY) developed with phosphate buffer (pH 8, 100 mM EDTA). Under these conditions, the labeled antibody remains at the baseline and

unbound ^{64}Cu travels at the solvent front. The number of available chelators was then calculated from the concentrations of immunoprotein, cold copper, and degree of incorporation.

Radiolabeling with ^{64}Cu

Three volume equivalents of sodium acetate buffer (0.1 M, pH 5.0) were added to 2 mCi ^{64}Cu in 5-10 μL HCl (0.04 N), and a solution of the antibody in acetate buffer (250 $\mu\text{g}/75 \mu\text{L}$) was then added to the ^{64}Cu solution. After 30 min incubation at 25 $^{\circ}\text{C}$, the degree of radiolabeling was assessed by TLC using the same conditions as described above. If the TLC assay showed that the radiochemical purity was <95% the product was purified using centrifugal filter units and the post-filtration purity was confirmed by TLC. The radioimmunoconjugate was diluted with saline to give a final protein concentration of 0.5 mg/mL and sterile filtered (0.2 μm) before injection.

Biodistribution Studies

The ^{64}Cu -labeled antibody (50 μg antibody, 5.5 ± 1.5 MBq ($149 \pm 41 \mu\text{Ci}$)) was injected into the tail vein of mice (athymic (nu/nu) females (The Jackson Laboratory; Bar Harbor ME; 6-8 weeks of age and 20-25 g in weight). The mice were then anesthetized using isoflurane (1-4% in a flow of oxygen) and PET data were collected for 30 min. at 24 and 48 h post injection (p.i.). At 48 h p.i., CT images were obtained. After CT imaging, the mice were euthanized by CO_2 inhalation, and an ex vivo biodistribution analysis was carried out. Tissues were collected and weighed, and the radioactivity was assayed. PET and CT images were registered manually using AMIDE software (35). Data from volumes of interest (VOIs) were used to calculate the biodistribution in selected tissues for the small animal PET imaging studies.

Statistical analysis

Statistical analysis was carried out using SPSS V 19.0 for Windows. The 48 h *ex vivo* biodistribution data were analyzed by ANOVA with *post hoc* analysis using the Bonferroni correction. Differences were considered significant at the 5% level ($p < 0.05$).

Metastatic Model of Neuroblastoma

An imaging study was carried out to evaluate the ability of ^{64}Cu -labeled ch14.18- $\Delta\text{C}_{\text{H}2}$ to localize to neuroblastoma (NB) liver metastases *in vivo*. IMR32 NB cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with antibiotics (100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin), L-glutamine (2 mM), and 10% FCS (Atlantic Biologicals; Miami, FL) as a semicontinuous adhesion culture, with passaging by trypsinization every 3-4 days. Cells were used after 2 weeks of initiation of culture, and were grown *in vitro* for no longer than 12 weeks. For tumor implantation, cells were trypsinized (0.05% Trypsin, 0.53 mM EDTA in HBSS), pelleted by centrifugation, resuspended in medium, counted using a hemocytometer, recentrifuged and resuspended in PBS at a concentration of 20×10^6 cells/ml. Athymic (nu/nu) female mice (The Jackson Laboratory; Bar Harbor ME; 6-8 weeks of age and 20-25 g in weight) were anesthetized (1-4% isoflurane in oxygen), the spleen was exteriorized through an incision on the left side, and 1×10^6 cells in 50 μl were injected. Two minutes after injection of the tumor cells the splenic vessels were tied off and the spleen was removed. The inner wound was sutured and the outer wound was closed using metal clips which were removed approximately 9 days post-surgery. Meloxicam (5 mg/kg subcutaneous) was administered perioperatively for analgesia.

Three weeks post-surgery the mice were injected with ^{64}Cu -NOTA-ch14.18- $\Delta\text{C}_{\text{H}2}$ (50 μg protein, mean of 75.7 μCi (range 55 - 90 μCi)) and were imaged at 1, 4, 24 and 48 h post

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3 injection. After the final imaging session the mice were euthanized by CO₂ asphyxiation and
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5 samples of the liver were taken for histological analysis. Tissue was frozen in isopentane cooled
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7 in a slurry of isopropanol and frozen carbon dioxide and then stored at -80 °C. Tissues were
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9 sectioned (10 μm thickness) using a cryotome, air dried, fixed in methanol, and then exposed to
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11 phosphor storage screens. The screens were scanned using a Fujifilm BAS-5000, and the
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13 resulting digital images of radionuclide distribution within the tissue were compared with the
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15 sections following hematoxylin and eosin staining to demonstrate general morphology.
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RESULTS

