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Supporting Information

Orthogonal Cysteine Protection Enables Homogeneous Multi-Drug Antibody–Drug Conjugates

Matthew R. Levengood, Xinqun Zhang, Joshua H. Hunter, Kim K. Emmerton, Jamie B. Miyamoto, Timothy S. Lewis, and Peter D. Senter*

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Supporting Information

EXPERIMENTAL METHODS

Synthesis of drug linkers and drug carriers

Unless otherwise noted, all solvents and reagents were purchased from commercial sources in the highest purity possible and not further purified prior to use. Anhydrous dimethylformamide (DMF) and CH_2Cl_2 were purchased from Aldrich. Fmoc-protected amino acids and 2-Cl-tritylchloride resin (substitution 1 mmol/g, 200-300 mesh, 1% DVB) were purchased from Novabiochem. Fmoc-protected amino-dPEG$_{24}$-COOH was purchased from Quanta Biodesign. MDpr was prepared as described previously.[1] Maleimodocaproyl-MMAF (**1**), maleimidocaproyl-Val-Cit-PAB-MMAF (**2**), and maleimidocaproyl-Val-Cit-PAB-MMAE (**3**) were synthesized as previously described.[2] [3] Solid phase synthesis was performed in plastic syringes (National Scientific Company) fitted with a filter cut out of fritware PE medium grade porous sheet (Scienceware). Small molecule LC-MS was performed on a Waters Xevo G2 ToF mass spectrometer interfaced to a Waters Acquity H-Class Ultra Performance LC equipped with an Acquity UPLC BEH C18 2.1 x 50 mm, 1.7µm reverse phase column. The acidic mobile phase (0.1% formic acid) consisted of a gradient of 3% acetonitrile/97% water to 100% acetonitrile (flow rate = 0.7 mL/min). Preparative reverse-phase HPLC was performed on a Varian ProStar 210 solvent delivery system configured with a Varian ProStar 330 PDA detector. Products were purified over a Phenomenex Synergy MAX-RP 30.0 x 250 mm, 4 µm, 80 Å reverse phase column eluting with 0.05 % trifluoroacetic acid in water (solvent A) and 0.0.5 % trifluoroacetic acid in acetonitrile (solvent B). The purification methods generally consisted of linear gradients of solvent A to solvent B, ramping from 90% aqueous solvent A to 10% solvent A. The flow rate was 5.0 mL/min with monitoring at 220 nm.

Solid-phase peptide synthesis:

Both maleimidocaproyl-Cys(Acm) (mc-Cys(Acm)) and drug carrier **4** were synthesized on the solid phase using Fmoc chemistry.

General procedure for peptide synthesis:

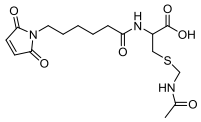
Resin loading: In a 10 mL solid phase reaction vessel (plastic syringe with PET frit) was added 0.15 g of 2-Cl-Tritylchloride resin (0.225 mmol) followed by a solution of Fmoc-amino acid or Fmoc-amino-PEG$_{24}$-COOH (0.225 mmol, 1.0 equiv) and N,N-diisopropylethylamine (DIPEA) (0.338 mmol, 1.5 equiv) in 3 mL of dry CH_2Cl_2. The vessel was shaken for 5 min, then more DIPEA (0.225 mmol, 1.0 equiv) was added, and the vessel was shaken for additional 30 min. Unreacted resin was quenched by adding MeOH (1.0 mL) for 5 min. Resin was then washed with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL), diethyl ether (5 x 5 mL) and dried *in vacuo*.

Fmoc removal procedure: Resin containing Fmoc-protected peptide was treated with 20 % piperidine in DMF (5 x 3 mL) for 30 min total. The resin was then washed with DMF (5 x 5 mL) prior to further manipulation.

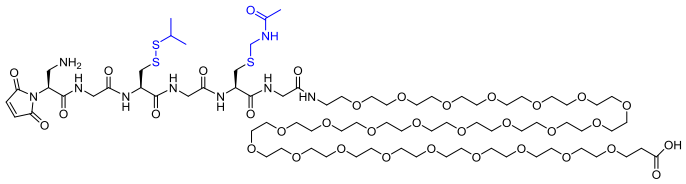
Standard coupling procedure: To the resin deprotected N-terminus amino acid (1 equiv), a solution containing Fmoc-amino acid or maleimido-acid (3 equiv), HATU (3 equiv), and DIPEA (6 equiv) in DMF (5 mL) was added. The reaction vessel was shaken for 1 hr. The resin was then washed with DMF (5 x 5

