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# Reagents for astatination of biomolecules. 6. An intact antibody conjugated with a maleimido-*closo*-decaborate(2-) reagent via sulfhydryl groups had considerably higher kidney concentrations than the same antibody conjugated with an isothiocyanato-*closo*-decaborate(2-) reagent via lysine amines

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## Abstract

We are investigating the use of an $^{211}$At-labeled anti-CD45 monoclonal antibody (mAb) as a replacement of total body irradiation in conditioning regimens designed to decrease the toxicity of hematopoietic cell transplantation (HCT). As part of that investigation, dose-escalation studies were conducted in dogs using $^{211}$At-labeled anti-canine CD45 mAb, CA12.10C12, conjugated with a maleimido-*closo*-decaborate(2-) derivative, **4**. Unacceptable renal toxicity was noted in the dogs receiving doses in the 0.27 – 0.62 mCi/kg range. This result was not anticipated, as no toxicity had been noted in prior biodistribution and toxicity studies conducted in mice. Studies were conducted to understand the cause of the renal toxicity and to find a way to circumvent it. A dog biodistribution study was conducted with $^{123}$Ilabeled CA12.10C12 that had been conjugated with **4**. The biodistribution data showed that 10-fold higher kidney concentrations were obtained with the maleimido-conjugate than had been obtained in a previous biodistribution study with $^{123}$I-labeled CA12.10C12 conjugated with an amine-reactive phenylisothiocyanato-CHX-A" derivative. The difference in kidney concentrations observed in dogs for the two conjugation approaches led to an investigation of the reagents. SE-HPLC analyses showed that the purity of the CA12.10C12 conjugated via reduced disulfides was lower than that obtained with amine-reactive conjugation reagents, and non-reducing SDS-PAGE analyses indicated protein fragments were present in the disulfide reduced conjugate. Although we had previously prepared *closo*-decaborate(2-) derivatives with amine-reactive functional groups (e.g. **6** & **8**), a new easily synthesized, amine-reactive (phenylisothiocyanate) derivative, **10**, was prepared for use in the current studies. A biodistribution was conducted with co-administered $^{125}$I- and $^{211}$At-labeled CA12.10C10 conjugated with **10**. In that study, lower kidney concentrations were obtained for both radionuclides than had been obtained in the earlier study of the same antibody conjugated with **4** after reduction of disulfide bonds.

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**Supporting Information Available:** A table (Table S1) containing biodistribution data from CA12.10C12 conjugates [$^{123}$I]**5b** and [$^{125}$I]**11b**/[$^{211}$At]**11c** and a graph (Figure S1) plotting the blood clearance of [$^{123}$I]**5b** and [$^{125}$I]**11b**/[$^{211}$At]**11c** are provided. This material is available free of charge via the Internet at http://pubs.acs.org/BC.

## INTRODUCTION

We are investigating the use of monoclonal antibody (mAb[1])-targeted α-emitting radionuclides as a replacement for the total body irradiation (TBI) to decrease the toxicity of hematopoietic cell transplantation (HCT) conditioning regimens.[1] In our prior studies, we found that stable engraftment could be obtained in a dog model when either an anti-CD45 or anti-TCRαβ mAb labeled with the α-emitting radionuclide bismuth-213 ($^{213}$Bi) was used to replace TBI in the conditioning regimen.[2, 3] While successful, the translation of the $^{213}$Bi-labeled mAbs to clinical studies was not practical due to the high cost[2] and low availability of the parent radionuclide actinium-225. Therefore, we are presently evaluating the use of another α-emitting radionuclide, astatine-211 ($^{211}$At) in the place of the $^{213}$Bi in the conditioning regimen. Importantly, $^{211}$At is readily available at our institution, and at a cost[2] that will allow translation to a clinical study.

As part of the transition from $^{213}$Bi to $^{211}$At, studies were conducted in mice to determine the best method to use for labeling mAbs with $^{211}$At, and to compare the efficacy of $^{211}$At-labeled anti-CD45 mAb to the same mAb labeled with $^{213}$Bi. Due to concern about the in vivo stability of $^{211}$At-labeled mAbs,[4] biodistributions of $^{211}$At-labeled anti-CD45 mAbs, 30F11, obtained from two $^{211}$At-labeling approaches were conducted. Those labeling approaches (**A** & **B**) are depicted in Figure 1. In the studies, the often used mAb-labeling approach, employing a N-succinimidyl ester of *meta*-trialkylstannylbenzoic acid **1** to prepare N-succinimidyl *meta*-[$^{211}$At]astatobenzoate **2**, was compared with conjugation of a maleimido-*closo*-decaborate(2-) derivative **4**, which had been previously shown to be stable to in vivo deastatination.[5] The comparison studies demonstrated that $^{211}$At-labeled maleimido-*closo*-decaborate(2-) mAb conjugate, [$^{211}$At]**5c** had a higher in vivo stability than the $^{211}$At-labeled benzoate mAb conjugate [$^{211}$At]**3b**. Importantly, conjugation of **4** to the mAb allowed direct labeling of the conjugate **5a**, which made the labeling procedure much simpler and provided higher radiochemical yields than the 2-step labeling procedure used to prepare [$^{211}$At]**3b**. Subsequently, studies were conducted in mice to compare the hematopoietic cell-killing efficacy of [$^{213}$Bi]30F11 with [$^{211}$At]30F11, labeled after conjugation with **4**. Those studies demonstrated that the $^{211}$At-labeled mAb was equal or superior to the $^{213}$Bi-labeled mAb in depleting hematopoietic cells.[6] From the data obtained, it was estimated that 50 μCi of [$^{211}$At]30F11 would provide a higher dose to the target cells in the spleen (294 Gy) than 500 μCi [$^{213}$Bi]30F11 (117 Gy) when delivered on 10 μg mAb, while the radiation dose to the other tissues was similar for the two radiolabeled mAb doses.

Encouraged by the results obtained in mice, a dose-escalation study of $^{211}$At-labeled anti-canine CD45 mAb was conducted using [$^{211}$At]CA12.10C12, [$^{211}$At]**5c**, labeled after

[1]Abbreviations: ChT, chloramine-T; cpm, counts per minute; DTPA-CHX-A", DIPE, diisopropylether, DMSO, dimethyl sulfoxide, DTPA derivative with cyclohexane in backbone; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; HCT, hematopoietic cell transplantation; IEF, isoelectric focusing; mAb, monoclonal antibody; MWCO, molecular weight cut-off; PBS, phosphate buffered saline; %ID/g, percent injected dose/gram; pI, isoelectric point; rt, room temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE-HPLC, size-exclusion HPLC; TBI, total body irradiation; TCDI, 1,1′-thiocarbonyldiimidazole; TFA, trifluoroacetic acid

[2]The cost of radionuclide in an early clinical study using $^{213}$Bi-labeled anti-CD45 for conditioning in hematopoietic cell transplantation was estimated at $225,000. That amount is based on injection of 2 mCi/kg with an average 70 kg patient (probably higher) making the total injected 140 mCi/70 kg patient. Due to decay during labeling and QC ($^{213}$Bi half-life 45.6 min) one can expect a 50% yield at best, so one would have to start with a minimum of 280 mCi $^{213}$Bi. That amount of $^{213}$Bi activity could be obtained from a 150 mCi $^{225}$Ac generator if two injections were made (more injections would be problematic). At present the cost of $^{225}$Ac is $1500/mCi so that generator would cost $225,000. In contrast, the longer half-life of $^{211}$At makes it possible to treat with 0.2 mCi/kg. Therefore, a 70 kg patient might require 14 mCi of $^{211}$At-labeled anti-CD45 for haploidentical transplantation. Currently the (internal) cost for cyclotron time is ~$600/hour and about 60 mCi could be obtained in a 3 h irradiation. With the current isolation procedure, 60%, or 36 mCi, could be isolated from the irradiated target. With labeling yields and QC time, one can estimate that at least 50% of the activity could be isolated as $^{211}$At-labeled anti-CD45 mAb, providing at least 18 mCi for patient treatment. The estimated cost of $^{211}$At might be $1800 for cyclotron time + $1200 for target, personnel and other costs, resulting in a total of ~$3000 for $^{211}$At used in the treatment of a patient.