Synthesis

The complex $[\text{Cu}(\text{NH}_3)(\text{CO}_2\text{CH}_3)\text{sar}](\text{CF}_3\text{SO}_3)_3$ was acylated with 2-sulfobenzoic anhydride (**Fig. 2, top**). The presence of the methyl ester ensured that the sulfonated Cu^{II} complex carried a positive charge suitable for application to cation exchange resin for purification. The ester was hydrolysed upon treatment with strong acid and the product, $\text{Cu}(\text{SO}_3)(\text{CO}_2)\text{sar}$ was isolated upon neutralization with Dowex 1 $\times$ 8 anion exchange resin (acetate form) and precipitation with methanol. Crystals of $\text{Cu}(\text{SO}_3)(\text{CO}_2)\text{sar}\cdot 9(\text{H}_2\text{O})$ were analysed by X-ray crystallography (**Fig. 3**, see also supplementary data **Table S1**). The structural analysis shows the complex to be neutral with deprotonated sulfonate and carboxylate groups. The Cu-N bond lengths of the distorted octahedral $\text{Cu}(\text{SO}_3)(\text{CO}_2)\text{sar}$ complex range from 2.244(3)-2.107(4) Å. Perhaps because of the constraints imposed by the sarcophagine cage, there is no apparent pattern to the differences in Cu-N bond lengths. The aliphatic carboxylate functional group extends away from the macrobicyclic cage, which is present in the $1e/3$ conformation, where the C-C bonds of the five-membered chelate rings are parallel to the pseudo- C_3 axis. The metal ion was removed from $\text{Cu}(\text{SO}_3)(\text{CO}_2)\text{sar}$ by reducing the Cu^{II} with NaBH_4 and Pd/C followed by purification by cation exchange chromatography to give the free ligand, $(\text{SO}_3)(\text{CO}_2\text{H})\text{sar}$.

Conjugation, Antigen Binding and Radiolabeling

Examples of the ELISA results are presented in **Figure 4**. The study demonstrated that all of the immunoconjugates retained antigen binding following conjugation.

The number of available chelators attached to each antibody fragment was found to range from 0.18 ± 0.008 to 2.0 ± 0.4 (means of three separate determinations $\pm$ E.S.D.). The

immunoconjugates were labeled to specific activities of 0.073-0.15 MBq (1.98-4.03 μ Ci) per μ g protein.

Imaging and Biodistribution

Examples of images from data collected at 48 h p.i. for the Sar-based chelators are presented in **Fig. 5**. For ^{64}Cu -SarAr-ch14.18- $\Delta\text{C}_{\text{H}2}$ (**Fig. 5**), the kidney dominates the image, while kidney uptake was lower for the ^{64}Cu -(NH_2)(CO_2)sar-ch14.18- $\Delta\text{C}_{\text{H}2}$ study (**Fig. 5B**). For ^{64}Cu -(CH_3)(CO_2)sar-ch14.18- $\Delta\text{C}_{\text{H}2}$ (**Fig. 5C**), kidney uptake was lower than for ^{64}Cu -SarAr-ch14.18- $\Delta\text{C}_{\text{H}2}$, and kidney uptake of ^{64}Cu -(SO_3)(CO_2)sar-ch14.18- $\Delta\text{C}_{\text{H}2}$ (**Fig. 5D**) was further decreased, with some liver uptake evident.

The results of the analysis of the PET data are presented in **Fig. 6**. Generally, normal tissue uptake of the radioimmunoconjugates (RICs) incorporating Sar-based BFCs is higher than that of the PACs, particularly in the liver and kidney. Liver uptake of the Sar-based BFCs was within the range 10-20% ID/g. Within the Sar-based BFCs, the greatest and most significant change was in kidney uptake. Interestingly, changing the apical primary amine, which is likely to be protonated at physiological pH *in vivo*, of the sarcophagine cage (SarAr) to a methyl group ((CH_3)(CO_2H)sar) led to a decrease in kidney uptake; and a further decrease was achieved when the apical group was an aromatic sulfonate. Among the PAC BFCs, there is an overall pattern of low uptake in most tissues as well as rapid clearance. The greatest difference was seen in the liver data, i.e., using DOTA as the BFC resulted in high hepatic uptake relative to the other two PAC BFCs. Kidney uptake for the PAC BFCs is comparable to that of the lowest of the Sar-based chelators.

The results of the 48 h biodistribution study are summarized in **Table 1**. Aside from the high kidney uptake of SarAr- and $(\text{CH}_3)(\text{CO}_2)\text{sar}$ -labeled protein (46.87% and 28.37% ID/g, respectively), the tissue distributions of ch14.18- $\Delta\text{C}_{\text{H}2}$ labeled via these BFCs are similar to those of the PAC BFCs. At 48 h p.i., the blood concentration of the ^{64}Cu -DOTA-protein (2.12% ID/g) was significantly higher than that of the other RICs except for that incorporating $(\text{SO}_3)(\text{CO}_2)\text{sar}$ (1.66% ID/g). Uptake of the ^{64}Cu -labeled proteins using Sar-based BFCs in the heart was generally higher than that seen with the PAC-labeled proteins. In the lung, the $(\text{SO}_3)(\text{CO}_2)\text{sar}$ and DOTA RIC concentrations were both relatively high, possibly because of their higher blood concentration. The liver concentrations of the BAT-6- and NOTA-conjugated proteins were lower than those of the other BFCs. Overall, gut uptake was low, with similar numbers for all the BFCs, with DOTA being the highest uptake (possibly because of its higher liver concentration) and NOTA and BAT-6 being the lowest. Brain uptake was low (as expected for a large protein), with DOTA showing the highest uptake (0.34% ID/g).

