Published in final edited form as:

Bioconjug Chem. 2011 June 15; 22(6): 1089–1102. doi:10.1021/bc1005625.

Reagents for Astatination of Biomolecules. 5. Evaluation of hydrazone linkers in ^{211}At - and ^{125}I -labeled *closo*-decaborate(2-) conjugates of Fab' as a means of decreasing kidney retention

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Abstract

Evaluation of monoclonal antibody (MAb) fragments (e.g. Fab', Fab or engineered fragments) as cancer-targeting reagents for therapy with the α -particle emitting radionuclide astatine-211 (^{211}At) has been hampered by low in vivo stability of the label and a propensity of these proteins localize to kidneys. Fortunately, our group has shown that the low stability of the ^{211}At label, generally a *meta*- or *para*-[^{211}At]astatobenzoyl conjugate, on MAb Fab' fragments can be dramatically improved by use of *closo*-decaborate(2-) conjugates. However, the higher stability of radiolabeled MAb Fab' conjugates appears to result in retention of the radioactivity in kidneys. This investigation was conducted to evaluate whether the retention of radioactivity in kidney might be decreased by the use of acid-cleavable hydrazone between the Fab' and the radiolabeled *closo*-decaborate(2-) moiety. Five conjugation reagents containing sulfhydryl-reactive maleimide groups, a hydrazone functionality and a *closo*-decaborate(2-) moiety were prepared. In four of the five conjugation reagents, a discrete polyethylene glycol (PEG) linker was used, and one substituent adjacent to the hydrazone was varied (phenyl, benzoate, anisole or methyl) to provide varying acid-sensitivity. In the initial studies, the five maleimido-*closo*-decaborate(2-) conjugation reagents were radioiodinated (^{125}I or ^{131}I), then conjugated with an anti-PSMA Fab' (107-1A4 Fab'). Biodistributions of the five radioiodinated Fab' conjugates were obtained in nude mice at 1, 4 and 24 h post injection (pi). In contrast to *closo*-decaborate(2-) conjugated to 107-1A4 Fab' through a non-cleavable linker, two conjugates containing either a benzoate or a methyl substituent on the hydrazone functionality displayed clearance rates from kidney, liver and spleen that were similar to those obtained with directly radioiodinated Fab' (i.e. no conjugate). The maleimido-*closo*-decaborate(2-) conjugation reagent containing a benzoate substituent on the hydrazone was chosen for study with ^{211}At . That reagent was conjugated with 107-1A4 Fab', then labeled (separately) with ^{125}I and ^{211}At . The radiolabeled Fab' conjugates were coinjected into nude mice bearing LNCaP human tumor xenografts, and biodistribution data was obtained at 1, 4 and 24 h pi. Tumor targeting was achieved with both ^{125}I - and ^{211}At -labeled Fab', but the ^{211}At -labeled Fab' reached a higher concentration (25.56 ± 11.20 vs. 11.97 ± 1.31 %ID/g). Surprisingly, while the ^{125}I -labeled Fab' was cleared from kidney similar to earlier studies, the ^{211}At -labeled Fab' was not (i.e. kidney conc. for ^{125}I vs. ^{211}At ; 4h: 13.14 ± 2.03 ID/g vs. 42.28 ± 16.38 %D/g, 24h: 4.23 ± 1.57 ID/g vs. 39.52 ± 15.87 %ID/g). Since the Fab' conjugate is identical in both cases except for the radionuclide, it seems likely that the difference in tissue clearance seen is due to an

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Supporting Information Available: Four tables (Table S1 – S4) containing biodistribution data for Fab' conjugates [^{125}I]4, [^{131}I]5, [^{125}I]6a, [^{211}At]6b, [^{125}I]7 and [^{131}I]8 are provided. This material is available free of charge via the Internet at <http://pubs.acs.org/BC>.

effect that ^{211}At has on either the hydrazone cleavage or on the retention of a metabolite. Results from other studies in our laboratory suggest that the latter case is most likely. The hydrazone linkers tested do not provide the tissue clearance sought for ^{211}At , so additional hydrazones linkers will be evaluated. However, the results support the use of hydrazone linkers when Fab' conjugated with *closo*-decaborate(2-) reagents are radioiodinated.

INTRODUCTION

The α -particle-emitting radionuclide astatine-211 (^{211}At), when combined with tumor-targeting agents, has great potential in development of new radiopharmaceuticals for treatment of cancer¹, particularly for blood-borne (e.g. lymphoma;² leukemia^{3,4}), compartmentalized cancer (e.g. ovarian cancer⁵⁻⁷), minimal residual disease after surgical tumor resections⁸, and metastatic cancer⁹⁻¹¹). Although most of the current investigations use intact monoclonal antibodies (MAB1) or their F(ab')₂ fragments for delivery of ^{211}At to cancer cells in vivo, some studies have shown that cancer-targeting agents with biological half-lives that are matched to the physical half-life of ^{211}At ($t_{1/2} = 7.21$ h) can be effective therapeutic agents, even in solid tumors¹⁰. This latter fact is of particular interest, as metastatic cancer can form tumors where barriers to penetration of intact MABs have become present.^{12,13} Therefore, we believe that the optimal cancer-targeting agents for carrying ^{211}At may be antibody Fab' fragments, divalent genetically engineered MAB fragments such as diabodies,^{14,15} or nanomolecular constructs that have biological half-lives closely matched to the half-life of ^{211}At .

Until recently, development of endoradiotherapy agents based on MAB Fab' (or Fab) fragments, or small genetically engineered MAB fragments has been problematic due to an inherent instability of ^{211}At label in vivo.¹⁶ However, we have demonstrated that conjugates of ^{211}At -labeled MAB Fab' fragments can be stabilized to deastatination by using nonahydro-*closo*-decaborate(2-) conjugates.¹⁷ Despite this advancement, there is another major impediment to overcome before ^{211}At -labeled MAB Fab' fragments can be effectively used in cancer therapy. That impediment is the inherent kidney localization of Fab' and similar molecular weight engineered MAB fragments. Unlike intact MABs or their F(ab')₂ fragments, Fab' fragments have a molecular size and shape that allows them to be filtered by the kidney glomerulus.^{18,19} Like other proteins, once filtered Fab' undergo reabsorption into the proximal tubule cells, presumably by binding megalin and cubulin receptors,²⁰⁻²² where they are ultimately degraded in the lysosomes.²³ There is evidence that complete degradation of the protein occurs by lysosomal peptidases, however, the radiolabeled metabolite, an amino acid adduct,^{24,25} can have a slow release from kidney. The retention of radiolabeled metabolites in kidneys is a critical issue when α -emitting radionuclides are used. Although therapy with ^{213}Bi -labeled Fab²⁶ and ^{211}At -labeled diabodies¹⁰ has been shown to be effective despite kidney localization, such reagents must be considered non-optimal as therapeutic agents due to potential kidney toxicity. To address this issue, we have incorporated pH-sensitive hydrazone functionalities between the MAB Fab' fragment and the *closo*-decaborate(2-) radiolabeling moiety as a potential means of obtaining a more rapid release of ^{211}At from kidneys. Although the hydrazone functionality has been used to release drugs within cells,²⁷ examples of its use in releasing radioactivity from kidneys were not readily found.

¹Abbreviations: ChT, chloramine-T; cpm, counts per minute; DTT, dithiothreitol; MAB, monoclonal antibody; MPS-EDA.TFA, trifluoroacetate salt of N-(2'-aminoethyl)-3-(maleimido)-propionamide NCS, N-chlorosuccinimide; NEM, N-ethylmaleimide; %ID/g, percent injected dose / gram; PBS, phosphate buffered saline; PEG, polyethylene glycol; *pi*, post injection; rt, room temperature; tBoc, *tert*-butoxycarbonyl; TCDI, 1,1'-thiocarbonyldiimidazole; TFA, trifluoroacetic acid

In this investigation, five hydrazone-containing *closo*-decaborate(2-) conjugates of anti-PSMA antibody, 107-1A4,²⁸ Fab' (**4** – **8**) were prepared and evaluated in vivo to compare the release of ¹²⁵I or ²¹¹At in kidney and other tissues. To better assess the effect of the hydrazone linker, biodistribution data previously reported for the same Fab' directly radioiodinated, [¹²⁵I]**1**, radioiodinated with N-hydroxysuccinimidyl *para*-[¹²⁵I]iodobenzoate, **2**, and radioiodinated with a non-cleavable *closo*-decaborate(2-) reagent, **3**¹⁷ were included in the comparison. Depictions of the radiohalogenated Fab' conjugates are provided in Figure 1. The key feature of the Fab' conjugates prepared in this study is that they contain hydrazone functionalities in molecular linkers between the Fab' and the *closo*-decaborate(2-) moiety used for radiolabeling. Initial in vivo studies were conducted with ¹²⁵I- or ¹³¹I-labeled Fab' conjugates. In the studies, sulfhydryl-reactive, hydrazone-containing, *closo*-decaborate(2-) derivatives were radiolabeled prior to conjugation with Fab'-SH to assure that the radioiodine was on the *closo*-decaborate(2-) cage moiety. In a later biodistribution study, Fab'-SH was conjugated prior to radiolabeling with the hydrazone-containing *closo*-decaborate(2-) derivative found in the first biodistribution studies to have the most favorable in vivo characteristics. That Fab' conjugate was directly labeled with ²¹¹At and (separately) ¹²⁵I, then the in vivo distribution of the coadministered ²¹¹At and ¹²⁵I-labeled Fab' was evaluated. The experiments conducted and results obtained in the investigation are reported herein.