conjugation with **4**. The dose-escalation study was stopped when blood samples showed that renal function was impaired in some of the dogs. This toxicity was unanticipated, as no toxicity had been previously observed in the $^{211}$At-labeled anti-CD45 mAb mouse studies. While the cause of the kidney toxicity was unknown, it was suspected that the toxicity might be caused by the nature of the mAb conjugate. To determine if that suspicion was correct, HPLC, IEF and SDS-PAGE analyses were run on the mAb conjugates **5a, 7a, 9a** and **11a**, prepared by conjugation of **4** after reduction of disulfides or by conjugation of the amine-reactive reagents, **6, 8** and **10** with mAb lysine amines. The amine-reactive *closo*-decaborate(2-) conjugation reagents, **6** and **8** had been previously reported, while a synthesis of **10** was carried out. Additionally, biodistributions of radiolabeled mAb-conjugates [$^{123}$I]**5a** and [$^{125}$I]**11b**/[$^{211}$At]**11c** were conducted in dogs to compare concentrations of the radionuclides in blood and tissues at time of necropsy. The results of the investigation of mAb-*closo*-decaborate(2-) conjugates and the synthesis of the new amine-reactive *closo*-decaborate(2-) reagent, **10**, used for conjugation are described herein.

## EXPERIMENTAL PROCEDURES

### Reagents

Chemicals purchased from commercial sources were analytical grade or better and were used without further purification. Decaborane ($B_{10}H_{14}$) was obtained from Alfa Aesar (Ward Hill, MA). 4-(2-Aminoethyl)aniline, **13**, DL-dithiothreitol (DTT), chloramine-T (ChT) and phosphate buffered saline (PBS) were obtained from Sigma-Aldrich (Sigma, St. Louis, MO). 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; GE Healthcare) and Vivaspin 6 centrifugation filters (30 kDa molecular weight cut-off (MWCO); GE Healthcare) were obtained from Fisher Scientific (Houston, TX).

### Radioactive Materials

All radioactive materials were handled according to approved protocols at the University of Washington and Fred Hutchinson Cancer Research Center. Standard methods for safely handling radioactive samples were employed.[7] Na[$^{123}$I]I was purchased from Nordion (Vancouver, BC) as a radiochemical in 0.1 N NaOH. Na[125I]I was purchased from PerkinElmer Life Sciences (Billerica, MA) as high concentration/high specific activity radioiodide in 0.1 N NaOH. $^{211}$At was produced by irradiation of a thin layer (0.007 inch; 0.18 mm) of bismuth metal (99.999%, Aldrich) with 29.0 MeV α-particles on a Scanditronix MC-50 cyclotron using the conditions previously described.[8] The $^{211}$At was isolated from the bismuth target material using a “wet chemistry” isolation procedure (described below). 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 on the glovebox exhaust and subsequently through a second charcoal-filtered Plexiglas enclosure (12 × 16 × 21”; Biodex 112-038) within a radiochemical fume hood.

All radiohalogenation reactions were conducted in a charcoal-filtered Plexiglas enclosure (radioiodine fume hood, 20 × 24 × 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.

Measurement of $^{123}$I, $^{125}$I and $^{211}$At were conducted on a Capintec CRC-15R Radioisotope Calibrator using the preprogrammed $^{123}$I button and calibration numbers 319 and 44 for $^{125}$I and $^{211}$At respectively (designated by Capintec Technical Services). Tissue samples were

counted in a Packard Cobra II gamma counter (PerkinElmer Life and Analytical Services, Wellesley, MA). The tissue samples containing $^{211}$At and $^{125}$I were counted within 24 h of necropsy to obtain both $^{211}$At and $^{125}$I counts, and were recounted after 3-5 days (0.1% or less $^{211}$At remaining) to obtain $^{125}$I counts. Radioactivity counts were imported into an Excel Spreadsheet (Microsoft Corp., Redmond, WA) where calculations were made. The $^{211}$At counts were obtained by subtracting $^{125}$I counts from total counts. Individual tissue counts for $^{211}$At were corrected for decay from the time of counting the first standard (at the beginning of the tissue counting process).

## Isolation of Na[$^{211}$At]At

$^{211}$At was isolated from the irradiated bismuth target by a "wet chemistry" approach, as follows: An irradiated aluminum-backed bismuth target containing 18-22 mCi[3] of $^{211}$At was placed flat (with Bi down) in a 1L polypropylene dissolution chamber, and 10 mL of concentrated $HNO_3$ was added. This dissolution process was allowed to continue for 10 min at room temperature. The $HNO_3$ solution was removed from the chamber using a pipette 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). 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 305°C was used to distill the $HNO_3$ leaving a white residue (~20 min) in the flask. After cooling, 8 mL of 8M HCl was added to the flask with stirring to dissolve the white residue. The resultant clear solution was removed and placed in a 20 mL scintillation vial. The flask was rinsed with additional 2 mL aliquot of 8M HCl, and that was added to the scintillation vial. An 8 mL quantity of freshly distilled diisopropylether (DIPE), pre-equilibrated with 8M HCl[4], was added to the scintillation vial and that mixture was stirred at room temperature for 10 min. The 8M HCl was removed by pipette to waste, and an additional 5 mL of 8M HCl was added and stirred for 5 min. The 8M HCl was removed to waste. This rinse was repeated 3 more times to minimize the amount of bismuth remaining in the DIPE[5]. To the DIPE was added 1.2 mL of 4 M NaOH to make the solution basic. The basic solution was brought to near neutral (pH 6.5-7.5) with 4N HCl and 0.5N HCl[6]. The final volume of the $^{211}$At solution was 1.5-2 mL with 12-15 mCi[7]. The Na[$^{211}$At]At vial was tightly sealed, placed in sealed (Ziploc) plastic bag, then transferred from the glove box to the Biodex radiolabeling enclosure within a radiochemical fume hood.

## Monoclonal Antibodies

The anti-canine CD45 mAb, CA12.10C12, an $IgG_1$ isotype mAb[9], was used for targeting hematopoietic cells in the dog model.[2, 10] For flow cytometry, mAbs against canine CD45 (CA12.10C12, IgG1), CD4 (CA13.1.E4, IgG1), CD8 (CA9.JD3, IgG2a)[11] were used. The mAb 31A (IgG1) directed at the mouse Thy-1 receptor served as an irrelevant isotype-matched control, because it did not cross-react with canine cells.[12] All mAbs were produced and purified at the Biologics Production Facilities of the Fred Hutchinson Cancer Research Center (Seattle, WA).

[3]Measurement of $^{211}$At activity in the target must be considered an approximation due to attenuation and geometry.
[4]Pre-equilibration of the DIPE with 8M HCl, and also pre-equilibration of 8M HCl with DIPE, appears to provide better control of the amounts of reagents added and removed during the wet chemistry processing.
[5]Analysis of the amount of bismuth remaining in the 8M HCl provided data that indicates 4 washes are required to minimize it.
[6]The neutralization process was begun with 4N HCl to minimize the solution volume, but 0.5 N HCl was used when the pH was lower than 11 so that the targeted pH was not passed (i.e. becoming too acidic).
[7]In 48 $^{211}$At-isolation experiments using this wet chemistry approach, radiochemical yields of 82.0% ± 18.1% (decay corrected; or 61.1% ± 14.3% actual) were obtained from targets containing 20.5 ± 2.2 mCi.

### Spectral Analyses

$^{1}H$ NMR and $^{11}B$ NMR spectra of compounds **10** and **14** 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 $BF_3OEt_2$ 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 dissolved in MeOH and were introduced by an integral syringe infusion pump (Cole Parmer Series 74900).

### Chromatography

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 μm, 250 × 4.5 mm) using a gradient solvent system at a flow rate of 1 mL/min. A gradient of MeOH and 0.05M $Et_3NHOAc$ was used. Starting with 20% 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 nonradioactive compounds. 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 ($Et_3NHOAc$) in water. A gradient was used which started with 100% $Et_3NHOAc$ 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.