Metastatic Model of Neuroblastoma

Imaging of three mice bearing intrahepatic metastases revealed high multifocal uptake of ^{64}Cu -NOTA-ch14.18- $\Delta\text{C}_{\text{H}2}$ in the liver metastases throughout the imaging time course (1 to 48 h). Typical images are shown in **Figure 7**. Histological analysis of the H&E stained tissue sections (**Fig. 7E**) revealed small tumors with a diameter of approximately 1 mm throughout the liver with some concentration towards the outer edge. The corresponding autoradiographs (**Fig. 7F**) indicated that the concentration of the radiolabeled antibody was much higher in the tumors than in the normal liver with a mean count ratio of approximately 10:1.

DISCUSSION

In this study, we investigated the distribution of ch14.18- ΔC_H2 , an engineered immunoprotein, labeled with ^{64}Cu via a series of sarcophagine (sar) and polyaza polycarboxylic (PAC) bifunctional chelators. This study was prompted by our observation that the kidney concentration of ^{64}Cu -SarAr-ch14.18- ΔC_H2 was extremely high. The result is consistent with the report by Bartolo *et al.* who observed high kidney uptake (>200% ID/g) when using SarAr to label B72.3 F(ab')₂ (36). These results led to the hypothesis that the high renal concentration was a consequence of the ionic positive charge associated with the Cu^{II} -SarAr moiety, and a series of sar BFCs were prepared with different ionic charges. The biodistribution of the ^{64}Cu -labeled RICs prepared using these BFCs showed a large range of uptake by the kidneys, from 46.9% ID/g for $^{64}\text{Cu}^{\text{II}}$ -SarAr (net local charge increase¹: +4) to 28.4% ID/g for $^{64}\text{Cu}^{\text{II}}$ -(CH₃)(CO₂)sar (net local charge increase: +1) to 6.64% ID/g for $^{64}\text{Cu}^{\text{II}}$ -(SO₃)(CO₂)sar (net local charge increase: 0). Interestingly, $^{64}\text{Cu}^{\text{II}}$ -(NH₂)(CO₂)sar-ch14.18- ΔC_H2 (net local charge increase: +2; renal uptake: 5.48% ID/g) does not seem to fit this pattern, since it has a higher apparent net charge and lower renal uptake.

The reason that $^{64}\text{Cu}^{\text{II}}$ -(NH₂)(CO₂)sar-ch14.18- ΔC_H2 does not fit this pattern can be understood when one considers the chemistry of the conjugation reaction between the BFC and the protein. In this study the sar BFCs were conjugated to the protein using EDC which reacts with carboxylates to form an O-acylisourea intermediate that can react with amines to form an amide

¹ Net local change in charge: To calculate this we considered: the apical charge on the BFC at physiological pH (e.g. for SarAr = +1); the copper (+2); and the effect of reacting with the protein group (in the case of SarAr a loss of CO₂⁻, so a net +1), giving a total of +4. We assumed that the secondary amines of the Sar cage would not be protonated following radiolabeling as the coordination of the metal ion would increase the acidity of the donor atoms.

bond. Thus EDC will react with the carboxylic acid groups on $(\text{NH}_2)(\text{CH}_3)\text{sar}$ and $(\text{SO}_3\text{H})(\text{CO}_2\text{H})\text{sar}$ and form amide bonds with amine residues (e.g., lysine) on the protein. Similarly, EDC will react with carboxylic acid residues (e.g., glutamate) on the protein to form amide bonds with the aromatic or alkyl amine on SarAr. In the case of $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$, both an alkyl amine and a carboxylic acid are present on the chelator allowing the formation of an amide bond either between the amine on the $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ and a carboxylic acid on the protein or the carboxylic acid on the $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ and an amine on the protein. If the carboxylic acid on the chelator reacts with an amine on the protein, the result is that the amine on the chelator is exposed, presenting a positive charge to the environment. Conversely, if the amine on the chelator reacts with a carboxylate on the protein, the net result is that the carboxylate is exposed, presenting a negative charge to the environment. Nakajima and Ikada (37) examined the mechanism of the EDC conjugation reaction and present a strong argument in favor of the reaction between the primary amine of the $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ chelator and a carboxylate group on the protein. The results of the present study are in agreement with their conclusion in that the renal uptake of $^{64}\text{Cu}-(\text{NH}_2)(\text{CO}_2\text{H})\text{sar-ch14.18-}\Delta\text{C}_{\text{H}2}$ is similar to that of $^{64}\text{Cu}-(\text{SO}_3)(\text{CO}_2)\text{sar-ch14.18-}\Delta\text{C}_{\text{H}2}$, which presents a negatively charged sulfonate to the environment, suggesting that the outer charge following conjugation with $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ is negative. The only previous use of $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ to radiolabel an antibody compared its performance in radiolabeling rituximab with seven other BFCs.(38) In that study, $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ was activated using EDC and then stabilized using sulfonated *N*-hydroxysuccinimide (sulfo-NHS), which was then reacted with the amine groups of the protein. In this case, EDC can only activate the carboxylate on the BFC because the protein is not yet present in the solution and the resulting sulfo-NHS will react with an amine on the protein leaving the amine of the $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ ligand exposed to the environment. Consistent with this premise, the kidney uptake of $^{64}\text{Cu}-(\text{NH}_2)(\text{CO}_2\text{H})\text{sar-rituximab}$ was the highest of the BFCs investigated in that study.