EXPERIMENTAL PROCEDURES

General

Chemicals purchased from commercial sources were analytical grade or better and were used without further purification. Decaborane (B₁₀H₁₄) was obtained from Alfa Aesar (Ward Hill, MA). DL-Dithiothreitol (DTT), chloramine-T (ChT) and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich (Sigma, St. Louis, MO). MPS-EDA.TFA (TFA salt of N-(2'-aminoethyl)-3-(maleimido)propionamide), **13**, and MAL-dPEG[®]₄-t-boc-hydrazide, **16**, were obtained from Quanta BioDesign (Powell, OH; quantabiodesign.com). N-Chlorosuccinimide (NCS), 4-aminobenzoic hydrazide, **10**, acetyl chloride, trifluoroacetic acid (TFA) and most other chemicals were obtained from Sigma-Aldrich (Milwaukee, WI). Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μm) prior to use. Sephadex G-25 desalting columns (PD-10 and NAP-10) were obtained from Amersham Biosciences (Piscataway, NJ). Melting points were obtained in open capillary tubes on an EZ-Melt automated melting point apparatus (Stanford Research Systems, Sunnyvale, CA), and are uncorrected.

Radioactive Materials

All radioactive materials were handled according to approved protocols at the University of Washington. Standard methods for safely handling radioactive samples were employed.²⁹ Na[¹²⁵I]I was purchased from PerkinElmer Life Sciences (Billerica, MA) as high concentration / high specific activity radioiodide in 0.1 N NaOH. Measurement of ¹²⁵I and ²¹¹At were conducted on a Capintec CRC-15R Radioisotope Calibrator using calibration numbers 319 and 44 respectively (designated by Capintec Technical Services). Tissue samples containing ²¹¹At and ¹²⁵I were counted in a Wallac 1480 Wizard[™] gamma counter (PerkinElmer Life and Analytical Services, Wellesley, MA). The tissue samples were initially counted to obtain both ²¹¹At and ¹²⁵I counts, then were recounted after 3–5 days (0.1% or less ²¹¹At remaining) to obtain ¹²⁵I counts. Radioactivity counts were imported into an Excel Spreadsheet (Microsoft Corp., Redmond, WA) where calculations were made. The ²¹¹At counts were obtained by subtracting ¹²⁵I counts from total counts. Individual tissue counts for ²¹¹At were corrected for decay from the time of counting the first standard (at the beginning of the tissue counting process).

^{211}At was produced by irradiation of a thin layer (0.007 inch; 0.18 mm) of bismuth metal (99.999%, Aldrich) with 28.0 MeV α -particles on a Scandatronix MC-50 cyclotron using the conditions previously described.³⁰ All handling and processing of the irradiated target was done in a glovebox (Innovative Technologies, Inc., radioisotope glovebox) which was vented through a charcoal filter.

“Wet Chemistry” Isolation of $\text{Na}[^{211}\text{At}]\text{At}$

Briefly, an irradiated aluminum-backed bismuth target containing ~ 19.4 mCi of ^{211}At was placed in a 1L polypropylene dissolution chamber and 10 mL of conc. HNO_3 was added. The HNO_3 solution was removed from the chamber using a pipette after 10 min, and was placed in a 50 mL round-bottom flask. An additional 5 mL of conc. HNO_3 was added to the dissolution chamber containing the irradiated target (as a rinse). After 5 min, the HNO_3 solution was removed and placed in the round-bottom flask with the other HNO_3 solution. The flask containing HNO_3 solution was connected to a distillation apparatus. A hot plate (preheated) at 260°C was used to distill the HNO_3 and leaving a white residue (~ 50 min). After cooling the flask containing the white residue, 8 mL of 8M HCl was added with stirring to dissolve it. The resultant clear solution was removed and placed in a 20 mL scintillation vial. The flask was rinsed with an additional 2 mL 8M HCl , and that was placed in the scintillation vial. An 8 mL quantity of diisopropylether (DIPE) was added to the scintillation vial and that mixture was stirred at room temperature for 10 min. The 8 M HCl was removed by pipette to waste, and an additional 2 mL of 8M HCl was added and stirred for 5 min. The 8M HCl was removed to waste, and then 3 mL of 4 M NaOH was added. The NaOH solution was placed in a distillation apparatus and the H_2O was removed. The resulting residue was dissolved in 1 mL of H_2O and was adjusted to pH 9-10 by addition of 4M HCl . A white gel was obtained so an additional 1 mL of H_2O was added. That solution was filtered through a 0.2 mm Acrodisc to obtain a colorless solution (5.37 mCi), with 3.46 mCi remaining in the flask (did not rinse) and 8.6 mCi ^{211}At being retained on the filter. This was an early attempt at the wet chemistry approach to isolation of $\text{Na}[^{211}\text{At}]\text{At}$. Optimization was not done at that time³, as the amount recovered was much more than required for the labeling experiment. The $\text{Na}[^{211}\text{At}]\text{At}$ vial was tightly sealed, placed in sealed (Ziploc) plastic bag, then transferred from the glove box to a radiolabeling enclosure within a fume hood.

All radiohalogenation reactions were conducted in a charcoal-filtered Plexiglas enclosure (radioiodine fume hood, $20 \times 24 \times 36$ in, Biodex Medical Systems Inc., Shirley, NY) housed within a radiochemical fume hood. The radiohalogenation reactions were conducted in vials capped with Teflon-coated septa, and were vented through a 10 mL charcoal filled syringe. Additions of reagents to, or removal of materials from, the radiohalogenation vessel were conducted by passing a syringe needle through the septa.

Spectral Analyses

^1H NMR and ^{11}B NMR spectra were obtained on a Bruker AV 500 (500 MHz) instrument. Proton chemical shifts are expressed as ppm using tetramethylsilane as an internal standard ($\delta = 0.0$ ppm). Boron chemical shifts used $\text{BF}_3 \cdot \text{OEt}_2$ as an external standard. High-resolution mass spectral (HRMS) data were obtained on a Bruker APEX III 47e Fourier Transform Mass Spectrometer using electrospray ionization. For analysis, the samples were

²Measurement of ^{211}At activity in the target must be considered an approximation due to attenuation and geometry.

³The wet chemistry isolation procedure has been modified since the described experiments were conducted. The modified wet chemistry ^{211}At isolation procedure provides higher radiochemical yields (85 ± 11 % decay corrected yields). A description of the modified ^{211}At isolation procedure will be published elsewhere.

dissolved in 50/50 MeOH/H₂O and were introduced by an integral syringe infusion pump (Cole Parmer Series 74900).

Chromatography

All synthetic reactions were monitored by HPLC. Analytical samples were assessed on a system that contained a Hewlett-Packard quaternary 1050 gradient pump, a variable wavelength UV detector (254 nm), and an Alltech ELSD 2000 evaporative light-scattering detector (Deerfield, IL). Analyses of HPLC data were conducted using Hewlett-Packard HPLC ChemStation software. Reversed-phase HPLC chromatography was carried out on an Alltech Altima C-18 column (5 μ m, 250 $\times$ 4.5 mm) using a gradient solvent system at a flow rate of 1 mL/min. A gradient of MeOH and 0.05M Et₃NHOAc was used. Starting with 0% MeOH, the initial solvent mixture was increased to 100% MeOH over the next 15 min, then held at 100% MeOH for 5 min. Retention times (t_R) obtained under these conditions are provided in the experimental procedures.

Preparative chromatography was conducted on most nonradioactive compounds for purification. Purification was accomplished using a Biotage SP Flash Purification System (Charlottesville, VA) on a reversed-phase C18 Flash 25+M or 40+M column, eluting with a mixture of MeOH and 0.05 M triethylammonium acetate (Et₃NHOAc) in water. A gradient was used which started with 100% Et₃NHOAc and was increased over 20 min to 100% MeOH. Fractions containing the desired product were combined and evaporated to dryness under vacuum on a rotary evaporator.