### Halogen-Reactive closo-Decaborate(2-) Conjugation Molecules

The sulfhydryl-reactive maleimido-*closo*-decaborate(2-) derivative, **4**, and amine-reactive biotin-containing, isothiocyanatophenyl-*closo*-decaborate(2-) derivative **8** were obtained as previously described.[5]

### $[Et_3NH]_2B_{10}H_9$-NCO, 6

This compound was synthesized using the method described by Hawthorne et al.[13] Briefly, oxalyl chloride (0.838 mL, 9.76 mmol) was added dropwise to a solution of $[Et_3NH]_2B_{10}H_{10}$, **12** (3.0 g, 9.30 mmol)[14] and anhydrous $CH_3CN$ (80 mL) at 0°C. The resultant solution was stirred at 0°C for 0.5 h. After the ice-bath was removed the solution was stirred for another 0.5 h. To that solution was added solid $NaN_3$ (1.27 g, 19.53 mmol), and the solution was stirred at room temperature for 24 h. The mixture was filtered, then the volume of the filtrate was reduced to ~ 40 mL by rotary evaporator under vacuum. $Et_2O$ (200 mL) was added slowly to the solution with stirring, and the mixture was stirred at ice-bath temperature for 2 h. The solvents were decanted, and the light-brown product was dried under vacuum overnight to yield 2.66 g (79%) of **6** as tacky light brown solid. HPLC: $t_R$ = 4.4 min.

### $[Et_3NH]_2B_{10}H_9$-NHCONH-$CH_2CH_2$-Ph-$NH_2$, 14

A solution of **6** (0.50 g, 1.375 mmol), 4-(2-aminoethyl)aniline, **13** (0.187 g, 1.375 mmol), $Et_3N$ (0.288 mL, 2.063 mmol), and anhydrous dimethylformamide (4 mL) was stirred and heated by microwave (Biotage Initiator; Biotage, LLC, Charlotte, N.C.) at 150 °C for 5 min.

To the reaction mixture was added 5 mL $H_2O$, then the crude product solution was purified via Biotage Flash Chromatography (C18 FLASH 25+M column) via a gradient elution. The isolated fractions containing product were evaporated on a rotary evaporator under vacuum to yield 0.38 g (55%) of **14** as a light-yellow tacky solid. $^1$H NMR [includes $Et_3NH$] ($CD_3CN$, 500 MHz): δ 0.18-0.95 (m, 9H), 1.22 (t, J = 7.2 Hz, 18H), 2.66 (t, J = 7.3 Hz, 2H), 3.10 (q, J = 7.2 Hz, 12H), 3.26 (m, 2H), 6.60 (d, J = 8.5 Hz, 2H), 7.03 (d, J = 8.5 Hz, 2H), 7.14 (s, 1H). $^{11}$B NMR ($CD_3CN$, 160.4 MHz): δ 0.25 (1B), −4.67 (1B), −14.64 (1B), −23.89 (2B), −24.36 (2B), −26.57 (1B), −27.28 (1B), −29.87 (1B). HRMS (ES-) $C_9H_{22}B_{10}N_3O$ (M+H)-calcd: 298.2699. Found: 298.2692. HPLC: $t_R$ = 6.5 min.

### $[Et_3NH]_2$ $B_{10}H_9$-NHCONH-$CH_2CH_2$-Ph-NCS, 10

Solid thiocarbonyldiimidazole (0.086 g, 0.48 mmol) and **14** (0.20 g, 0.40 mmol) were dissolved in anhydrous $CH_3CN$ (10 mL). The resultant solution was stirred at room temperature for 1 h. To the reaction solution 10% EtOAc/hexanes (100 mL) was slowly added with stirring, and the mixture was allowed to stir at room temperature for 1 h. The solvents were decanted, the residue was washed with 10% EtOAc/hexanes (20 mL), dried under vacuum for overnight to yield 0.21 g (97%) of **10** as a tacky light-yellow solid. $^1$H NMR [includes $Et_3NH$]($CD_3CN$, 500 MHz): δ −0.15-0.98 (m, 9H), 1.23 (t, J = 7.3 Hz, 18H), 2.83 (t, J = 7.0 Hz, 2H), 3.12 (q, J = 7.3 Hz, 12H), 3.38 (m, 2H), 7.21 (d, J = 8.6 Hz, 2H), 7.35 (d, J = 8.6 Hz, 2H). $^{11}$B NMR ($CD_3CN$, 160.4 MHz): δ 0.45 (1B), −4.61 (1B), −14.67 (1B), −23.73 (2B), −24.22 (2B), −26.54 (1B), −27.25 (1B), −29.71 (1B). HRMS (ES-) $C_{10}H_{18}B_{10}N_3OS$ (M-H)-calcd: 338.2107. Found: 338.2100. HPLC: $t_R$ = 12.9 min.

### CA12.10C12 sulfhydryl conjugation of 4

To a 1.5 mL solution of 6.1 mg/mL CA12.10C12 in PBS was added 200 μL of 100 mM dithiothreitol. After 1 h of gentle mixing at room temperature, the reduced protein was eluted over a PD-10 column with PBS (pH 6.5 containing 1 mM EDTA). The protein-containing fractions were pooled to give 6 mL of eluate at a concentration of 1.4 mg/mL (UV 280 nm). To that solution was added 39 μL (10 equiv.) of a 10 mg/mL solution of **4** in DMSO. The reaction solution was gently mixed for 30 min at room temperature before quenching with 1.8 mg of sodium tetrathionate. The protein was concentrated in a Vivaspin 30K MWCO concentrator and washed 5× with 10 mL each of PBS pH 7.2, concentrating between each wash. The isolated CA12.10C12-**4** was obtained in 2.0 mL solution at a concentration of 3.9 mg/mL (85% protein recovery).

### CA12.10C12 lysine conjugation of 6

To a 250 μL solution of 1.0 mg/mL CA12.10C12 in PBS was added 250 μL of 100mM sodium bicarbonate, pH 9.0. To that solution was added 10 equivalents (1.8 μL) of a 3.2 mg/mL solution of **6** in DMSO. The mixture was tumbled gently overnight at room temperature. The reaction mixture was then placed on a size-exclusion column (PD-10) and was eluted with PBS. The protein-containing fractions were pooled and concentrated to give 150 μL of 1.0 mg/mL (62% protein recovery) solution of the mAb conjugate. A second conjugation reaction, in which the quantities and conditions employed were the same except 20 equivalents (13.6 μL) of a 3.2 mg/mL solution of **6** in DMSO was added, resulted in 120 μL of 1.1 mg/mL (54% protein recovery) solution of the mAb conjugate.

### CA12.10C12 lysine conjugation of 8

To a 200 μL solution of 5.4 mg/mL CA12.10C12 in PBS was added 200 μL of 100mM HEPES buffer, pH 8.5. To that solution was added 5 equivalents (5.3 μL) of an 8.3 mg/mL solution of **8** in DMSO. The mixture was tumbled gently overnight at room temperature. The reaction mixture was then placed on a size-exclusion column (PD-10) and was eluted

with PBS. The protein-containing fractions were pooled and concentrated to give 135μL of 5.2 mg/mL (70% protein recovery) solution of the mAb conjugate. A second conjugation reaction, in which the quantities and conditions employed were the same except 10 equivalents (10.5 μL) of a 3.2 mg/mL solution of **8** in DMSO was added, resulted in 170μL of 4.5 mg/mL (77% protein recovery) solution of the mAb conjugate. HABA analysis[7] indicated that there were 0.5 and 1.2 biotins per antibody respectively for the isolated mAb conjugated with **8**. [Note: running the conjugation in sodium bicarbonate pH 9.0 produced similar results]

### CA12.10C12 lysine conjugation of 10

**analytical scale—**To 180 μL of CA12.10C12 at 5.5 mg/mL in PBS was added 180 μL HEPES buffer (100 mM with 150 mM NaCl, pH 8.6) followed by 5, 10, or 20 eqivalents (18, 36, or 72 μg) of **10** at 7 mg/mL in dimethyl sulfoxide. The reactions were run overnight at room temperature with gentle tumbling. In the morning, each reaction mixture was eluted over a separate size-exclusion (PD-10) column using PBS as eluant. The protein containing fractions were combined and concentrated in Vivaspin 6 centrifugal filters (30kDa MWCO) to yield 700-880 μg of each of the three conjugates.