The sarcophagine chelator SarAr was introduced as a BFC for labeling antibodies with ^{64}Cu as an alternative to the widely used DOTA, which lacks kinetic stability.⁽¹⁾ Accordingly, we compared the performance of the new sar chelators to three polyaza polycarboxylate (PAC) BFCs (DOTA, NOTA, and BAT-6) that are also frequently used to label antibodies and other biomolecules with ^{64}Cu (Table 1). Among the three PAC chelators, the highest liver uptake was observed for DOTA (13.6% ID/g) while both BAT-6 (3.6% ID/g) and NOTA (4.0 % ID/g) were significantly lower. Similarly, the concentration of ^{64}Cu -DOTA-ch14.18- $\Delta\text{C}_{\text{H}2}$ in the blood and lung at 48 h p.i. was also higher than that of the BAT-6 and NOTA conjugates. Comparing the PAC BFCs to the new sar chelators, the renal concentration of the three PAC conjugates is generally lower than that of all of the sar-based chelators, although the differences are statistically significant only for SarAr and $(\text{CH}_3)(\text{CO}_2\text{H})\text{sar}$. The liver concentrations of the BAT-6 and NOTA conjugates are lower than those of the other conjugates. These results suggest that the preferred BFC for labeling ch14.18- $\Delta\text{C}_{\text{H}2}$ with ^{64}Cu is *p*-NCS-Bn-NOTA. This BFC contains three anionic carboxylate functional groups and thus forms mono anionic copper complexes resulting in an RIC with lower retention in the kidneys compared to the positively-charged conjugates that utilise cationic sarcophagine complexes.

The nature of the immunoprotein is also important in assessing these results. The $\Delta\text{C}_{\text{H}2}$ format was generated for targeting GD2 because the (monovalent) Fab portion of the antibody ch14.18 failed to bind, suggesting that divalent binding was necessary for tumor targeting (8). Moreover, ch14.18- $\Delta\text{C}_{\text{H}2}$ has higher affinity for GD2 than does the whole antibody ch14.18 and has much faster clearance from the blood and other normal tissues (8). The rapid clearance from the blood is due, at least in part, to the absence of the $\text{C}_{\text{H}2}$ domain, which contains three of the six amino acid residues required for interaction with the neonatal Fc receptor (FcRn) in the kidney,

part of the recycling system that ensures antibodies in the blood are not continually excreted (39). As the ΔC_H2 cannot be returned to the blood, it is excreted into the urine, facilitating its clearance from non-target tissues.

This study is part of our continuing effort to develop an imaging agent for the detection and characterization of neuroblastoma metastases. Although several agents are currently available for imaging neuroblastoma, Yeh *et al.* (21) and Reuland *et al.* (18) observed that anti-GD2 antibodies may give an earlier indication of response-to-therapy than does $^{123/131}\text{I}$ -mIBG. A radiolabeled anti-GD2 antibody may also prove important in the evaluation of patients who are being considered for immunotherapy with non-radioactive hu14.18, the humanized version of the ch14.18 antibody.

To demonstrate the potential of ^{64}Cu -labeled ch14.18- ΔC_H2 for imaging neuroblastoma metastases, we carried out a preclinical imaging study in an orthotopic model of intrahepatic metastases using NOTA as the BFC. Focal uptake of ^{64}Cu -NOTA-ch14.18- ΔC_H2 was observed in the liver with minimal uptake in other areas (**Fig. 7**). At necropsy small lesions (<1 mm) were observed in the liver that were confirmed to be tumor by histological analysis and autoradiography. The apparently larger focal areas observed in the PET images of tumors detected by the PET camera are due to the physical limitations of the microPET system. The system resolution is approximately 1.4 mm, so partial volume averaging causes the lesions to appear much larger than they actually are and with lower RIC uptake. Interestingly, the images are similar throughout the two-day imaging study with the lesions being clearly seen as early as 4 h post-injection. This suggests that shorter half-life radionuclides, such as ^{68}Ga ($t_{1/2} = 68$ min.), could be used with NOTA-ch14.18- ΔC_H2 , providing the same information while greatly reducing the time required between injection and imaging and the radiation dose to the patient.

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5 In conclusion, these results support the hypothesis that the high renal uptake of ^{64}Cu -SarAr-
6 ch14.18- $\Delta\text{C}_\text{H}2$ was due to its high net positive charge with Sar analogs having lower net positive
7 charges showing lower uptake in the kidneys. However, the PAC BFC conjugates all showed
8 low tissue accumulation compared with the Sar-based chelators, with ^{64}Cu -NOTA-ch14.18-
9 $\Delta\text{C}_\text{H}2$ offering the greatest potential as an imaging agent for neuroblastoma. Future studies will
10 focus on the use of the RIC to detect and characterize neuroblastoma metastases and monitor
11 therapy response.
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Conflict of Interest Statement

The authors declare no competing financial interest.