The product mixtures from radioiodination reactions of **14** and **17a-17d** were analyzed by, and radioactive peaks containing [¹²⁵I]**15**, [¹³¹I]**18a**, [¹²⁵I]**18b**, [¹²⁵I]**18c** and [¹³¹I]**18d** were isolated from, a radio-HPLC using a C-18 column (Altima C-18, 5 mm, 250 $\times$ 4.5 mm; Alltech, Deerfield, IL) eluting at a flow rate of 1 mL/min using the same gradient conditions as described for non-radioactive iodination products. It should be noted that the pH of the Et₃NHOAc elution buffer was brought to 7.2 due to concerns for cleaving the hydrazone moiety during isolation. The HPLC equipment used in the analyses consisted of a Hewlett-Packard quaternary 1050 gradient pump, Waters 601 UV detector, and a Beckman model 170 radioisotope detector. Analysis of the HPLC data was conducted on Hewlett-Packard HPLC ChemStation software. The retention times (t_R) of the radioiodinated compounds are provided in the experimental procedures.

Size exclusion HPLC (SE-HPLC) separations of the conjugates of Fab' were obtained on Hewlett-Packard isocratic system consisting of a Model 1050 pumping system and a Model 1050 Multiple Wavelength Detector (280 nm). Proteins were evaluated on a Protein Pak 300 SW glass size exclusion column (10 mm, 8 mm $\times$ 30 cm, Waters Corporation, Milford, MA) run at 1.0 mL/min eluting with 50 mM sodium phosphate buffer (pH 6.8) which contained 300 mM NaCl, 1 mM EDTA, and 1 mM sodium azide. Analyses of the HPLC data were conducted using Hewlett-Packard HPLC ChemStation software. Retention times (t_R) for Fab' and their conjugates were ~11 min.

Compound Syntheses

Preparations of the following compounds were conducted as previously described: [Et₃NH]₂B₁₀H₁₀³¹; [Et₃NH]₂B₁₀H₉-CO-C₆H₅, **9a**³²; [Et₃NH]₂B₁₀H₉-CO-C₆H₄CO₂H, **9b**³², [Et₃NH]₂B₁₀H₉-CO-C₆H₄OMe, **9c**.³²

[Et₃NH]₂B₁₀H₉-CO-CH₃, **9d**

A solution of [Et₃NH]₂B₁₀H₁₀ (2g; 6.2 mmol) and acetyl chloride (10 mL; 141 mmol) in anhydrous CH₃CN (25 mL) was stirred at room temperature for 4 min. After that time, the

solution was triturated with diethyl ether (100 mL) and filtered. The crude product was dissolved in MeOH/H₂O (1:1) and purified via Biotage (C18 FLASH 40+M column) to yield 1.55 g (69%) of a red tacky solid. ¹H NMR (CD₃OD, 500 MHz): δ -0.20–1.06 (m, 9H), 1.31 (t, J = 7.5 Hz, 18H), 1.95 (s, 3H), 3.22 (q, J = 7.5 Hz, 12H). ¹¹B NMR (CD₃OD, 106.4 MHz): δ 5.10 (1B), 2.42 (1B), -19.30 (1B), -22.90 (1B), -25.38 (1B), -26.14 (1B), -26.98 (1B), -27.90 (1B), -29.14 (1B), -29.98 (1B). [Attempts to obtain HRMS data were unsuccessful.] HPLC: t_R = 7.1 min.

[Et₃NH]₂B₁₀H₉-C(C₆H₅)=NNH-CO-C₆H₄-NH₂, **11**

A solution of [Et₃NH]₂B₁₀H₉-CO-C₆H₅, **9a** (200 mg, 0.469 mmol), 4-aminobenzoic hydrazide, **10** (85 mg, 0.562 mmol) and MeOH (8 mL) was stirred at room temperature for 16 h. The crude product solution was purified directly via Biotage (C18 FLASH 25+M column) to yield 242 mg (92%) of **11**. ¹H NMR (CD₃OD, 500 MHz): δ -0.08–1.13 (m, 9H), 1.23 (t, J = 7.4 Hz, 18H), 3.09 (q, J = 7.4 Hz, 12H), 6.65 (d, J = 8.8 Hz, 2H), 7.15–7.17 (m, 3H), 7.61–7.63 (m, 2H), 7.69 (d, J = 8.8 Hz, 2H). ¹¹B NMR (CD₃OD, 160.4 MHz): δ 0.69 (2B), -23.24 (1B), -26.65 (7B). HRMS (ES⁻) C₁₄H₂₂B₁₀N₃O (M+H)⁻ calcd: 358.2693. Found: 358.2688. HPLC: t_R = 10.5 min.

[Et₃NH]₂B₁₀H₉-C(C₆H₅)=NNH-CO-C₆H₄-NCS, **12**

A solution of **11** (100 mg, 0.18 mmol), TCDI (46.7 mg, 0.262 mmol) and anhydrous DMF (4 mL) was stirred at room temperature for 1 h. The solution was washed with 20% EtOAc/hexanes (5 × 15 mL), dried under vacuum for 2 h to yield 106 mg (99%) of a light-yellow tacky solid. ¹H NMR (CD₃OD, 500 MHz): δ -0.12–1.34 (m, 9H), 1.25 (t, J = 7.3 Hz, 18H), 3.14 (q, J = 7.3 Hz, 12H), 7.19–7.22 (m, 3H), 7.36 (d, J = 8.8 Hz, 2H), 7.60–7.62 (m, 2H), 7.98 (d, J = 8.8 Hz, 2H). ¹¹B NMR (CD₃OD, 160.4 MHz): δ 2.69 (1B), 1.78 (1B), -21.78 (1B), -25.80 (7B). HRMS (ES⁻) C₁₅H₂₀B₁₀N₃OS (M+H)⁻ calcd: 400.2258. Found: 400.2250. HPLC: t_R = 13.7 min.

[Et₃NH]₂B₁₀H₉-C(C₆H₅)=NNH-CO-C₆H₄-EDA-MAL, **14**

MPS-EDA.TFA, **13** (85 mg, 0.26 mmol) was added to a solution of **12** (100 mg, 0.17 mmol), NEt₃ (56 μL, 0.40 mmol) and anhydrous DMF (6 mL). The resultant solution was stirred at room temperature for 2 h, then volatile materials were removed under vacuum by rotary evaporation. The crude product was dissolved in MeOH/water (1/1) and purified via Biotage (C18 FLASH 25+M column) to provide 92 mg (68%) of a colorless solid. ¹H NMR (CD₃CN, 500 MHz): δ -0.08–0.93 (m, 9H), 1.16 (t, J = 7.4 Hz, 18H), 2.96 (q, J = 7.4 Hz, 12H), 3.29–3.33 (m, 2H), 3.41–3.45 (m, 2H), 3.50–3.53 (m, 2H), 3.54–3.65 (m, 2H), 6.72 (s, 2H), 7.16–7.21 (m, 3H), 7.46–7.50 (m, 2H), 7.54–7.57 (m, 2H), 7.88 (d, J = 8.7 Hz, 2H). ¹¹B NMR (CD₃CN, 160.4 MHz): δ 2.65 (1B), 1.82 (1B), -21.73 (1B), -25.72 (7B). HRMS (ES⁻) C₂₄H₃₂B₁₀N₆O₄S (M+H)⁻ calcd: 611.3215. Found: 611.3236. HPLC: t_R = 11.2 min.

[Et₃NH]₂B₁₀H₉-C(R)=NNH-CO-dPEG₄-MAL, **17a-17d**

A solution of MAL-dPEG₄-CONHNH-BOC, **16** (1 g, 1.885 mmol) and neat TFA (4 mL) was stirred at room temperature for 3 min. After volatile materials were evaporated by a stream of argon, the acyl hydrazine derivative was washed with 10% EtOAc/hexanes (2 × 15 mL), dried under vacuum overnight to provide a colorless oil. HPLC: t_R = 9.4 min. This material was used in the next step without further purification.

A methanol solution of MAL-dPEG₄-CONHNH₂, **16** (TFA salt; 100 mg, 0.184 mmol) and [Et₃NH]₂B₁₀H₉-CO-R (0.184 mmol) was stirred at room temperature for 1–4 h, then the volatile materials were removed on a rotoevaporator under vacuum. The crude products

(**17a-17d**) were dissolved in MeOH/water (1/1) and purified via Biotage (C18 FLASH 25+M column). The gradient mixture was composed of MeOH and 0.05 M triethylammonium acetate. Starting with 100% 0.05 M triethylammonium acetate, increased to 100% MeOH over the next 20 min).

17a—R = C₆H₅; reaction time = 4h; 118 mg (77%) yield. ¹H NMR (CD₃CN, 500 MHz): δ -0.22–0.99 (m, 9H), 1.22 (t, J = 7.2 Hz, 18H), 2.36–2.46 (m, 4H), 3.03 (q, J = 7.2 Hz, 12H), 3.27–3.35 (m, 2H), 3.47–3.52 (m, 2H), 3.54–3.64 (m, 16H), 3.66–3.81 (m, 7H), 6.79 (s, 2H), 7.11–7.22 (m, 3H), 7.50–7.63 (m, 2H). ¹¹B NMR (CD₃CN, 106.4 MHz): δ 1.44 (2B), -22.79 (1B), -26.27 (7B). HRMS (ES⁻) C₂₅H₄₃B₁₀N₄O₈ (M+H)⁻ calcd: 637.4017. Found: 637.4048. HPLC: t_R = 11.6 min.