**preparative scale—**To 1.0 mL of CA12.10C12 at 9.68 mg/mL in PBS was added 1.0 mL HEPES buffer (100mM with 150mM NaCl, pH 8.6) followed by 10 equivalents (0.350 mg) of **10** at 7 mg/mL in DMSO (50μL). The reaction was run overnight at room temperature with gentle tumbling. In the morning, the reaction was split into two fractions and was eluted over two size-exclusion (PD-10) columns using PBS as eluant. The protein containing fractions were combined and concentrated in a Vivaspin 6 centrifugal filter (30kDa MWCO) to yield 8.9 mg of conjugate at 3.5 mg/mL (92%).

### mAb Conjugate Characterization

mAb conjugates were evaluated by size-exclusion (SE)-HPLC. Non-radioactive mAb conjugates were also evaluated on IEF gels to determine changes in charge (isoelectric point, pI) as described below. Additionally, non-radioactive mAb conjugates were characterized by non-reducing and reducing SDS-PAGE analyses to determine molecular weights of conjugates and whether the interchain disulfide bonds were intact in those conjugates (non-reducing). Cell binding was evaluated for the mAb conjugate **11a** by flow cytometry. A brief description of the methods and equipment used in these assays is provided below.

**Isoelectric Focusing—**IEF analyses were conducted on a Novex PowerEase 500 instrument with the XCell II chamber using Invitrogen (Novex) precast gels, pH 3-10 or pH 3-7 (1.0 mm thick × 12 wells), running under the standard IEF program. The protein was stained with GelCode Blue Stain (Pierce). IEF standards were from Serva Electrophoresis GmbH: pH 10.7, cytochrome C; 9.5, ribonuclease A; 8.3, 8.0, 7.8, lectin; 7.4, 6.9, myoglobin; 6.0, carbonic anhydrase; 5.3, 5.2, γ-lactoglobulin; 4.5, trypsin inhibitor; 4.2, glucose oxidase; 3.5, amyloglucosidase.

**SDS-PAGE—**Reducing and non-reducing SDS-PAGE analyses were conducted as instructed for use with the Invitrogen (Carlsbad, CA) Novex Tris-Glycine Gels in the XCell SureLock Mini-Cell apparatus. Briefly, the test samples were denatured in aqueous Tris-Glycine SDS Sample Buffer, with or without NuPAGE reducing agent by heating at 85°C for 2 min. The samples and SeeBlue Plus2 prestained standards were loaded on the gel. The electrophoresis was run at 125 V for ~90 min. After that time, the sample proteins were visualized by gentle rocking in a GelCode Blue Stain Reagent solution. Following staining the gel was placed in a rack to air dry, then it was scanned for digital archiving. Molecular

weight (kDa) standards for Tris-Glycine Gels are: myosin, 250; phophorylase, 148; BSA, 98; glutamic dehydrogenase, 64; alcohol dehydrogenase, 50; carbonic anhydrase, 36; lysozyme, 16 and aprotinin, 6.

**Flow Cytometry—**Flow cytometry was used to evaluate the antigen binding of CA12.10C12 after the conjugation reaction using 5, 10 or 20 equivalents of reagent **10**. Two hundred thousand normal dog peripheral blood mononuclear cells (PBMC) in 50 μL of 15% horse serum (HS)-1× PBS were incubated with 50 μg/mL of the CA12.10C12 or one of the three **11a** conjugates on ice for 20 minutes. After washing, cells were resuspended in 50 μL of 15% HS-PBS and were added 10 μg/mL of the secondary antibody, goat anti-mouse fluorescein isothiocyanate (FITC)-labeled $F(ab')_2$ (Biosource, Camarillo, CA), and incubated on ice for 20 min. To test the blocking ability of the antibody, 10 μg/mL FITC-conjugated CA12.10C12 was added to cells incubated with CA12.10C12 or the **11a** conjugates. After incubation, cells were washed once with 2% HS-PBS, then once with 1× PBS and resuspended in 200 μL of 1% paraformaldehyde. Cells were analyzed on a FACScan Flow cytometer (Becton Dickinson, San Jose, CA).

### Radioiodination to prepare [$^{123}$I]5b

To 640 μL of a 3.9 mg/mL solution of CA12.10C12 sulfhydryl conjugate **5a** in PBS was added 300 μL of sodium phosphate (0.5 M, pH 7.2) followed by 82 μL of Na[$^{123}$I]I (3.92 mCi in 0.1 N NaOH). To that solution was added 85 μL of a 1 mg/mL solution of chloramine-T in $H_2O$. After 2 min at room temperature the reaction was quenched with 85 μL of a 1 mg/mL solution of sodium metabisulfite in $H_2O$. The reaction mixture was then eluted over a Sephadex G-25 size-exclusion column (PD-10) using PBS as the eluant. The protein fractions were combined to yield 2.76 mCi (73% radiochemical yield). The purified [$^{123}$I]**5b** was diluted with a calculated amount of **5a** to prepare an injectate that contained 5.15 mg of labeled [$^{123}$I]**5b** in 3.7 mL PBS.

### Radioiodination to prepare [$^{125}$I]11b

To 286 μL of a 3.5 mg/mL solution of CA12.10C12 lysine conjugate **11a** in PBS was added 50 μL of sodium phosphate (0.5 M, pH 7.2) followed by 3 μL of Na[$^{125}$I]I (1.42 mCi in 0.1 N NaOH). To that solution was added 3.5 μL of a 10 mg/mL solution of chloramine-T in $H_2O$. After 1 min at room temperature the reaction was quenched with 3.5 μL of a 10 mg/mL solution of sodium metabisulfite in $H_2O$. The reaction mixture was then eluted over a Sephadex G-25 size-exclusion column (PD-10) using PBS as the eluant. Protein fractions were combined to yield 0.55 mCi (39% radiochemical yield) on 850 μg of [$^{125}$I]**11b**. The [$^{125}$I]**11b** was mixed with [$^{211}$At]**11c** for injection as described below.

### Astatination to prepare [$^{211}$At]11c

To 571 μL of a 3.5 mg/mL solution of CA12.10C12 lysine conjugate **11a** in PBS was added 300 μL of sodium phosphate (0.5 M, pH 7.2) followed by 500 μL of Na[$^{211}$At]At (11 mCi at pH 7). To the resultant solution was added 14 μL of a 10 mg/mL solution of chloramine-T in $H_2O$. After 2 min at room temperature, the reaction was quenched with 14 μL of a 10 mg/mL solution of sodium metabisulfite in $H_2O$. The reaction mixture was then eluted over a Sephadex G-25 size-exclusion column (PD-10) using PBS as the eluant. Protein fractions were combined to yield 6.58 mCi (62% radiochemical yield) on 1.7 mg of [$^{211}$At]**11c**. The [$^{211}$At]**11c** was combined with [$^{125}$I]**11b** and a calculated quantity of **11a** to prepare an injectate that contained a total of 5.08 mg of **11a/b/c** in 5.4 mL PBS.

### Dogs

Dogs were selected from litters of beagles and mini-mongrel-beagle crossbreeds raised at the Fred Hutchinson Cancer Research Center (Seattle, WA) or purchased from other commercial kennels in the United States. The dogs were quarantined for 1 month and judged to be disease-free before study. They were immunized against distemper, leptospirosis, hepatitis, papilloma virus, and parvovirus. The experimental protocol for the biodistributions and dose-escalation studies were approved by the Institutional Animal Care and Use Committee of the Fred Hutchinson Cancer Research Center. The study was performed in accordance with the principles outlined in the © 2004 Lippincott Williams & Wilkins Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources). The kennels are certified by the American Association for Accreditation of Laboratory Animal Care.