References

- (1) Maheshwari, V., Dearling, J. L. J., Treves, S. T., and Packard, A. B. (2012) Measurement of the rate of copper(II) exchange for ^{64}Cu complexes of bifunctional chelators. *Inorg. Chim. Acta* 393, 318-323.
- (2) Smith, S. V. (2004) Molecular imaging with copper-64. *J Inorg Biochem* 98, 1874-901.
- (3) Dearling, J. L., and Pedley, R. B. (2007) Technological advances in radioimmunotherapy. *Clin. Oncol. (R. Coll. Radiol.)* 19, 457-69.
- (4) Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* 52, 3402-8.
- (5) Gillies, S. D., and Wesolowski, J. S. (1990) Antigen binding and biological activities of engineered mutant chimeric antibodies with human tumor specificities. *Hum Antibodies Hybridomas* 1, 47-54.
- (6) Slavin-Chiorini, D. C., Kashmiri, S. V., Schlom, J., Calvo, B., Shu, L. M., Schott, M. E., Milenic, D. E., Snoy, P., Carrasquillo, J., Anderson, K., and Horan Hand, P. (1995) Biological properties of chimeric domain-deleted anticarcinoma immunoglobulins. *Cancer Res.* 55, 5957s-5967s.
- (7) Chinn, P. C., Morena, R. A., Santoro, D. A., Kazules, T., Kashmiri, S. V., Schlom, J., Hanna, N., and Braslawsky, G. (2006) Pharmacokinetics and tumor localization of (^{111}In) -labeled HuCC49DeltaC_H2 in BALB/c mice and athymic murine colon carcinoma xenograft. *Cancer Biother. Radiopharm.* 21, 106-16.
- (8) Mueller, B. M., Reisfeld, R. A., and Gillies, S. D. (1990) Serum half-life and tumor localization of a chimeric antibody deleted of the CH2 domain and directed against the disialoganglioside GD2. *Proc Natl Acad Sci U S A* 87, 5702-5.
- (9) Kushner, B. H. (2004) Neuroblastoma: a disease requiring a multitude of imaging studies. *J. Nucl. Med.* 45, 1172-88.
- (10) Howman-Giles, R., Shaw, P. J., Uren, R. F., and Chung, D. K. (2007) Neuroblastoma and other neuroendocrine tumors. *Semin. Nucl. Med.* 37, 286-302.
- (11) Hero, B., Hunneman, D. H., Gahr, M., and Berthold, F. (2001) Evaluation of catecholamine metabolites, mIBG scan, and bone marrow cytology as response markers in stage 4 neuroblastoma. *Med. Pediatr. Oncol.* 36, 220-3.
- (12) Matthay, K. K., Edeline, V., Lumbroso, J., Tanguy, M. L., Asselain, B., Zucker, J. M., Valteau-Couanet, D., Hartmann, O., and Michon, J. (2003) Correlation of early metastatic response by ^{123}I -metaiodobenzylguanidine scintigraphy with overall response and event-free survival in stage IV neuroblastoma. *J. Clin. Oncol.* 21, 2486-91.
- (13) Schulz, G., Cheresch, D. A., Varki, N. M., Yu, A., Staffileno, L. K., and Reisfeld, R. A. (1984) Detection of ganglioside GD2 in tumor tissues and sera of neuroblastoma patients. *Cancer Res.* 44, 5914-20.
- (14) Wu, Z. L., Schwartz, E., Seeger, R., and Ladisch, S. (1986) Expression of GD2 ganglioside by untreated primary human neuroblastomas. *Cancer Res.* 46, 440-3.
- (15) Lode, H. N., Handgretinger, R., Schuermann, U., Seitz, G., Klingebiel, T., Niethammer, D., and Beck, J. (1997) Detection of neuroblastoma cells in CD34+ selected peripheral stem cells using a combination of tyrosine hydroxylase nested RT-PCR and anti-ganglioside GD2 immunocytochemistry. *Eur. J. Cancer* 33, 2024-30.
- (16) Cheung, I. Y., Sahota, A., and Cheung, N. K. (2004) Measuring circulating neuroblastoma cells by quantitative reverse transcriptase-polymerase chain reaction analysis. *Cancer* 101, 2303-8.