17b—R = C₆H₄-CO₂H; reaction time = 1h; 107 mg (66%) yield. ¹H NMR (CD₃CN, 500 MHz): δ -0.33–0.84 (m, 9H), 1.18 (t, J = 7.3 Hz, 18H), 2.31–2.44 (m, 4H), 2.51, 2.55 (2 × d, J = 3.8 Hz, 1H), 2.78, 2.81 (2 × d, J = 8.4 Hz, 1H), 3.13 (q, J = 7.3 Hz, 12H), 3.20–3.32 (m, 3H), 3.45 (q, J = 5.0 Hz, 3H), 3.52–3.59 (m, 9H), 3.63–3.67 (m, 4H), 3.69–3.73 (m, 1H), 3.93 (dd, J = 3.8, 8.4 Hz, 1H), 5.21 (s, 1H), 6.75 (s, 2H), 6.92 (s, 1H), 7.54 (d, J = 8.7 Hz, 1H), 7.79 (d, J = 8.7 Hz, 1H), 8.22 (s, 1H). ¹¹B NMR (CD₃CN, 106.4 MHz): δ 1.60 (2B), -22.79 (1B), -26.01 (7B). HRMS (ES⁻) C₂₆H₄₃B₁₀N₄O₁₀ (M+H)⁻ calcd: 681.3915. Found: 681.3897. HPLC: t_R = 9.8 min.

17c—R = C₆H₄-OCH₃; reaction time = 1h; 129 mg (81%) yield. ¹H NMR (CD₃CN, 500 MHz): δ -0.27–0.93 (m, 9H), 1.24 (t, J = 7.4 Hz, 18H), 2.37–2.49 (m, 4H), 3.13 (q, J = 7.4 Hz, 12H), 3.21–3.25 (m, 2H), 3.40–3.45 (m, 2H), 3.48–3.61 (m, 16H), 3.64–3.72 (m, 4H), 3.80–3.89 (m, 3H), 6.74 (s, 1H), 6.75 (s, 1H), 6.88 (d, J = 8.8 Hz, 1H), 6.94 (d, J = 8.8 Hz, 1H), 7.68 (d, J = 8.8 Hz, 1H), 7.78 (d, J = 8.8 Hz, 1H). ¹¹B NMR (CD₃CN, 106.4 MHz): δ 5.36 (1B), 3.03 (1B), -19.33 (1B), -21.51 (1B), -24.75 (2B), -25.48 (2B), -26.15 (2B). HRMS (ES⁻) C₂₆H₄₅B₁₀N₄O₉ (M+H)⁻ calcd: 667.4123. Found: 667.4128. HPLC: t_R = 11.8 min.

17d—R = CH₃; reaction time = 1h; 72 mg (50%) yield. ¹H NMR (CD₃CN, 500 MHz): δ -0.34–0.97 (m, 9H), 1.21 (t, J = 7.4 Hz, 18H), 1.88 (s, 3H), 2.32–2.45 (m, 4H), 3.05 (q, J = 7.4 Hz, 12H), 3.21–3.31 (m, 2H), 3.41–3.46 (m, 2H), 3.48–3.59 (m, 16H), 3.61–3.69 (m, 4H), 6.74 (s, 1H), 6.75 (s, 1H). ¹¹B NMR (CD₃CN, 160.4 MHz): δ 4.30 (1B), 0.26 (1B), -20.08 (1B), -24.18 (1B), -26.64 (2B), -27.45 (2B), -28.18 (2B). HRMS (ES⁻) C₂₀H₄₁B₁₀N₄O₈ (M+H)⁻ calcd: 575.3860. Found: 575.3868. HPLC: t_R = 10.5 min.

General Procedure for preparing iodinated HPLC standards; [Et₃NH]₂B₁₀H₈I-C(R)=NNH-CO-dPEG₄-MAL, **18a** – **18d**

A 12 μmol quantity (159 μL; 10 mg/mL in MeOH) of N-chlorosuccinimide (NCS) was added to a solution containing 24 μmol of the *closo*-decaborate(2-) derivative (**17a-17d**), 12 μmol of NaI (17.9 μL; 100 mg/mL in deionized H₂O) and 5% HOAc/MeOH (0.5 mL). The reaction solution was stirred at room temperature for 1 min, then 12 μmol Na₂S₂O₅ (113 μL; 20 mg/mL in deionized H₂O) was added and the resultant solution was stirred at room temperature for 1 min. A small quantity of iodinated product was isolated from an analytical HPLC column for mass spectral analysis and to be used as a HPLC retention time standard for the radioiodination reactions. The identity of each standard was confirmed by high-resolution mass spectral data.

18a—R = C₆H₅; HRMS (ES⁻) C₂₅H₄₂IB₁₀N₄O₈ (M+H)⁻ calcd: 763.2978. Found: 763.3029. HPLC: t_R = 12.5 min.

18b—R = C₆H₅-CO₂H; HRMS (ES⁻) C₂₆H₄₂IB₁₀N₄O₁₀ (M+H)⁻ calcd: 807.2876. Found: 807.2867. HPLC: t_R = 11.7 min.

18c—R = C₆H₅-OCH₃; HRMS (ES⁻) C₂₆H₄₄IB₁₀N₄O₉ (M+H)⁻ calcd: 793.3084. Found: 793.3100. HPLC: t_R = 12.3 min.

18d—R = CH₃; HRMS (ES⁻) C₂₀H₄₀IB₁₀N₄O₈ (M+H)⁻ calcd: 701.2821. Found: 701.2809. HPLC: t_R = 11.9 min.

General procedure for preparing [¹²⁵I]**15**, [¹³¹I]**18a**, [¹²⁵I]**18b**, [¹²⁵I]**18c**, [¹³¹I]**18d**

To 50 μL of 1.0 mg/mL solution of the **14**, **17a**, **17b**, **17c** or **17d** dissolved in a MeOH/2.5% HOAc mixture was added 1.1–1.4 mCi of Na[¹²⁵I]I or Na[¹³¹I]I in 0.1 N NaOH. To this mixture was added 20 μL of 1 mg/mL N-chlorosuccinimide solution. The reaction was allowed to proceed at room temperature for 1 min, then 20 μL of 1 mg/mL sodium metabisulfite solution was added to quench. To this solution was added 50 μL of PBS and the volume was reduced to 50% under a stream of argon. The solutions containing radioiodinated derivatives ([¹²⁵I]**15**, [¹³¹I]**18a**, [¹²⁵I]**18b**, [¹²⁵I]**18c** or [¹³¹I]**18d**) were used directly in the subsequent conjugation reactions.

Preparation of 107-1A4 and its' F(ab')₂ fragment

The monoclonal antibody 107-1A4, an IgG₁ MAb, which is reactive with the prostate-specific membrane antigen found in prostate tissue and upregulated in prostate cancer,³³ was obtained and purified as previously described.²⁸ The F(ab')₂ fragment was prepared from intact MAb by digestion using pepsin (5.2 μg/mL in 80 mM NaOAc, pH 3.9) at 37°C for 24 h. The F(ab')₂ produced was purified on a Protein A column followed by a Fractogel TMAE column (EM Science, Gibbstown, NJ). The purified F(ab')₂ was stored at refrigerator temperature. The 107-1A4 F(ab')₂ was converted to Fab'-SH just prior to conjugation as described in the experimental procedures below.

Preparation of Fab'-SH conjugate, **19**

To 1.5 mL of 2.9 mg/mL F(ab')₂ in PBS was added 60 μL of 100 mM DTT to make a final concentration of 4 mM DTT. This was gently mixed for 45 min at room temperature before passing through a PD-10 column eluting with PBS pH 6.5 with 1 mM EDTA. Protein containing fractions were combined to give 5 mL at 0.77 mg/mL Fab'-SH.

To 2.5 mL (1.9 mg; 0.038 μmol) of the Fab'-SH was added 30 μL of 11 mg/mL (0.33 mg; 0.48 μmol) **17b** in DMF. After 30 min at room temperature, a 10 μL aliquot of a 23.8 mg/mL N-ethylmaleimide (NEM) solution in DMF was added to react with the remaining sulfhydryl groups. After 10 min at room temperature, the mixture was split in half and each half was run over a PD-10 column. The protein fractions, containing **19**, were eluted in PBS and combined to yield 1.3 mg (64% protein recovery).