mL). Fmoc-Cys amino acids (1 equiv) were coupled by adding a solution containing Fmoc-Cys (4 equiv), hydroxybenzotriazole (HOBT) (4 equiv), and N,N'-diisopropylcarbodiimide (DIC) (4 equiv).
Cleavage from resin: To dried resin was added 2 mL of a solution of 10 % trifluoroacetic acid(TFA) in CH_2Cl_2 that also contained 2.5 % H_2O and 2.5 % triisopropyl silane in a 5 mL plastic syringe. After 1 min, the reaction mixture was transferred to a 20 mL borosilicate glass scintillation vial. This procedure was repeated three times. The cleavage solution was dried under a stream of N_2, washed 3x with 0.5 mL diethyl ether, then dried *in vacuo*. The crude products were either used without subsequent purification or purified by reverse-phase HPLC using the procedure described above.

Drug carrier characterization:
Maleimidocaproyl-Cys(Acm):
Expected exact mass: 385.13; observed m/z: 384.3 $(M+H)^+$, LC-MS t_R = 0.66 min. The crude product was judged to be > 95% pure and was used for antibody conjugation with no subsequent purification.



Drug carrier **4**:
Expected exact mass: 1833.9; observed m/z: 1835.1 $(M+H)^+$, LC-MS t_R = 0.88 min, preparative LC t_R = 24 min.



Conjugation methods

Materials and general methods
Chimeric anti-CD30 monoclonal antibody cAC10 was prepared as described previously.[4] Protein LC-MS data were acquired on a Waters Xevo GS-S QTOF coupled to a Waters Acquity H-Class UPLC system. Samples were reduced with 10 mM dithiothreitol (DTT) for 10 min at 37 °C and then chromatographed over an analytical reversed-phase column (Agilent Technologies, PLRP-S, 300Å, 2.1 mm ID x 50 mm, 3 µm) at 80°C and eluted with a linear gradient of 0.01% TFA in acetonitrile from 25% to 65% in 0.05% aqueous TFA over 5 minutes, followed by isocratic 65% 0.01% TFA in acetonitrile for 0.5 min at a flow rate of 1.0 mL/min. Mass spectrometry data was acquired in ESI+ mode using a mass range of 500-4000 m/z and were deconvoluted using MaxEnt1 to determine masses of the resulting conjugates. The extent of aggregation of the conjugates was determined by size-exclusion chromatography (SEC) using an analytical SEC column (Sepax SRT-C 300 7.8 mm ID x 30 cm, 5µm) on a Waters 2695 HPLC system. The injected material was eluted using an isocratic mixture of 92.5% 25 mM sodium phosphate (pH 6.8), 350 mM NaCl, and 7.5% isopropyl alcohol at a flow rate of 1 mL/min

ADCs were prepared by reduction of antibody interchain disulfides followed by addition of a 25-100% excess maleimide as described previously.[5] Full reduction of 8 thiols per antibody was accomplished by

addition of 12 equivalents of tris(2-carboxyethyl)-phosphine (TCEP) to an antibody solution (1-10 mg/mL in PBS, pH 7.4). The extent of antibody reduction was monitored by reverse-phase LC-MS and additional TCEP was added as needed to complete the reaction. TCEP was then removed by ultrafiltration (3x, 10-fold dilution into PBS, pH 7.4 containing 1 mM EDTA, centrifugation at 4000 x *g* through a 30-kDa MWCO filter). Fully reduced antibodies in PBS-EDTA were conjugated with 10-16 molar equivalents (25-100 % excess) of drug-linker or drug-carrier as a 10 mM DMSO stock. The resulting solution was vortexed and left at room temperature for 10-20 minutes. The extent of conjugation was assessed by reverse-phase LC-MS as described above, and additional drug-linker or drug-carrier was added as needed. Once all available Cys thiols were alkylated, the crude ADC solution was purified by buffer exchange into PBS using either a Nap-5 desalting column (GE Healthcare) or through 3-5 rounds of ultrafiltration. The final ADC concentration was determined spectrophotometrically.