### Dose-escalation

Five dogs (H034, G530, G982, H117 and H115)[8] were treated with escalating quantities of $^{211}$At-labeled on 0.5 mg/kg of modified CA12.10C12, [$^{211}$At]**5c**, conjugated via sulfhydryl groups produced by disulfide reduction. An initial injection of 10% of the total mAb (0.05 mg/kg) was given as a predose to diminish non-specific uptake in liver.[15] The remaining 0.45 mg/kg of mAb was injected with the radiolabel at 1-1.5 h post the predose injection. Peripheral blood samples were obtained daily until hematological recovery was noted, and continued bi-weekly until the end of the study. Hepatic and renal functions were assessed weekly in the first month, then bi-weekly to monthly until the end of the study.

### Biodistribution Studies

Two biodistribution studies were conducted. In the first biodistribution study, a 10.8 kg dog (G996)[8] was first injected with 0.05 mg/kg **5a** followed 1 hour later by injection of a solution containing 2.56 mCi [$^{123}$I]**5b** (0.45 mg/kg CA12.10C12). In the second biodistribution a 11.3 kg dog (H137) was first injected with 0.57 mg (0.05 mg/kg) of **11a** followed 1 hour later by injection of a solution containing a mixture of 0.55 mCi [$^{125}$I]**11b** and 6.33 mCi [$^{211}$At]**11c** on a total of 0.45 mg/kg of CA12.10C12. In each study, blood samples were taken at 5 min, 15 min, 30 min, 1h, 2h, 3h, 4h and 21 or 24 h post injection to obtain blood clearance data. The dog injected with [$^{123}$I]**5b** was euthanized at 24 h post injection and the dog injected with [$^{125}$I]**11b**/[$^{211}$At]**11c** was euthanized at 21 h post injection. Necropsies were performed to evaluate the concentrations of radioactivity in blood and selected tissues. Triplicate samples of tissues were excised, blotted free of blood, weighed, and counted. Calculation of percent injected dose (%ID) and percent injected dose per gram (%ID/g) for the collected tissues was accomplished using injection standards for $^{123}$I, $^{125}$I or $^{211}$At counts. The full data set obtained in each biodistribution study is provided as Tables S1 & S2 in the Supporting Information.

## RESULTS

### Syntheses of *closo*-decaborate(2-) conjugates

The sulfhydryl-reactive protein conjugation reagent **4** and the lysine amine-reactive protein conjugation reagents **6** and **8** were obtained as previously described.[5] The amine-reactive reagent **10** was prepared in three synthetic steps from the readily accessible triethyl ammonium salt of decahydro-*closo*-decaborate(2-), **12**, as shown in Scheme 1. Reaction of **12** with oxalyl chloride at 0°C for 30 minutes, followed by reaction with $NaN_3$ at room temperature for 24 h as previously described by Shelly et al.[13] provided the isocyanate-

[8]Numbers in parentheses are those used to identify the dog.

derivatized *closo*-decaborate(2-) **6** in 79% yield. Reactivity of the isocyanate functionality in **6** with amine nucleophiles is low, presumably due to the electron donation from the dianionic *closo*-decaborate(2-) moiety. Therefore, reaction with aminoethylaniline **13** was conducted in dimethylformamide at 150°C for 5 minutes using a microwave reactor. After purification, a 55% yield of the adduct **14** was obtained. Conversion of the aniline **14** to the protein-reactive phenylisothiocyanate derivative **10** was accomplished in nearly quantitative yield using thiocarbonyldiimidazole (TCDI) in $CH_3CN$ at room temperature for 1 h.

## mAb conjugates

In this study, conjugations of CA12.10C12 were conducted with the sulfhydryl-reactive reagent **4** and amine-reactive reagents **6, 8** and **10** as depicted in Figure 1. Conjugation of maleimido-*closo*-decaborate(2-) **4** with dithiothreitol-reduced disulfides on CA12.10C12 at pH 6.5 was rapid, being complete within 30 min at room temperature as previously described.[4] After size-exclusion chromatography (PD-10) purification, an 85% recovery of **5a** was obtained. Conjugation of the lysine amines on CA12.10C12 with **6** or **8** at pH 8.5 (50 mM $NaHCO_3$) was considerably slower, taking up to 16 h at room temperature for completion. Similarly, conjugation of CA12.10C12 with **10** at room temperature in HEPES buffer at pH 8.6 was allowed to react overnight to obtain 72-92% protein recovery after purification by size-exclusion chromatography.

Conjugates of CA12.10C12 (**5a, 7a, 9a** and **11a**) were characterized by size-exclusion (SE) HPLC, SDS-PAGE, IEF electrophoresis and flow cytometry. A comparison of SE-HPLC chromatograms for conjugations of the anti-CD45 mAbs CA12.10C12 and 30F11 are shown in Figure 2. The HPLC chromatograms show that reduction with dithiothreitol and subsequent conjugation with the maleimido-*closo*-decaborate(2-) reagent **4** with either mAb produces some higher molecular weight species, but appears to produce only a small amount of lower molecular weight species. In contrast, conjugations of the mAbs with the isothiocyanate derivative **8** or **10** under basic conditions show only very minor changes in the chromatograms from the observed for the unmodified mAb.

Based on previous studies, most conjugation reactions were conducted using 10 equivalents of the protein-reactive molecule for each equivalent of the mAb. Evaluation of the mAb conjugate obtained from this ratio of reagents by IEF often showed a significant change in the pI, indicating that all of the mAb molecules had at least one pendant group (e.g. Fig. 2, ref. #4; Fig. 3, ref. #5). However, since conjugation reagent **10** had not previously been used, conjugation reactions of CA12.10C12 with **10** were conducted using three molar quantities (5, 10 and 20 equivalents) to determine the best ratio of reagents. An IEF gel (Figure 3) shows how the pI (and thus number of conjugates) varies with the number of equivalents offered. While the actual number of conjugates is not readily obtained, the shift of bands from unmodified CA12.10C12 (pI 6.1-6.5) is made increasingly more acidic as more equivalents of **10** are added to the conjugation reaction mixture; 5 equiv. (pI~5.4-6.4), 10 equiv. (pI~5.2-6.3) and 20 equiv. (pI~5.1-6.1).

Analysis of samples on non-reducing SDS-PAGE provided evidence of the presence of protein species having molecular weights significantly different from the unmodified mAb. Figure 4 shows the migration of denatured proteins on reducing and non-reducing SDS-PAGE gels for CA12.10C12 conjugates formed by reaction with maleimide **4** (**5a**), isocyanate **6** (**7a**), isothiocyanate **8** (**9a**), or isothiocyanate **10** (**11a**). It is readily apparent that the disulfide reduction followed by conjugating with maleimide-containing reagent **4** results in having several smaller protein species present (lane marked **5a**). In contrast, mAb conjugations with amine-reactive reagent **10**, under basic conditions, do not produce protein fragments, as shown in Figure 4, right panel.

The effect on binding with normal dog PBMC expressing the CD45 antigen brought about by conjugation of CA12.10C12 with 5, 10 and 20 equivalents of **10** was assessed by flow cytometry. Binding histograms from the FACS analysis of those conjugates are shown in Figure 5. The histograms indicated that there was a minimal effect on dog peripheral blood mononuclear cell (PBMC) binding after conjugation of **10** with CA12.10C12 (upper panel), and that an effective blocking of FITC-labeled CA12.10C12 and the CA12.10C12 conjugates, **11a**, could be obtained in the presence of excess CA12.10C12 (lower panel). However, when considering which of the reactions (5, 10 or 20 equivalents offered) to use in future studies, the fact that the binding histogram for conjugate **11a** prepared from 20 equivalents of **10** is broader than the other histograms (left panel), and does not overlie the other histograms in the competition assay, was concerning. Conjugate **11a**, obtained from reaction of CA12.10C12 with 10 equivalents of **10**, was chosen as the best candidate for future studies, as we had previously observed that higher radiolabeling yields could be obtained when an mAb conjugate had a higher number of pendant *closo*-decaborate(2-) moieties attached.

## Radiohalogenation of CA12.10C12 conjugates, 5a and 11a

Direct radioiodination of the CA12.10C12 conjugate **5a** with Na[$^{123}$I]I was accomplished in 2 minutes at room temperature using chloramine-T as the oxidant to provide [$^{123}$I]**5b** in 73% radiochemical yield after purification. Under similar radioiodination conditions, reaction of CA12.10C12 conjugate **11a** with Na[$^{125}$I]I gave a 39% purified radiochemical yield of [$^{125}$I]**11b**, while labeling with Na[$^{211}$At]At provided a 62% radiochemical yield of [$^{211}$At]**11c**.