General Procedure for conjugating [¹²⁵I]**15**, [¹³¹I]**18a**, [¹²⁵I]**18b**, [¹²⁵I]**18c** or [¹³¹I]**18d** with 107-1A4 Fab'-SH

To 357 μL of 8.4 mg/mL 107-1A4 F(ab')₂ in PBS was added 14.3 μL of 100 mM DTT to make a final concentration of 4 mM DTT. This was gently mixed for 45 min at room temperature before eluting over a PD-10 column (Sephadex G-25) with PBS pH 6.5 with 1 mM EDTA. Protein containing fractions were combined to give 2.5 mL at 1.25 mg/mL Fab'-SH.

A 0.5 mL aliquot of the Fab'-SH solution was added to a PBS solution containing [¹²⁵I]**15**, [¹³¹I]**18a**, [¹²⁵I]**18b**, [¹²⁵I]**18c** or [¹³¹I]**18d**. After 30 minutes at room temperature, 13 μ L of 2 mg/mL N-ethylmaleimide in DMSO was added to cap the remaining sulfhydryl groups. After another 10 min at room temperature, the reaction mixture was passed over a PD-10 column by eluting with PBS. The protein-containing fractions were combined to give 85% recovery of protein with 30–40% radiochemical yields for [¹²⁵I]**15**, [¹³¹I]**18a**, [¹²⁵I]**18b**, [¹²⁵I]**18c** or [¹³¹I]**18d**.

Assessment of Fab'-Hydrazone Cleavage with Acid

Fab' conjugates [¹²⁵I]**3** and [¹²⁵I]**6a** were prepared by radioiodination of *N*-(15-(aminoacyldecaborate)-4,7,10-trioxatri-decanyl)-3-maleimidopriopionamide¹⁷ and **17b** respectively, followed by conjugation with Fab' and purification as described above. Aliquots of the purified radioiodinated Fab' conjugates (100 μ L, 20 μ g/2–6 μ Ci) were placed in three vials containing 200 μ L of 50 mM sodium acetate solutions adjusted to pH 7, 4.5 and 2.0 using HCl. The radioiodinated Fab' conjugate solutions were incubated at 37°C for 26 hours. Samples taken at 1, 4 and 26 h were placed in ultracentrifugation filters (Microcon YM-10), spun at 10,000 rpm for 10 min, then washed 3x with 200 μ L PBS. The retentate and filtrate were counted in a gamma counter to provide the percent radioactivity bound to the protein.

Preparation of [¹²⁵I]**6a** by direct labeling

To 25 μ L of 500 mM sodium phosphate pH 7.4 was added ~1 μ L (157 μ Ci) Na[¹²⁵I]I in 0.1 N NaOH. To this, 22 μ L of **19** (6.9 mg/mL) was added, followed by 10 μ L of a solution containing 1 mg/mL chloramine-T in water. After 1 min at room temperature, the reaction was quenched with 10 μ L of 1 mg/mL sodium metabisulfite in H₂O solution. The mixture was eluted on a NAP-10 column (Sephadex G-25) with PBS. The protein fractions were combined to give 149 μ Ci (95% radiochemical yield) of [¹²⁵I]**6a**.

Preparation of [²¹¹At]**6b**

To 200 μ L of 500 mM sodium phosphate pH 7.4 was added 300 μ L (573 μ Ci) of Na[²¹¹At]At solution, pH 9. To this, 22 μ L of **19** (6.9 mg/mL) was added, followed by 50 μ L of 1 mg/mL chloramine-T in H₂O. After 1 min at room temperature, the reaction was quenched with 50 μ L of 1 mg/mL sodium metabisulfite in water. The mixture was run over a PD-10 column eluting with PBS. The protein fractions were combined to give 367 μ Ci (64% radiochemical yield) of [²¹¹At]**6b**.

Biodistribution Studies

Biodistribution studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Washington. Male nude (nu/nu) and C.B.-17 SCID mice, obtained from Charles River Laboratories (Hollister, CA), were housed for 1 week in the isolator facility prior to beginning the study. The biodistribution experiments were conducted, when possible, by evaluation of two compounds (dual labeled; ¹²⁵I & ¹³¹I, or ¹²⁵I & ²¹¹At) in a single set of mice to decrease the number of mice required for the study. In the initial biodistribution studies, nude mice were injected with [¹²⁵I]**4**, an admixture containing [¹³¹I]**5**/[¹²⁵I]**6** or an admixture containing [¹²⁵I]**7**/[¹³¹I]**8**. The admixtures were prepared to provide a total of 30 μ g of Fab' conjugate having preset amounts of μ Ci of each radiolabeled compound. A later experiment evaluated an admixture of the directly labeled [¹²⁵I]**6a** and [²¹¹At]**6b** in mice bearing the LNCaP tumor xenografts. The radioactive admixtures were diluted with phosphate buffered saline (PBS) to prepare an injection volume of ~100 μ L and were injected into each of the nude mice via the lateral tail vein. The actual amount of injectate each animal received was determined by weighing the

administering syringe before and after injection. Groups of 3–5 mice were sacrificed by cervical dislocation (under anesthesia) at 1 and 4 h post injection. The tissues were excised, blotted free of blood, weighed, and counted. Blood weight was estimated to be 6% of the total body weight.³⁴ Calculation of percent injected dose (%ID) and percent injected dose per gram (%ID/g) for the collected tissues was accomplished using internal standards for ¹²⁵I, ¹³¹I or ²¹¹At counts. The full data obtained in each biodistribution study, including amounts injected, animal weights, and results of statistical significance (using Student's-t test) are provided as Tables S1-S4 in the Supporting Information.

RESULTS

Syntheses of maleimido-*closo*-decaborate(2-) derivatives

Five *closo*-decaborate(2-) derivatives containing the maleimide functionality were targeted for conjugation with Fab'-SH. The synthetic pathways used to prepare the maleimido-*closo*-decaborate(2-) derivatives **14**, **17a**, **17b**, **17c**, and **17d** are shown in Schemes 1 and 2.

The initial synthesis was conducted to prepare the maleimide derivative **14**, as shown in Scheme 1. The first step to prepare **14** involved formation of the hydrazone functionality by reaction of *para*-aminobenzoic hydrazide, **10**, in MeOH at room temperature for 16 hours with the readily prepared triethylammonium salt of the benzoyl adduct of *closo*-decaborate(2-) **9a**.³² A 92% yield of the adduct **11** was obtained. Following that step, the aniline was converted to an isothiocyanate by reaction with thiocarbonyldiimidazole (TCDI) in anhydrous DMF. The crude isolated isothiocyanate derivative **12** was reacted with commercially available N-(2'-aminoethyl)-3-(maleimido)propionamide, **13**, to provide 68% of **14** after purification by reversed-phase flash chromatography.

In the structures of maleimido-*closo*-decaborate(2-) derivatives **17a**, **17b**, **17c** and **17d**, the hydrazone functionality was changed from a phenacyl hydrazone (in **14**) to an alkyl acyl hydrazone to alter the sensitivity to acid cleavage. Within the group of compounds the nature of the functional group (R) bonded with the ketone carbon of the hydrazone was varied (i.e. phenyl, benzoate, anisole and methyl) for the same purpose. A polyethylene glycol (PEG) linker was incorporated to improve the aqueous solubility of the conjugation molecule. To simplify the syntheses, a commercially available PEG derivative (**16**) that contained both maleimide and acyl hydrazone functionalities was used. Thus, a one pot, two-step, synthesis could be used to prepare *closo*-decaborate(2-)-containing maleimide derivatives **17a**, **17b**, **17c** and **17d**, as shown in Scheme 2. In those syntheses, the commercially available tBoc protected maleimido-dPEG₄-acylhydrazide, **16**, was deprotected in neat TFA, then that crude product was directly reacted with the readily prepared acyl derivatives of *closo*-decaborate(2-), **9a** – **9d**,³² in MeOH at room temperature for 1–4 h to provide 50–81% yields of **17a** – **17d**.

Radioiodination of maleimido-*closo*-decaborate(2-) derivatives

Prior to incorporation of radioiodine, **14**, **17a**, **17b**, **17c** and **17d** were iodinated by reaction of N-chlorosuccinimide (NCS) and NaI in 5% HOAc/MeOH solution. Small quantities of the iodinated product were obtained for use as HPLC retention time standards in the radioiodination studies. The non-radioactive iodinated derivatives were isolated using analytical reversed-phase HPLC. The identities of the isolated iodinated products were confirmed by high-resolution mass spectrometry.

Radioiodination reactions were conducted by reacting 50 µg quantities of the maleimido-*closo*-decaborate(2-) derivatives **14**, **17a**, **17b**, **17c** and **17d** with 1–1.4 mCi of Na[¹²⁵I]I or Na[¹³¹I]I in MeOH/2.5% HOAc and N-chlorosuccinimide to form [¹²⁵I]**15**, [¹³¹I]**18a**, [¹²⁵I]**18b**, [¹²⁵I]**18c** and [¹³¹I]**18d**. After 1 min reaction time, sodium metabisulfite was used

to quench the reaction. The MeOH solutions were diluted with phosphate buffered saline and that solution was reduced to 50% volume under a stream of argon to remove organics. The crude isolated radioiodinated products were used directly in the Fab' conjugation reactions.