- (17) Fonti, R., Cheung, N. K., Bridger, G. J., Guo, H. F., Abrams, M. J., and Larson, S. M. (1999) ^{99m}Tc -monoclonal antibody radiolabeled via hydrazino nicotinamide derivative for imaging disialoganglioside G_{D2} -positive tumors. *Nucl. Med. Biol.* **26**, 681-6.
- (18) Reuland, P., Geiger, L., Thelen, M. H., Handgretinger, R., Haase, B., Muller-Schauenburg, W., Niethammer, D., and Bares, R. (2001) Follow-up in neuroblastoma: comparison of metaiodobenzylguanidine and a chimeric anti-GD2 antibody for detection of tumor relapse and therapy response. *J. Pediatr. Hematol. Oncol.* **23**, 437-42.
- (19) Voss, S. D., Smith, S. V., DiBartolo, N., McIntosh, L. J., Cyr, E. M., Bonab, A. A., Dearling, J. L., Carter, E. A., Fischman, A. J., Treves, S. T., Gillies, S. D., Sargeson, A. M., Huston, J. S., and Packard, A. B. (2007) Positron emission tomography (PET) imaging of neuroblastoma and melanoma with ^{64}Cu -SarAr immunoconjugates. *Proc. Natl. Acad. Sci. USA* **104**, 17489-93.
- (20) Yu, A. L., Gilman, A. L., Ozkaynak, M. F., London, W. B., Kreissman, S. G., Chen, H. X., Smith, M., Anderson, B., Villablanca, J. G., Matthay, K. K., Shimada, H., Grupp, S. A., Seeger, R., Reynolds, C. P., Buxton, A., Reisfeld, R. A., Gillies, S. D., Cohn, S. L., Maris, J. M., Sondel, P. M., and Children's Oncology, G. (2010) Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *The New England journal of medicine* **363**, 1324-34.
- (21) Yeh, S. D., Larson, S. M., Burch, L., Kushner, B. H., Laquaglia, M., Finn, R., and Cheung, N. K. (1991) Radioimmunodetection of neuroblastoma with iodine-131-3F8: correlation with biopsy, iodine-131-metaiodobenzylguanidine and standard diagnostic modalities. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **32**, 769-76.
- (22) Dearling, J. L., Voss, S. D., Dunning, P., Snay, E., Fahey, F., Smith, S. V., Huston, J. S., Meares, C. F., Treves, S. T., and Packard, A. B. (2011) Imaging cancer using PET--the effect of the bifunctional chelator on the biodistribution of a (^{64}Cu)-labeled antibody. *Nucl. Med. Biol.* **38**, 29-38.
- (23) Kume, M., Carey, P. C., Gaehle, G., Madrid, E., Voller, T., Margenau, W., Welch, M. J., and Lapi, S. E. (2012) A semi-automated system for the routine production of copper-64. *Applied radiation and isotopes : including data, instrumentation and methods for use in agriculture, industry and medicine* **70**, 1803-6.
- (24) Di Bartolo N.M., S. A. M., Donlevy T.M. and Smith S.V. (2001) Synthesis of a new cage ligand, SarAr, and its complexation with selected transition metal ions for use in radioimaging. *J. Chem. Soc., Dalton Trans.*, 2303-2309.
- (25) Paterson, B. M., Roselt, P., Denoyer, D., Cullinane, C., Binns, D., Noonan, W., Jeffery, C. M., Price, R. I., White, J. M., Hicks, R. J., and Donnelly, P. S. (2014) PET imaging of tumours with a ^{64}Cu labeled macrobicyclic cage amine ligand tethered to Tyr³-octreotate. *Dalton Trans.* **43**, 1386-96.
- (26) Ma, M. T., Karas, J. A., White, J. M., Scanlon, D., and Donnelly, P. S. (2009) A new bifunctional chelator for copper radiopharmaceuticals: a cage amine ligand with a carboxylate functional group for conjugation to peptides. *Chem. Commun (Camb)*, 3237-9.
- (27) CrysAlis CCD, O. D. L., [Version 1.171.32.5 (release 08-05-2007 CrysAlis171 .NET) (compiled May 8 2007,13:10:02
- (28) Sheldrick, G. M. (2008) A short history of SHELX. *Acta Crystallogr Sect. A* **64**, 112-22.
- (29) Farrugia, L. (1997) ORTEP-3 for Windows - a version of ORTEP-III with a Graphical User Interface (GUI). *J. Appl. Cryst.* **30**, 565.
- (30) Farrugia, L. J. (1999) WinGX suite for small-molecule single-crystal crystallography. *J. Appl. Cryst.* **32**, 837-838.
- (31) McCall, M. J., Diril, H., and Meares, C. F. (1990) Simplified method for conjugating macrocyclic bifunctional chelating agents to antibodies via 2-iminothiolane. *Bioconjug. Chem.* **1**, 222-6.
- (32) Vosjan, M. J., Perk, L. R., Visser, G. W., Budde, M., Jurek, P., Kiefer, G. E., and van Dongen, G. A. (2010) Conjugation and radiolabeling of monoclonal antibodies with zirconium-89 for PET