Conjugation and trial deprotection of mc-Cys(Acm):

To fully reduced cAC10 antibody in PBS-EDTA was added 10 equiv. of mc-Cys(Acm) from a 100 mM DMSO stock. The resulting solution was vortexed and left at room temperature for 15 min. At this time, reverse-phase LC-MS indicated full alkylation of antibody thiols with no loss of fidelity of the Acm protecting group. The conjugate was purified by ultrafiltration according to the procedures described above, and the chromatography and mass spectrometric characterization of the conjugate is shown in Supplementary Figure 1A. The resulting conjugate cAC10-mc-Cys(Acm) with 8 carriers per antibody was then subjected to deprotection conditions. To the conjugate in PBS, pH 7.4 was added 50 equiv. of aqueous mercury acetate ($Hg(OAc)_2$) as a 10 mM stock. The reaction mixture was incubated for 45 min at room temperature. To the reaction mixture was added an aqueous slurry of Quadrasil MP resin (Sigma Aldrich, 0.025 mmol/g thiol capacity, 1 equiv. of resin to 1 equiv. mercury acetate added), and the mixture vortexed vigorously for 15 min. At this time, the mixture was centrifuged at 13,200 x *g* for 2 min and the supernatant removed. To the supernatant was added 10-20 equiv. of N-ethylmaleimide (NEM) to cap the liberated cysteine thiols. The extent of modification was observed by LC-MS. As shown in Supplementary Figure 1B, a conjugate was produced where all 8 Acm groups had been removed and each liberated thiol was capped with NEM.

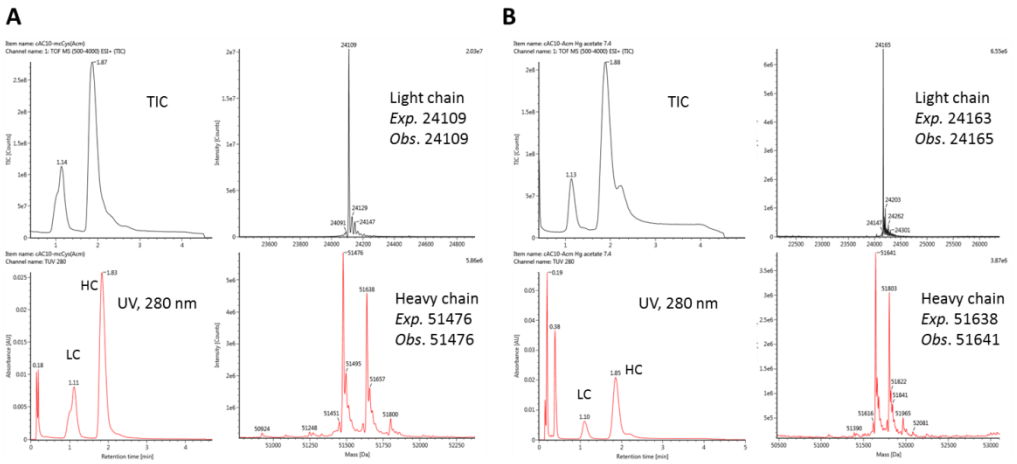


Figure S1. Conjugation (A) and deprotection (B) of cAC10-mc-Cys(Acm). Shown are the total ion chromatogram (TIC) and UV chromatogram (280 nm) after reverse-phase separation of light and heavy chain. The deconvoluted mass spectra for the main light and heavy chain species are shown to the right of the chromatograph, with the expected and observed masses shown. Note that multiple heavy chain mass species are present due to heterogeneity in the N-linked glycan. Only the G0 glycoform mass is noted for the heavy chain. LC = light chain, HC = heavy chain

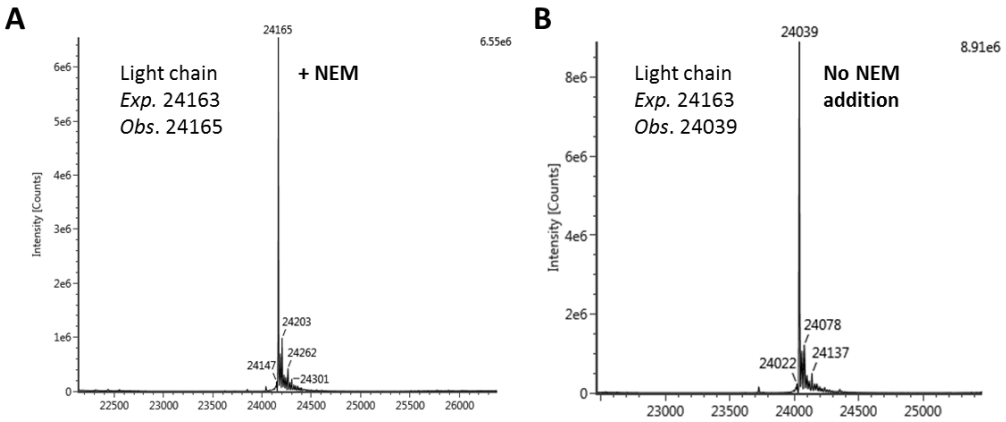


Figure S2. Removal of mercury from liberated Acm-protected cysteines using QMP resin is required for thiol-maleimide conjugation. Shown is the deconvoluted light chain mass of cAC10-mc-Cys after Acm deprotection and subsequent NEM addition, which either included prior treatment with QMP resin (A) or did not (B). Addition of NEM to the liberated thiol was only observed in (A).