Conjugation of maleimido-*c/oso*-decaborate(2-) derivatives

The protein used in the investigation was a Fab' fragment of the anti-PSMA antibody, 107-1A4.²⁸ To prepare the Fab' fragment, the intact antibody was digested following standard procedures employing pepsin at 37°C for 24 h to form the F(ab')₂ fragment. The F(ab')₂ fragment was purified on a Fractogel column and was stored at refrigerator temperature until used. Just prior to running the conjugation reactions, the F(ab')₂ was converted to the Fab'-SH fragment by reducing with 4 mM dithiothreitol (DTT) for 45 min at room temperature. After reduction, the Fab' was purified over a size exclusion column (PD-10).

Radioiodinated maleimido-*c/oso*-decaborate(2-) derivatives [¹²⁵I]**15**, [¹³¹I]**18a**, [¹²⁵I]**18b**, [¹²⁵I]**18c** and [¹³¹I]**18d** were reacted with Fab'-SH for 30 min at room temperature, then N-ethylmaleimide (NEM) was added to cap any unreacted thiol groups. The radioiodinated Fab' conjugates were purified by elution on size exclusion columns. This overall radiolabeling procedure provided isolated radiochemical yields of 30–40% for [¹²⁵I]**4**, [¹³¹I]**5**, [¹²⁵I]**6**, [¹²⁵I]**7** and [¹³¹I]**8**.

A non-radioactive Fab' conjugate was prepared by reaction with the maleimido-*c/oso*-decaborate(2-) derivative **17b** with 107-1A4 Fab'-SH. The selection of Fab' conjugated with maleimide derivative containing a benzoic acid moiety adjacent to the hydrazone, **17b**, was made based on the favorable biodistribution of its radioiodinated counterpart (see Figure 2). The conjugation reaction was conducted with a ratio of 1 Fab'-SH to 13 equivalents of **17b**. The conjugation was allowed to run for 30 min at room temperature, then the remaining thiols were capped with NEM and the conjugate was purified by size-exclusion chromatography (PD-10). The isolated Fab' conjugate was analyzed by size-exclusion HPLC to determine the peak shape, retention time, and number of species present.

Fab'-Hydrazone Cleavage with Acid

Fab' conjugates [¹²⁵I]**3** and [¹²⁵I]**6a** were incubated at 37°C in 50 mM sodium acetate solutions adjusted to pH 7, 4.5 and 2.0 for 26 hours to assess the cleavage of the hydrazone functionality in [¹²⁵I]**6**. The control (non-cleavable) Fab' conjugate, [¹²⁵I]**3**, had no cleavage (97–98% protein-bound activity) after 26 h. However, acid promoted cleavage (99% protein-bound at pH 7.0; 81% protein-bound at pH 4.5; 70% protein bound at pH 2.0) was observed for the Fab' conjugate, [¹²⁵I]**6a**, after 26 hours of incubation time.

Radiohalogenation of Fab'-SH conjugate **19**

Direct radiohalogenation of Fab' conjugate **19** was accomplished with ¹²⁵I and ²¹¹At. Radioiodination of **19** was accomplished by reaction of Na[¹²⁵I]I and chloramine-T (ChT) for 1 min at room temperature. After quenching the reaction with sodium metabisulfite, elution of the labeled Fab' conjugate over a size-exclusion column provided a 95% isolated radiochemical yields of [¹²⁵I]**6a**. Similarly, astatination of **19** was accomplished by reaction of Na[²¹¹At]At with ChT for 1 min at room temperature to provide a 64% isolated radiochemical yield of [²¹¹At]**6b** after quenching and purification.

Biodistributions of Radiolabeled Fab'-SH Derivatives

Three biodistribution studies were conducted using hydrazone-containing radioiodinated Fab' conjugates to determine if the concentrations of radioiodine localized to kidney were

effected by the inclusion of the hydrazone moiety. Dual label biodistribution studies were conducted where possible to minimize the use of animals in the study. Thus, the initial three biodistribution studies evaluated the following Fab' conjugates: (1) [^{125}I]4; (2) [^{131}I]5 & [^{125}I]6a; (3) [^{125}I]7 & [^{131}I]8. The amount of protein injected into each mouse was kept constant throughout the groups (i.e. 30 $\mu\text{g}/\text{mouse}$). It was of high interest to compare the results obtained in this investigation with previous results obtained with a non-cleavable maleimido-*closo*-decaborate(2-) Fab' conjugate, [^{125}I]3. It was also of interest to compare the biodistribution results obtained in this study with results obtained from direct radioiodination, [^{125}I]1, and conjugation of a *para*[^{125}I]iodobenzoate derivative, [^{125}I]2 with the same Fab'. To highlight the comparison, the tissue concentrations (% injected dose / gram) of [^{125}I]4, [^{131}I]5, [^{125}I]6a, [^{125}I]7 and [^{131}I]8 have been plotted along with published data for [^{125}I]1, [^{125}I]2 and [^{125}I]3. The comparison graphs for selected tissues are provided in Figure 2. The radioiodine concentrations obtained for selected tissues and blood in this study are provided as Tables S1, S2 and S3 in Supporting Information.

When examining the tissue concentrations in Figure 2, the values for tissue concentrations of Fab' conjugates 1, 2 and 3 (from literature) should not be compared directly with those of Fab' conjugates 4 through 8 evaluated in this study, as the studies were not conducted under exactly the same conditions. In general, it appears that the reported concentrations of radioiodinated Fab' 1 through 3 are lower in most tissues than those obtained in this study for radioiodinated Fab' conjugates 4 through 8, except kidney and neck. The reasons for the differences are not known. While the relative concentrations are not evaluable, trends regarding clearance of radioiodine from tissues can be compared for all of the Fab' conjugates. The tissue of highest interest in this study is kidney, as the high localization of radioactivity to that tissue is dose limiting for radiolabeled Fab'. In the mouse model, radioiodinated Fab' [^{125}I]1 produced by direct labeling of tyrosine residues was highly localized in kidney, but was cleared from that tissue within 24 h. In contrast to this, Fab' conjugates [^{125}I]2 and [^{125}I]3 prepared by a two-step method using a *para*-[^{125}I]iodobenzoyl labeling reagent, or direct labeling after conjugation of a maleimido-*closo*-decaborate(2-) reagent, had little kidney clearance over a 24 h period.

It can be noted in Figure 2 that the kidney concentrations and clearance for Fab' conjugates [^{125}I]4, [^{131}I]5, [^{125}I]6a, [^{125}I]7 and [^{131}I]8 varied considerably. The concentration of [^{125}I]4 in kidney was low, and appeared to clear over the 24 h period. With exception of the liver, there was a lower concentration of [^{125}I]4 relative to [^{131}I]5, [^{125}I]6a, [^{125}I]7 and [^{131}I]8 in the other tissues. The lower tissue concentrations may be an indication that the hydrazone in [^{125}I]4 has a lower overall in vivo stability. The higher liver concentrations seen for [^{125}I]4 may be due to a higher lipophilicity of the conjugate metabolites. The kidney concentrations of [^{131}I]5 and [^{125}I]7 did not decrease significantly over the 24 hour period, indicating either that the hydrazone was not as readily cleaved or that the metabolites were retained. In contrast, kidney concentrations of [^{125}I]6a and [^{131}I]8 decreased dramatically over the 24 hour period. Indeed, the clearance from kidney of [^{125}I]6a and [^{131}I]8 was similar to that seen with directly labeled Fab' (i.e. [^{125}I]1).

A fourth biodistribution was conducted in nude mice bearing LNCaP human prostate cancer xenografts to compare the pharmacokinetics, tissue concentrations and tumor localization of [^{125}I]6a and [^{211}At]6b. The results of that study are depicted in the bar graphs shown in Figure 3, and the data are provided as Table S4 in Supporting Information. In the coinjected, dual labeled (^{125}I and ^{211}At) study, it was anticipated that the distribution of ^{125}I and ^{211}At would be very similar as that had been the case in most prior studies using *closo*-decaborate(2-) protein conjugates and small molecules. It is apparent in Figure 3 that kidney (and liver) retention is much higher in the ^{211}At -labeled Fab' conjugate. Interestingly, while

the tumor concentration appeared to stay constant in the ^{125}I -labeled Fab' conjugate, the concentration was higher and increased with time in the ^{211}At -labeled Fab' conjugate.