- imaging using the bifunctional chelate p-isothiocyanatobenzyl-desferrioxamine. *Nat. Protoc.* 5, 739-43.
- (33) Hamburger, A. W., and Salmon, S. E. (1977) Primary bioassay of human tumor stem cells. *Science* 197, 461-3.
- (34) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., and McTigue, M. (1984) Conjugation of antibodies with bifunctional chelating agents: isothiocyanate and bromoacetamide reagents, methods of analysis, and subsequent addition of metal ions. *Anal. Biochem.* 142, 68-78.
- (35) Loening, A. M., and Gambhir, S. S. (2003) AMIDE: a free software tool for multimodality medical image analysis. *Mol. Imaging* 2, 131-7.
- (36) Di Bartolo, N., Sargeson, A. M., and Smith, S. V. (2006) New ^{64}Cu PET imaging agents for personalised medicine and drug development using the hexa-aza cage, SarAr. *Org. Biomol. Chem.* 4, 3350-7.
- (37) Nakajima, N., and Ikada, Y. (1995) Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. *Bioconjugate Chem.* 6, 123-30.
- (38) Cooper, M. S., Ma, M. T., Sunassee, K., Shaw, K. P., Williams, J. D., Paul, R. L., Donnelly, P. S., and Blower, P. J. (2012) Comparison of (^{64}Cu)-Complexing Bifunctional Chelators for Radioimmunoconjugation: Labeling Efficiency, Specific Activity, and in Vitro/in Vivo Stability. *Bioconjugate Chem.* 23, 1029-39.
- (39) Kenanova, V., Olafsen, T., Crow, D. M., Sundaresan, G., Subbarayan, M., Carter, N. H., Ikle, D. N., Yazaki, P. J., Chatziioannou, A. F., Gambhir, S. S., Williams, L. E., Shively, J. E., Colcher, D., Raubitschek, A. A., and Wu, A. M. (2005) Tailoring the pharmacokinetics and positron emission tomography imaging properties of anti-carcinoembryonic antigen single-chain Fv-Fc antibody fragments. *Cancer Res.* 65, 622-31.

FIGURE LEGENDS

Figure 1. Structures of the whole antibody and the engineered form used in this study, ch14.18- ΔC_H2 (green arrows show the antigen-binding regions).

Figure 2. Synthetic scheme for $(SO_3H)(CO_2H)sar$ (*top*); (i) DIPEA, DMA, H^+/H_2O ; (ii) NaOH (0.2 M), $NaBH_4$, Pd/C, HCl. Structures of the Sar-based bifunctional chelators used in this study (*bottom*).

Figure 3. A thermal ellipsoid plot for $Cu(SO_3)(CO_2)sar \cdot 9(H_2O)$. Hydrogen atoms bonded to carbon and solvent have been omitted for clarity.

Figure 4. Example ELISA data for the ch14.18- ΔC_H2 antibody and three immunoconjugates. The data confirmed that all the immunoconjugates retained antigen binding following conjugation.

Figure 5. Coronal small animal PET/CT images of mice injected with ch14.18- ΔC_H2 labeled with ^{64}Cu -labeled via SarAr (**A**), $(NH_2)(CO_2H)sar$ (**B**), $(CH_3)(CO_2H)sar$ (**C**) and $(SO_3H)(CO_2H)sar$ (**D**) at 48 hours post injection. PET data in color scale, CT data in gray scale.

Figure 6. Bar graphs of small animal PET data for the BFCs incorporating the sarcophagine chelators (*top*) and the polyaminocarboxylate chelators (*bottom*). Data presented are for 24 (white) and 48 hours (black) post injection. Note different y-axes.

Figure 7. Uptake of ^{64}Cu -NOTA-ch14.18- ΔC_H2 in a model of neuroblastoma metastases. Typical small animal PET images of distribution of the imaging agent in mice at 4 h p.i. are shown. Images of mice without (*A and B*) and with metastases (*C and D, coronal top and transaxial bottom*) are presented for comparison, scales for each set of images at right (%ID/g). Panels E (*H&E stain of tissue section, 1 mm scale bar bottom left*) and F (*autoradiography, scale at right*) show small tumors (*purple*) throughout the liver (*pink*) with radionuclide concentrated in tumor.

Table 1. Summarizing 48h *ex vivo* biodistribution data (%ID/g mean, SD in parentheses) and the results of statistical analysis (ANOVA). Numbers in italics indicate significant difference ($p < 0.05$, Bonferroni correction) compared with the indicated chelate.

Supplementary data

Table S1. Supplementary crystallographic data for $Cu(SO_3)(CO_2)sar$.

Fig. 1. Structures of the whole antibody ch14.18 and the engineered form used in this study, ch14.18-ΔC_H2 (green arrows show the antigen-binding regions).

Fig. 2. Synthetic scheme for SulphSar-CO₂H (*top*); structures of the Sar-based bifunctional chelators used in this study (*bottom*).

Fig. 3. A thermal ellipsoid plot for $\text{Cu}(\text{SO}_3)(\text{CO}_2)\text{sar}\cdot 9(\text{H}_2\text{O})$. Hydrogen atoms bonded to carbon and solvent have been omitted for clarity.

Fig. 4. Example ELISA data for the ch14.18- ΔC_H2 antibody and three immunoconjugates. The data confirmed that all the immunoconjugates retained antigen binding following conjugation.