## Dose-escalation studies

Five dogs were administered a dose of 0.07 mCi/kg (H034), 0.12 mCi/kg (G530), 0.27 mCi/kg (G982), 0.57 mCi/kg (H117) or 0.62 mCi/kg (H115)[8] of the sulfhydryl-conjugate [$^{211}$At]**5c**. The two dogs receiving the lowest doses (0.07 & 0.12 mCi/kg) did not show any toxicity, and survived >33 weeks. The three dogs given higher doses (0.27-0.62 mCi/kg) had significantly elevated blood urea nitrogen (BUN) and creatinine concentrations indicating reduced kidney function. The BUN and creatinine concentrations for the 5 dogs in the study are plotted in Figure 6. The three dogs given higher doses were euthanized in poor condition due to renal failure between 10 and 27 weeks post administration of the [$^{211}$At]**5c**.

## Blood clearance and biodistribution studies

Two biodistribution studies were conducted in dogs to provide tissue concentration and blood clearance data. In the initial study, 2.56 mCi of $^{123}$I-labeled CA12.10C12 conjugated with **4**, i.e. [$^{123}$I]**5b**, was injected into a dog. Bood samples were obtained at 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 24 h (at time of necropsy). Serial images were obtained on a gamma camera (data not shown) and the dog was euthanized at 24 h post injection. Triplicate samples (most tissues) of 30 tissues, bone marrow biopsy and marrow “squeeze” were taken during necropsy. In the subsequent study, 0.55 mCi $^{125}$I-labeled CA12.10C12 conjugated with **10**, [$^{125}$I]**11b**, was mixed with 6.33 mCi $^{211}$At-labeled CA12.10C12 conjugated with **10**, [$^{211}$At]**11c**, and that mixture was injected[9]. No imaging was done, but the same blood and tissue samples were taken during necropsy (21 h) as in the initial study. Comparison of the blood clearance (graphs in Figure 7 & data in Figure 1S - Supporting Information) indicated that [$^{125}$I]**11b** and [$^{211}$At]**11c** cleared more slowly than [$^{123}$I]**5b**, perhaps due to localization in kidneys. However, the primary purpose of the second

[9]It should be noted that an amount of ~10-fold $^{211}$At as $^{125}$I was injected to obtain similar number of counts for each radionuclide at 21 h post injection due to the short half-life of $^{211}$At. After 21 h approximately 13% of $^{211}$At remains.

biodistribution was to determine if the tissue concentrations (particularly kidney) were changed in the amine-conjugated mAb relative to that seen in the thiol-conjugated mAb. Bar graphs showing the concentrations of radioactivity in a selected set of tissues are shown in Figure 7. Also included (left panel, balck bars) are data from a prior study. In that study, CA12.10C12 was conjugated with the DTPA derivative CHX-A" for subsequent chelation of $^{213}$Bi. It is apparent from the plotted data that the labeled mAbs targeted CD45-expressing cells to a similar extent (i.e. spleen concentrations), and that the kidney concentrations were much lower in the radiolabeled mAbs in which the labeling pendant groups were conjugated through a lysine amine. The complete biodistribution data are available in Supporting Information (Table S1).

## DISCUSSION

The method used to attach the short-lived ($t_{1/2}$ = 7.21 h) α-emitting radionuclide $^{211}$At to proteins can dramatically alter the outcome of therapy studies. The labeling method is particularly important with $^{211}$At, as in vivo stability can be problematic.[16] Indeed, rapidly metabolized $^{211}$At-labeled compounds, such as peptides and small molecules release free [$^{211}$At]astatide in vivo.[17, 18] We have investigated boron cage molecules to circumvent the instability, and have found that high in vivo stability of $^{211}$At-labeled compounds can be obtained using *closo*-decaborate(2-) pendant groups.[5, 17-19] Intact mAbs, which are slowly metabolized, are used to target $^{211}$At to hematopoietic cells in the conditioning regimens for HCT. The fact that the mAbs are slowly metabolized raised the question of whether a *closo*-decaborate(2-) conjugate was required in the studies. Therefore, our first study was a comparison of the often used $^{211}$At-labeling reagent, N-succinimidyl *meta*-[$^{211}$At]astatobenzoate, [$^{211}$At]**2b**[20, 21] with a maleimido-*closo*-decaborate(2-) derivative, **4**, that had been prepared for conjugation with sulfhydryl groups on Fab[5] and engineered mAb fragments containing a cysteine residue.[22] That study demonstrated that higher in vivo stability was obtained, but more importantly, $^{211}$At-labeling yields were much higher and the labeling process was much simpler when using the *closo*-decaborate(2-) mAb conjugate, **5a**, than obtained in the two-step conjugation procedure required for use of [$^{211}$At]**2b**.[4] In a subsequent study in mice directly comparing $^{211}$At-labeled anti-CD45 mAb (30F11 conjugated with **4**) and $^{213}$Bi-labeled anti-CD45 mAb (30F11 conjugated with CHX-A"-DTPA), it was noted that tissue distributions and therapeutic outcomes were similar. However, $^{211}$At-labeled 30F11 showed a more favorable therapeutic profile with less hepatic toxicity and somewhat superior myelosuppressive properties.[6] Based on the encouraging results from the studies in mice, we began dose-escalation studies of [$^{211}$At]**5c** in the dog model.

Dose-escalation of the $^{211}$At-labeled CA12.10C12 conjugated with **4** through reduced disulfide bonds, i.e. [$^{211}$At]**5c**, resulted in unexpected renal toxicity, as indicated by high BUN and creatinine concentrations in blood (Figure 6). The knowledge that radiolabeled protein fragments can be sequestered in kidneys[23] prompted us to evaluate whether the reduction of disulfides followed by conjugation resulted in protein fragments that localized to kidney. A biodistribution of [$^{123}$I]**5b** in a dog (Figure 7, left panel) supported that theory, as the kidney concentration was 10× higher than that obtained with $^{123}$I-labeled CA12.10C12 conjugated through lysine amines to the DTPA-CHX-A" chelate. This large difference in kidney concentration was noted even though both labeled mAb conjugates appear to target CD45-expressing cells in the spleen to about the same extent. It is believed that reduction with low concentrations of dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) can selectively cleave hinge-region disulfides for site-specific conjugations[24, 25] That was our belief when conjugating **4** to CA12.10C12, but there is evidence that the reduction is not that selective.[26] This is an important distinction as the hinge-region contains more than one disulfide, making it possible to conjugate there and

have other disulfides remain or reform resulting in no fragments being formed. However, if the conjugation occurs on a reduced disulfide that is between the light and heavy chains, conjugation of one sulfhydryl results in an inability to reform the disulfide bond between the light and heavy chains. If a radiolabeled non-covalently bonded light chain were to dissociate from the intact mAb, its small molecular size might cause it to be localized in kidney. Such a process could explain the higher kidney concentrations (cortex, 0.225, %ID/g; medulla, 0.167%ID/g) observed for [$^{123}$I]**5b**, versus those observed (cortex, 0.017%ID/g; medulla, 0.016%ID/g) when CA12.10C12 was conjugated with a DTPA derivative and labeled with $^{123}I^{2}$. Figure 7 (left panel) shows that comparison in a bar graph.