DISCUSSION

An objective in our laboratory is to obtain a tumor-targeting agent for use with ^{211}At that has a blood half-life similar (e.g. 6–8 h) to the physical half-life of ^{211}At (7.21 h), so that tumor masses can be readily penetrated and an efficient delivery of that radionuclide to the tumor can be obtained. Intact MABs have relatively long biological half-lives and do not penetrate tumors within a half-life of ^{211}At , making them unattractive as carriers of ^{211}At (for most cases). In contrast, small proteins (25 kDa or less) and peptides penetrate tumor masses readily, but are rapidly cleared from blood so only a small fraction of the injected radioactivity gets to the tumor site(s). This latter point is important, as cost and availability of ^{211}At must be considered in developing a reagent that uses it. This makes MAB fragments, such as Fab', of high interest as cancer targeting agents for ^{211}At since they are small enough to penetrate tumors and have a long enough biological half-life (e.g. 6.8 h in mice³⁵).

Our investigation of ^{211}At endoradiotherapy was hampered for a number of years by the fact that many ^{211}At -labeled tumor-targeting agents undergo deastatination in vivo, including Fab' fragments.¹⁶ However, more recently we demonstrated that switching from bonding of the ^{211}At with an aromatic carbon atom to bonding with a boron atom in an aromatic boron cage moiety provides high in vivo stability towards deastatination.^{17,31,36} Of the boron cage moieties evaluated, the nonahydro-*closo*-decaborate(2-) cage has become the ^{211}At -labeling moiety of choice, because it is; (a) easily prepared, (b) very reactive with electrophilic halogens, (c) quite stable in vivo and (d) appears to have minimal affect on the biodistribution of compounds studied. Although only small differences in biodistribution of radiohalogens are observed with *closo*-decaborate(2-) conjugates, it has been noted that the radiohalogens localized to kidney and/or liver can have slow clearance from those tissues.

In previous studies, we found that MAB Fab' fragments conjugated with a non-cleavable *closo*-decaborate(2-) moiety can have in vivo pharmacokinetics and tissue distributions that are quite different from the same protein directly labeled with radioiodine.¹⁷ The difference is particularly noticeable in kidney. That difference can be readily seen in Figure 2 (top right panel), where the concentration of radioiodine-labeled Fab', [^{125}I]1, decreases over a 24 h period, whereas in a radioiodinated Fab' containing a non-cleavable *closo*-decaborate(2-) conjugate, [^{125}I]3, the radioactivity is generally retained over that time period. It seems unlikely that the longer kidney retention is due to the dianionic *closo*-decaborate(2-) moiety, as similar kidney retention is observed when the same MAB Fab' is radioiodinated using N-hydroxysuccinimidyl *para*-[^{125}I]iodobenzoate (to give [^{125}I]2). The retention of radiohalogens in kidney prompted us to survey literature approaches for diminishing the uptake of ^{211}At -labeled Fab', or alternatively, for releasing metabolites from kidneys.

Several different approaches have been studied to reduce renal localization and retention of radiolabeled proteins, their enzymatically produced fragments and genetically engineered fragments.^{23,37,38} An attractive approach is to decrease uptake of ^{211}At -labeled Fab' without alteration of the Fab' or labeling molecule. Such an approach, that of infusing basic amino acids, has been investigated for Fab',^{39–44} however, that approach can be difficult to control and the decrease in kidney concentration may not be enough to be useful for ^{211}At -labeled Fab' (although it might be useful in combination with other approaches). Since it did not appear that infusion of basic amino acids would provide adequate clearance, approaches where the Fab' was modified were considered. One possible modification would be to conjugate another molecule (e.g. large polyethyleneglycol) to the Fab', such that its modified

size/shape would block filtration by the glomerulus. Although attractive as an approach, based on literature reports we surmised that approach would likely lead to loss of immunoreactivity of the Fab' before complete blockage of filtration was achieved.⁴⁵ Likewise, alteration of the pI on the Fab' by succinylation⁴⁶ or glycolation⁴⁷⁻⁴⁹ also seemed likely to have a negative affect its antigen binding.

An alternate approach to blocking uptake in kidney is to build functional groups into conjugation reagents that can be cleaved in the kidney to provide metabolites that are more readily cleared from kidneys. Several approaches have been described in the literature for decreasing the retention of radioactivity in kidney after administration of radiolabeled antibodies or their fragments.^{37,50} In one approach, investigators used enzymatic-based methods to decrease the retention of radiolabeled molecules in the kidney. Those methods are based on the use of conjugation linkers containing esters susceptible to esterases⁵¹⁻⁵⁵ or specific peptide sequences that can be cleaved by peptidases.⁵⁶⁻⁶¹ While enzyme-mediated release of radioactive metabolites from kidney functioned as designed, the results (kinetics and kidney concentrations) point to the need for exploring additional methods. An alternate method is the use the low pH in lysosomes (pH 4.5-5.0^{62,63}) for release of radioactivity from kidneys. That method was of particular interest as the cleavage process is non-specific and release kinetics can be optimized by chemical modification of the acid cleavable chemical functionality. It has been shown that lysosomal pH can be used for release of drugs from antibodies.²⁷ While pH-sensitive cis-aconityl⁶⁴ and acetal⁶⁵ linkages have been described, the use of readily prepared hydrazone linked antibody drug conjugates⁶⁶⁻⁷⁰ appeared most promising.

Prior to this study, we evaluated the tissue distributions of a series of radioiodinated *closo*-decaborate(2-) compounds in mice to determine which structural features provided the lowest tissue concentrations.³² In that study, compounds containing a hydrazone moiety had favorable pharmacokinetics and low tissue concentrations. While the study did not provide information that could be directly used to predict tissue distributions of metabolites of Fab' radioiodinated on a *closo*-decaborate (2-) moiety, it did provide information on distribution of radioiodinated hydrazone derivatives that were introduced into blood. The rapid clearance from blood and release of radioiodine from kidney encouraged us to prepare and evaluate radiolabeled Fab' conjugated with sulfhydryl-reactive *closo*-decaborate(2-) derivatives containing a hydrazone functionality. In this study, MAb Fab' fragments were used not only because they are of interest as a carrier molecule, but also as a model where differences in clearance rates between the radiohalogenated proteins could readily be observed.

Radioiodinated Fab'-hydrazone-*closo*-decaborate(2-) conjugates [¹²⁵I]**4**, [¹³¹I]**5**, [¹²⁵I]**6a**, [¹²⁵I]**7** and [¹³¹I]**8** were prepared for the study using a two-step labeling approach. Radioiodinations of the maleimido-derivatives (**14**, **17a**, **17b**, **17c** and **17d**) were conducted prior to conjugation with the Fab' to assure that all of the radioiodine was on the *closo*-decaborate(2-) moiety. By doing this, the interpretation of the tissue clearance was not confounded by having some of the radioactivity on protein tyrosine residues. In prior studies, thyroid and stomach concentrations of MAb fragments radioiodinated after conjugation with *closo*-decaborate(2-) indicated that some of the radioiodine reacted with protein tyrosine residues. Importantly, in those studies it appears that more than one *closo*-decaborate(2-) per antibody conjugate is required to assure that the radioiodine only reacts with that moiety.

The data depicted in tissue bar graphs shown in Figure 2 show that the hydrazone-containing Fab' conjugates, [¹²⁵I]**6a** and [¹³¹I]**8**, have kidney clearance rates similar to that of directly (tyrosine) labeled Fab', [¹²⁵I]**1**. Although the blood clearances are similar, Fab' conjugates [¹²⁵I]**4**, [¹³¹I]**5** and [¹²⁵I]**7** have kidney localization and retention profiles that are very

different from [^{125}I]**6a** and [^{131}I]**8**, or directly labeled [^{125}I]**1**. Tissue concentrations for [^{125}I]**4** are lower in general than for the other conjugates, with the exception of liver and spleen concentrations. Those concentrations are higher than for the other conjugates, perhaps indicating that some aggregation or serum protein binding occurred with the Fab' conjugate or its metabolites. Release of ^{125}I from liver also appears to be most efficient for [^{125}I]**6a** and [^{131}I]**8**. The only other major differences were seen in the neck (containing thyroid), where directly labeled [^{125}I]**1** was high at 1 and 4 h pi, as expected. As indicated previously, the high concentration of ^{125}I in neck for the non-cleavable *closo*-decaborate(2-) conjugate [^{125}I]**3** may be attributable to some of the ^{125}I reacting with tyrosine residues on the Fab' conjugate rather than the *closo*-decaborate(2-) cage. From these data it was concluded that Fab' conjugates [^{125}I]**6a** and [^{131}I]**8** have the most favorable in vivo characteristics for further study.

The ultimate goal in developing *closo*-decaborate(2-) conjugation reagents is to use them for direct labeling of the proteins with ^{211}At , as that is the simplest labeling procedure and provides the highest radiolabeling yields (up to 92% in our studies). Thus, conjugation of the maleimido-*closo*-decaborate(2-) reagent should be done prior to labeling. Although a two-step labeling procedure was used in the initial biodistribution studies to be certain that the radioiodine was bonded to the *closo*-decaborate(2-) moiety, ^{211}At does not stably bond to tyrosine residues so direct labeling only occurs on the *closo*-decaborate(2-). An important issue that arose was to make a decision on which hydrazone-containing conjugation reagent to use. Although both of the labeling reagents, [^{125}I]**6a** and [^{131}I]**8**, were of interest, the choice of hydrazone derivative to use was limited to the conjugate containing a benzoic acid moiety, **17b**. This choice was made as the acetyl-*closo*-decaborate(2-), **9d**, and its hydrazone-containing maleimido-*closo*-decaborate(2-) adduct, **17d**, were found to be somewhat unstable and difficult to purify.