Fig. 5. Coronal small animal PET/CT images of mice injected with ch14.18- $\Delta\text{C}_\text{H}2$ labeled with ^{64}Cu -labeled via SarAr (A), $(\text{NH}_2)(\text{CO}_2\text{H})\text{sar}$ (B), $(\text{CH}_3)(\text{CO}_2\text{H})\text{sar}$ (C) and $(\text{SO}_3\text{H})(\text{CO}_2\text{H})\text{sar}$ (D) at 48 hours post injection. PET data in color scale, CT data in gray scale.

Fig. 6. Bar graphs of small animal PET data for the BFCs incorporating the sarcophagine chelators (*top*) and the polyaminocarboxylate chelators (*bottom*). Data presented are for 24 (white) and 48 hours (black) post injection. Note different y-axes.

Fig. 7. Uptake of ^{64}Cu -NOTA-ch14.18. $\Delta\text{C}_\text{H}2$ in a model of neuroblastoma metastases. Typical small animal PET images of distribution of the imaging agent in mice at 4 h p.i. are shown. Images of mice without (A and B) and with metastases (C and D, coronal top and transaxial bottom) are presented for comparison, scales for each set of images at right (%ID/g). Panels E (H&E stain of tissue section, 1 mm scale bar bottom left) and F (autoradiography, scale at right) show small tumors (purple) throughout the liver (pink) with radionuclide concentrated in tumor.

	SarAr	(NH ₂) (CO ₂ H) sar	(CH ₃) (CO ₂ H) sar	(SO ₃ H) (CO ₂ H) sar	p-NH ₂ - Bn-DOTA	BAT-6	p-NCS- Bn-NOTA
Tissue	1	2	3	4	5	6	7
Blood	0.34 (0.04) <i>5</i>	0.67 (0.13) <i>5</i>	0.68 (0.07) <i>5</i>	1.66 (0.22) <i>7</i>	2.12 (1.20) <i>1,2,3,6,7</i>	0.66 (0.11) <i>5</i>	0.16 (0.04) <i>4,5</i>
Heart	2.89 (0.58) <i>6,7</i>	2.39 (0.82) <i>7</i>	3.42 (0.70) <i>5,6,7</i>	1.98 (0.50)	1.68 (0.21) <i>3</i>	1.01 (0.15) <i>1,2,3</i>	0.61 (0.15) <i>1,2,3</i>
Lung	1.63 (0.25) <i>5,7</i>	1.70 (0.06) <i>5,7</i>	2.31 (0.32) <i>6,7</i>	1.95 (0.49) <i>5,7</i>	2.99 (0.42) <i>1,2,4,6,7</i>	1.41 (0.11) <i>3,5</i>	0.57 (0.18) <i>1,2,3,4,5</i>
Liver	13.8 (1.8) <i>6,7</i>	15.7 (1.0) <i>3,6,7</i>	9.3 (1.5) <i>2,6</i>	10.2 (3.3) <i>6,7</i>	13.6 (2.1) <i>6,7</i>	3.6 (1.5) <i>1,2,3,4,5</i>	4.0 (1.1) <i>1,2,4,5</i>
Spleen	8.79 (4.44)	9.33 (3.38)	7.61 (2.06)	6.18 (3.03)	6.08 (2.53)	1.64 (0.38)	1.60 (0.10)
Kidney	46.9 (6.5) <i>2,3,4,5,6,7</i>	5.48 (0.40) <i>1,3</i>	28.4 (4.8) <i>1,2,4,5,6,7</i>	6.64 (2.08) <i>1,3</i>	4.22 (0.97) <i>1,3</i>	2.40 (0.39) <i>1,3</i>	1.88 (0.42) <i>1,3</i>
Gut	1.99 (0.34)	1.50 (0.08)	1.92 (0.37)	2.02 (1.69)	2.42 (0.21) <i>7</i>	1.00 (0.31)	0.57 (0.04) <i>5</i>
Brain	0.10 (0.05) <i>5</i>	0.09 (0.02) <i>5</i>	0.11 (0.02) <i>5</i>	0.11 (0.01) <i>5</i>	0.34 (0.09) <i>1,2,3,4,6,7</i>	0.11 (0.03) <i>5</i>	0.06 (0.02) <i>5</i>
Muscle	0.62 (0.13) <i>2,6,7</i>	1.02 (0.18) <i>1,4,5,6,7</i>	0.87 (0.24) <i>5,6,7</i>	0.57 (0.12) <i>2,7</i>	0.42 (0.13) <i>2,3</i>	0.24 (0.05) <i>1,2,3</i>	0.12 (0.03) <i>1,2,3,4</i>
Bone	2.33 (0.51) <i>7</i>	1.02 (0.18) <i>4,5,6,7</i>	1.92 (0.15)	1.53 (0.48) <i>2</i>	1.58 (0.41) <i>2</i>	1.23 (0.89) <i>2</i>	0.65 (0.21) <i>1,2</i>
n =	5	3	3	3	4	3	3

Table 1. Summarizing 48h ex vivo biodistribution data (%ID/g mean, SD in parentheses) and the results of statistical anlaysis (ANOVA). Numbers in italics indicate significant difference (p < 0.05, Bonferroni correction) compared with the indicated chelate.