With the belief that the renal toxicity could be caused by the process of conjugating the maleimide reagent, conjugates of two amine-reactive *closo*-decaborate(2-)-containing reagents, **6** and **8**,[5] with CA12.10C12 were prepared for comparison with **5a** and unmodified CA12.10C12. Evaluation of the conjugates by non-reducing SDS-PAGE analysis (Figure 4, left panel) clearly showed that there were protein fragments present after disulfide reduction and conjugation of **4**, whereas there were none if lysine amines were conjugated. From this data it was apparent that conjugations with lysine amines were more favorable, so a choice of reagent was required. The isocyanato-*closo*-decaborate(2-) reagent **6** has low reactivity and the biotin-containing phenylisothiocyanate reagent **8** is more complex and synthetically more difficult to obtain than desired. Thus, a new amine-reactive conjugation reagent, **10**, which is easily synthesized (Scheme 1), was prepared. Conjugation of 5, 10 and 20 equivalents of **10** with CA12.10C12 provided increased loading of reagent (Figure 3), but had minimal effect on the antigen binding of the conjugate (Figure 5). Comparison of the SE-HPLC chromatograms of CA12.10C12 conjugated with **4** or **10** to that of unmodified CA12.10C12 showed that amine conjugation with **10** has little effect on the protein (Figure 2). Evaluation of samples from earlier studies by SE-HPLC showed (Figure 2) the rat anti-mouse CD45 mAb, 30F11 conjugated with the same two reagents had similar chromatographic profiles.

A second biodistribution was conducted in the dog model to confirm our belief that conjugation of an amine-reactive reagent would alleviate the kidney localization problem. In that study, the CA12.10 conjugate, **11a**, was labeled with $^{125}$I and $^{211}$At. The dual label experiment was carried out to ascertain if the two radionuclides had the same in vivo distribution. The concentrations in the kidneys of [$^{125}$I]**11b** (cortex, 0.017%ID/g; medulla, 0.023%ID/g) and [$^{211}$At]**11c** (cortex, 0.029%ID/g; medulla, 0.026%ID/g) were low (Figure 7, right graph) compared to that observed for the $^{123}$I-labeled CA12.10C12 conjugated with maleimide reagent **4** (Figure 7, left graph, green bars). Interestingly, the concentration of radioactivity in the liver for [$^{125}$I/$^{211}$At]-labeled CA12.10C12 was about the same as the sulfhydryl conjugate, but lower than the DTPA-CHX-A” conjugate. The favorable biodistribution results led to resumption of dose-escalation studies using the $^{211}$At-labeled CA12.10C12 conjugate, [$^{211}$At]**11c**. The results of that investigation will be described elsewhere,[27] but no kidney toxicity was seen at the same dose levels that were problematic with the reduced disulfide conjugation and labeling approach.

## In conclusion

This investigation has demonstrated the importance of the method of conjugation when developing mAb-targeted α-emitting radiopharmaceuticals. The studies proved that conjugation of lysine amines with an amine-reactive *closo*-decaborate(2-) reagent, **10**, rather than using a method of conjugation that employs reduction of disulfide bonds on the mAb provides a more specific delivery of the $^{211}$At to the targeted CD45-expressing cells. Based on the high cytotoxicity of the α-emission from $^{211}$At, it is likely that even a small quantity of $^{211}$At-labeled mAb fragment localizing to kidneys can cause damage. The new $^{211}$At-

reactive protein conjugation reagent, **10**, is simple to use and has provided high labeling yields (up to 92%) for direct labeling of the mAb conjugate. These results make this labeling approach particularly attractive for our continuing studies in the dog model and for future clinical studies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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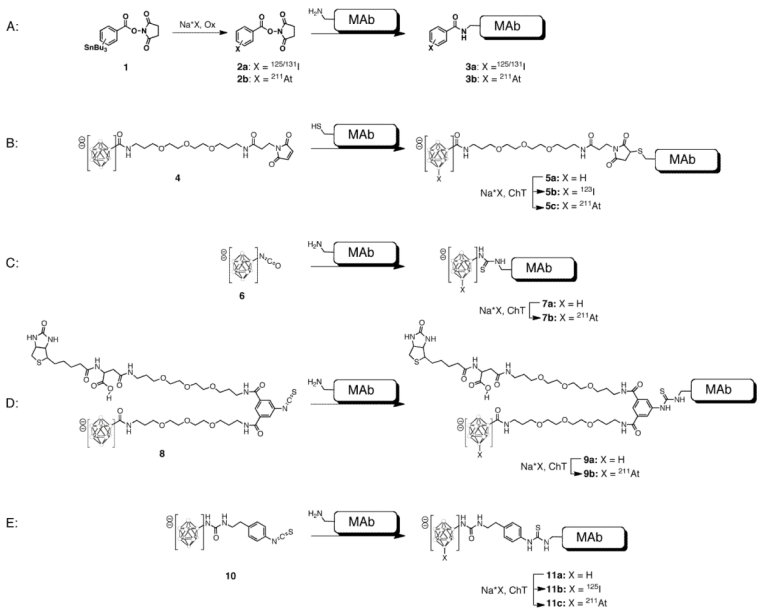



**Figure 1.**

Approaches to conjugation and radiolabeling mAbs with $^{211}$At. Approach **A** is a "2-step" labeling approach where a succinimidyl stannylbenzoate ester **1** is initially labeled, then the resultant $^{211}$At-labeled succinimidyl benzoate [$^{211}$At]**2b** is conjugated with lysine amines on a mAb. Approach **B** is direct labeling approach where sulfhydryl groups produced by reduction of disulfides on a mAb are conjugated with a maleimido-*closo*-decaborate(2-) reagent **4**, followed by $^{211}$At-labeling of the mAb-B10 conjugate to give [$^{211}$At]**5c**. **C, D** and **E** are direct labeling approaches where lysine amines on a mAb are conjugated with an isocyanato- or isothiocyanatophenyl-*closo*-decaborate(2-) reagent (**6, 8** or **10**), followed by $^{211}$At-labeling of the mAb-B10 conjugate to give [$^{211}$At]**7b**, [$^{211}$At]**9b** or [$^{211}$At]**11c**.

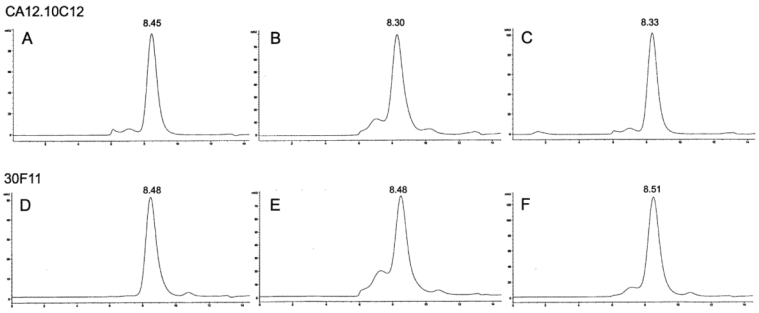



**Figure 2.**

SE-HPLC chromatograms of unmodified and conjugated anti-CD45 mAbs CA12.10C12 and 30F11. Unmodified mAbs are shown in panels **A** & **D**; mAbs modified by disulfide reduction and conjugation with **4** are shown in panels **B** and **E**; CA12.10C12 modified by conjugation of **10** is shown in panel **C** and 30F11 modified by conjugation of **10** is shown in panel **F**. X-axis shows elution time in minutes. Elution times are shown above the major peak in each chromatogram. Shorter elution times obtained in panels B & C are presumably due to differences in injections not in elution properties.

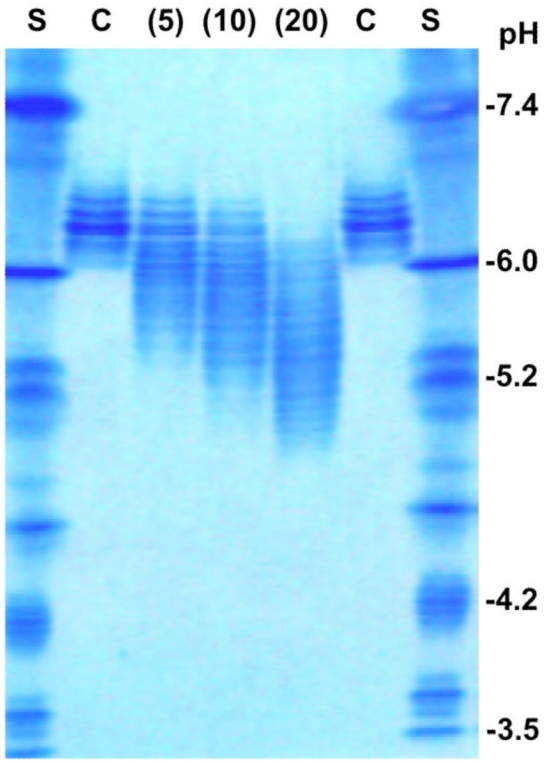



**Figure 3.**
Digitized isoelectric focusing gel (pH 3-7) showing protein bands of CA12.10C12 after reaction with 5, 10 and 20 equivalents of reagent **10**. The column markers correspond to: **S** = molecular weight standards; **C** = unmodified CA12.10C12; and **(5), (10)** and **(20)** indicate the number of equivalents of **10** reacted with CA12.10C12 to form the conjugate **11a**.