It was of interest to determine if the Fab' conjugate of choice, [^{125}I]**6a**, which contained a hydrazone functionality substituted with a *closo*-decaborate(2-) moiety and a *para*-benzoate moiety was cleaved under mild acid conditions. The use of hydrazone-containing linkers as a means of releasing radioactivity from kidney was based on previous literature reports that demonstrated antibody-drug conjugates containing hydrazone linkers released the drug under mild acid conditions (e.g. pH 4.5), but not at neutral pH.^{66,67,71-74} The rate of acid cleavage can be dependent on the electronic nature of substituents on the hydrazine and aldehyde or ketone reacted that form the hydrazone,⁷⁵ making it possible to form hydrazone linkers that are too rapidly cleaved for in vivo use, or alternatively, very slowly cleaved under in vivo conditions.⁷⁶ Importantly, none of the previous hydrazone conjugates contained the dianionic *closo*-decaborate(2-) moiety. The results obtained from the in vitro acid-catalyzed cleavage analysis indicate that acid catalyzed cleavage of the hydrazone functionality does occur for Fab' conjugate [^{125}I]**6a**. The observed acid-catalyzed cleavage supports our hypothesis for the mechanism of in vivo release of radioactivity from the kidney and liver (Figure 2), but the kinetics of the cleavage under the in vitro conditions used appear much slower than the release of radioactivity from tissues. It seems likely that, at least in part, the biological mechanism for release of radioactivity from tissue is acid-catalyzed cleavage of the hydrazone functionality. However, to be certain of the actual mechanism of release from the kidneys, studies of metabolites in blood, urine and feces would be required. Such metabolite studies are beyond the scope of this investigation.

All of the initial developmental studies were conducted with radioiodinated Fab' conjugates, but the ultimate goal is to use them for targeting ^{211}At to cancer cells in vivo. In the final Fab' study, **17b** was conjugated with 107-1A4 Fab', then directly labeled with ^{125}I to prepare [^{125}I]**6a** and (separately) with ^{211}At to prepare [^{211}At]**6b**. An important difference in this biodistribution study was that the radiohalogenated Fab' were evaluated in mice bearing

LNCAp human prostate tumor xenografts. The 107-1A4 is an anti-PSMA antibody that binds with the prostate-specific membrane antigen (PSMA), which is highly expressed on the LNCAp cells.²⁸ This model was used to demonstrate that tumor targeting could be achieved, and to assess whether the cleavable hydrazone group caused a decrease in tumor concentration at the later time points. The biodistribution data are plotted in Figure 3. It had been presumed that the tissue distribution of the Fab' conjugate [²¹¹At]**6b** would be very similar to that of [¹²⁵I]**6a**. This presumption was based on many prior studies, where the two radionuclides had similar distributions when the carrier molecules were conjugated with a *closo*-decaborate(2-) moiety. The results show that the use of ²¹¹At changes the retention of radioactivity in kidney, liver and tumor from that obtained with the *same Fab' conjugate* labeled with ¹²⁵I. The neck and stomach concentrations indicate that [²¹¹At]astatide is not being released, so it seems likely that another metabolite is being retained. Although the nature of that metabolite is not known for certain, it should be noted that we have obtained similar differences in kidney retention for ¹²⁵I- and ²¹¹At-labeled biotin derivatives that contain a ketone coupled to a *closo*-decaborate(2-) cage (unpublished results). Importantly, the kidney retention differences observed for that biotin derivative were alleviated by conversion of the ketone to an oxime derivative. That finding is suggestive that nature of the conjugated ketone or other metabolite is changed when the *closo*-decaborate(2-) is substituted with ²¹¹At rather than ¹²⁵I. It also suggests that alternate hydrazone structures might provide the faster renal clearance being sought.

In conclusion

This study has demonstrated that chemical linkers containing hydrazone moieties can be used to increase the rate of clearance of radioactivity (radioiodine) from kidneys and other tissues. The differences in tissue clearance data indicate that changing the nature of the hydrazone and substituents attached to it can change the tissue clearance rates. The striking differences in tissue clearance observed between radioiodinated and astatinated Fab' conjugate were surprising. That data indicates that additional studies must be conducted before effective hydrazone-containing linkers are found that function with ²¹¹At. In contrast, the data obtained indicate that use of a hydrazone containing *closo*-decaborate(2-) labeling moiety might be highly beneficial for radioiodinated proteins or peptides that are localized to kidney.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Quanta BioDesign (Powell, Ohio) for the gift of MPS-EDA.TFA and MAL-dPEG[®]₄-t-boc-hydrazide. We thank NIH (1 RO1 CA113431) for funding the studies.

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Figure 1.

Depiction of radiolabeled 107-1A4 Fab'-SH conjugates showing chemical structures of directly radioiodinated Fab', [^{125}I]**1**, versus Fab' radiolabeled by conjugation with a lysine amine, [^{125}I]**2**, or conjugation with a free sulfhydryl group, [^{125}I]**3**, [^{125}I]**4**, [^{131}I]**5**, [^{125}I]**6a**, [^{211}At]**6b**, [^{125}I]**7** and [^{131}I]**8**. Note that Fab' conjugates **4** – **8** contain hydrazone functionalities.

Figure 2.

Bar graphs representing tissue concentrations (% ID/g) of radioiodinated 107-1A4 Fab' and radioiodinated 107-1A4 Fab' conjugates in selected tissues. The numbers on the x-axis represent the radioiodinated Fab' derivatives depicted in Figure 1. Data plotted for directly labeled Fab'-NEM, [^{125}I]1, *para*-iodobenzoate-labeled Fab'-S-NEM, [^{125}I]2, and non-cleavable radioiodinated Fab'-SH conjugate [^{125}I]3 were obtained from the literature (17). Data plotted for hydrazone-containing conjugates [^{125}I]4, [^{131}I]5, [^{125}I]6a, [^{125}I]7 and [^{131}I]8 were obtained in this study. Identification of the tissue graphed is provided in the boxed legend within the graph. The percent injected dose/gram (%ID/g) values shown in each bar are average values in that tissue for 3–5 mice, and the error bars represent the standard deviation of those values. The first bar (red) represents values obtained at 1 h post injection (pi), the second bar (blue) represents values obtained at 4 h pi, and the third bar (green) represents values obtained at 24 h pi. Note that the range in %ID/g for the blood and kidney graphs (top) is 0 – 50 %ID/g whereas the range for the other 4 graphs is 0 – 20 % ID/g. The data plotted in the bar graphs are provided as Tables S1, S2 and S3 in Supporting Information.

Figure 3.

Bar graphs depicting the tissue concentrations (%ID/g) of [^{125}I]**6a** (left panel) and [^{211}At]**6b** (right panel). The Fab'-SH conjugates were prepared, then directly labeled with ^{125}I and (separately) ^{211}At . The labeled Fab' conjugates were mixed and co-administered so that tissue comparisons could be made in the same animal. Each bar represents the average %ID/g obtained in a group of 5 mice for that radionuclide in a selected tissue, and the error bars represent the standard deviation of those values. The red bars are tissue concentrations at 1 h post injection (pi), blue bars at 4 h pi and green bars at 24 h pi. The radionuclide being represented is shown in the boxed legend within the graph. The complete set of data for the values plotted are provided as table S4 in the Supporting Information.

Scheme 1.

^aMeOH, rt, 16h, 92%; ^bTCDI, anhyd. DMF, rt 1h, 99%; ^c**13**, Et₃N, anhyd. DMF, rt, 2h, 68%; ^dMeOH, HOAc, Na[¹²⁵I]I, NCS, rt, 1 min; ^eFab'-SH/PBS, 30 min, rt, NEM/DMSO, 10 min, rt.

Scheme 2.

^aTFA, rt, 3 min; ^bMeOH, **16**(TFA salt), **9a,9b,9c** or **9d**, rt, 1–4 h, 50–81%; ^cNaI or Na[¹²⁵/¹³¹I]I, MeOH, HOAc, NCS, rt, 1 min; ^dFab'-SH/PBS, 30 min, rt, NEM/DMSO, 10 min, rt, 30–40%; ^eFab'-SH/PBS, **17b**/DMF, 30 min, rt, NEM/DMF, 10 min, rt, 64%; ^fsodium phosphate (0.5 M, pH 7.4), Na[¹²⁵I]I or Na[²¹¹At]At, ChT, 1 min, Na₂S₂O₅, 95% & 64% respt.