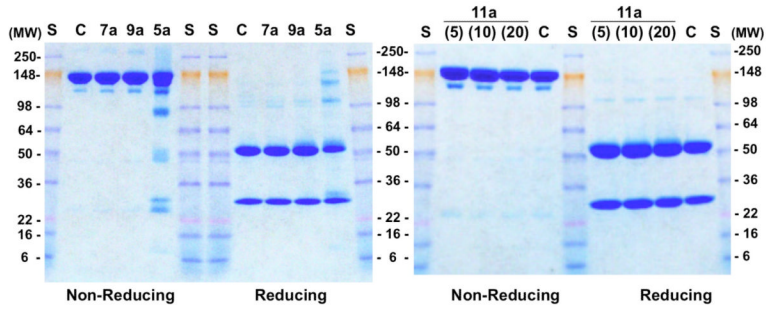



**Figure 4.**

SDS-PAGE gels showing molecular weights of conjugates under reducing and non-reducing conditions. In the left panel gel, the column markers correspond to: **S** = molecular weight standards; **C** = unmodified CA12.10C12; **5a**, CA12.10C12 conjugated with **4**; **7a**, CA12.10C12 conjugated with **6**; **9a**, CA12.10C12 conjugated with **8**. In the right panel gel, CA12.10C12 was conjugated with varying quantities of **10**. The column markers correspond to: **S** = molecular weight standards; **C** = unmodified CA12.10C12; and **(5), (10)** and **(20)** indicate the number of equivalents of **10** reacted with CA12.10C12 to form conjugate **11a**.

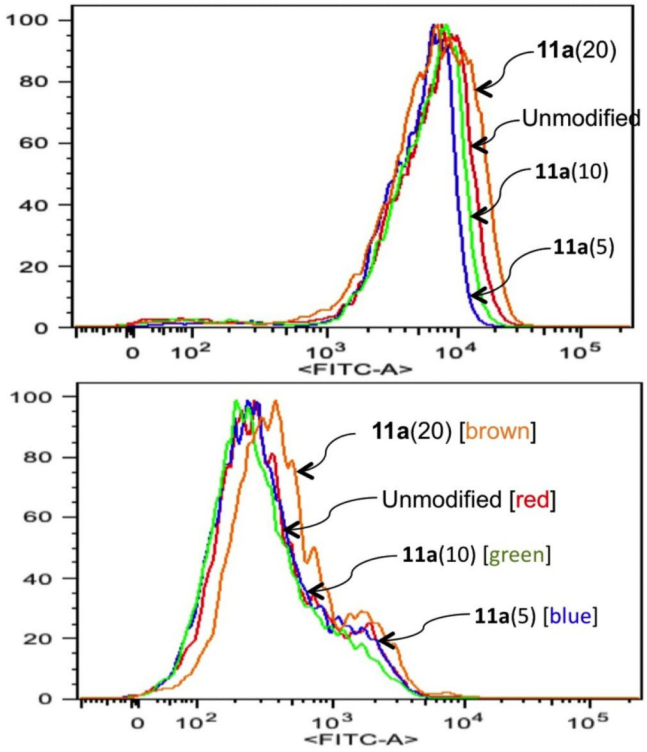



**Figure 5.**

Cell-binding histograms from flow cytometry (FACS) showing unmodified anti-canine CD45 mAb, CA12.10C12 (red lines) and conjugates of CA12.10C12 prepared by reaction with 5 (blue line), 10 (green line) or 20 (brown line) equivalents of conjugation reagent **10** with normal dog peripheral blood mononuclear cells (PBMC). [**Top Panel**] Shows binding of unmodified CA12.10C12 and **11a** conjugates with PBMC. [**Bottom Panel**] Shows blocked binding of FITC-conjugated CA12.10C12 and **11a** conjugates to PBMC in the presence of an excess of CA12.10C12.

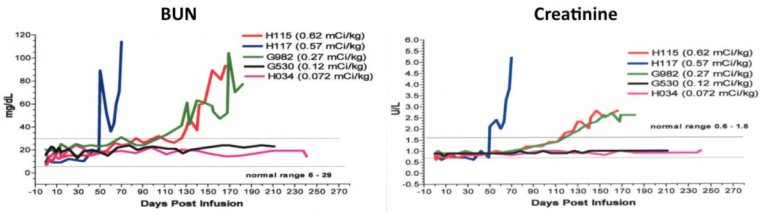



**Figure 6.**

Graphs of renal function as assessed by blood urea nitrogen (BUN) and serum creatinine in 5 dogs administered [$^{211}$At]**5c** on a dose-escalation protocol. Quantity of [$^{211}$At]**5c** administered to each dog is shown in the legends. Grey lines in graphs indicate the range of BUN and creatinine found in dogs with normal renal function.

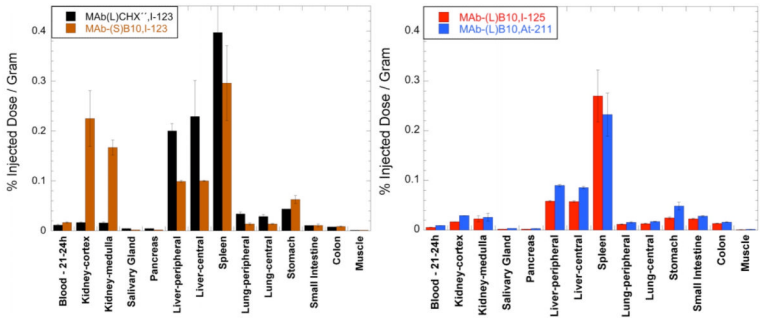
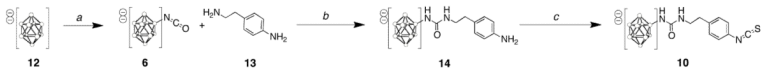



**Figure 7.**

Concentrations of [$^{123}$I]**5b** (left panel; brown bars) and [$^{125}$I]**11b**/[$^{211}$At]**11c** (right panel; red & blue bars, respt.) in selected tissues of two dogs at 24h post injection and 21 h post injection, respectively. [**Left Bar Graph**] Data plotted were obtained from two separate biodistribution studies. Error bars show ± standard deviation in 3 samples taken from different locations in the listed tissue. Black bars represent the tissue concentrations at 22 h post injection of CA12.10C12 conjugated with the chelate CHX-A" and radiolabeled with $^{123}$I. Data plotted as black bars were taken from Sandmaier et al.[2] Brown bars represent the tissue concentration in a dog (G996) at 24h post injection of CA12.10C12 conjugated with **4** and radiolabeled with $^{123}$I, [$^{123}$I]**5c**. [**Right Bar Graph**] Data were obtained from coadministered radiolabeled mAb in a single dog (H137) at 21 h post injection. Red bars represent tissue concentrations of CA12.10C12 conjugated with **10** and labeled with $^{125}$I, [$^{125}$I]**11b**. Blue bars represent tissue concentrations of CA12.10C12 conjugated with **10** and labeled with $^{211}$At, [$^{211}$At]**11c**. Full data set of tissue concentrations obtained in the dogs is provided in Supporting Information as Table S1.




[a](COCl)2, 0, 30 min, NaN3, rt, 24h, 79%; [b]Et3N, anh. DMF, 150°C (microwave), 5 min, 55%; [c]TCDI, CH3CN, rt, 1h, 97%

**Scheme 1.**

Reactions used to obtain isothiocyanatophenyl-decaborate(2-) derivative *closo* **10** for conjugation to MAb-lysine amines.