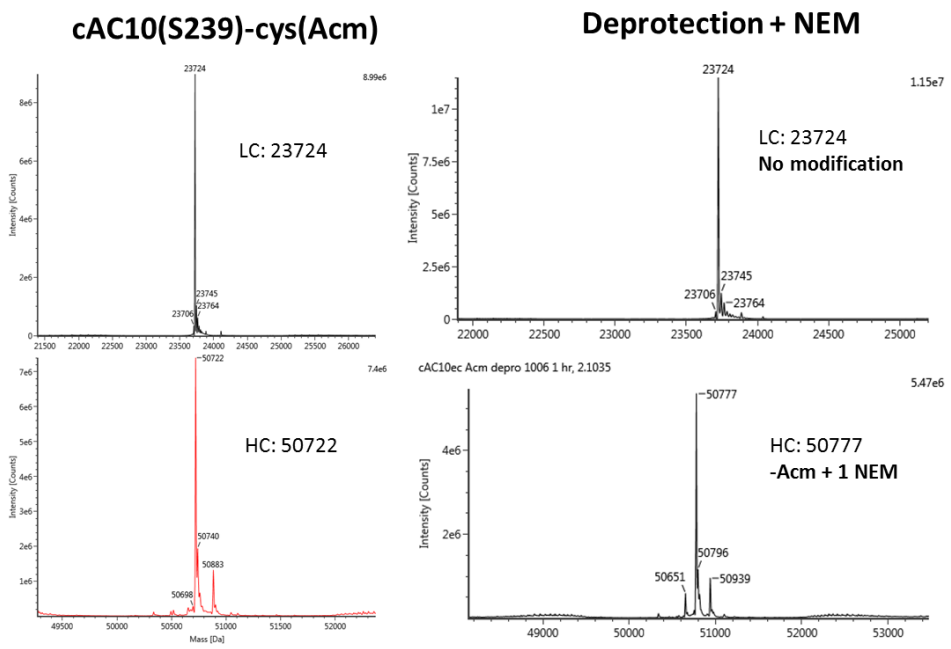


Figure S3. Mercury-mediated Acm deprotection does not affect antibody interchain disulfide integrity. Maleimidocaproyl-Cys(Acm) was conjugated to cAC10(S239C) at 2 carriers per antibody. This conjugate had all interchain disulfide bonds intact. The conjugate was subjected to mercury-mediated Acm deprotection conditions and subsequent NEM conjugation (ca. 20 molar equivalents). Shown are the deconvoluted light and heavy chain mass spectra following reverse-phase separation of light and heavy chains. This analysis demonstrates that only a single NEM molecule was added to the heavy chain and no modification of the light chain occurred. This indicates that mercury treatment does not affect antibody interchain disulfides. If mercury treatment disrupted the disulfide bonds, multiple NEM additions would be expected. Note that multiple heavy chain mass species are present due to heterogeneity in the N-linked glycan. Only the G0 glycoform mass is noted for the heavy chain. LC = light chain, HC = heavy chain

General procedure for dual-modified antibody conjugates.

Carrier **4** conjugation: To fully reduced cAC10 antibody in PBS-EDTA was added 16 equiv. of carrier **3** from a 10 mM DMSO stock. The resulting solution was vortexed and left at room temperature for 15 min. At this time, reverse-phase LC-MS indicated full alkylation of antibody thiols with no loss of fidelity of the Acm protecting group. The conjugate was purified by ultrafiltration according to the procedures described above.

Deprotection of Cys(SiPr): To cAC10-**4** (8 carriers/antibody) was added 10-12 equiv. of TCEP. The reaction mixture was incubated at 37 ^{o}C for 45 min, at which time reverse-phase LC-MS indicated that reduction was complete (by evaluation of the deconvoluted light and heavy chain masses, see Supplementary Figure 2 for an example). Upon completion of the reaction, excess TCEP and liberated isopropyl thiol was removed by 3 rounds of ultrafiltration into PBS-EDTA as described above.

First conjugation: To fully reduced cAC10-**4** was added 50 % molar excess of maleimide drug-linker from a 10 mM DMSO stock. The resulting solution was vortexed and left at room temperature for 15 min. At this time, reverse-phase LC-MS was used to judge reaction progress, and additional drug-linker or NEM was added as needed until all thiols had been alkylated. The conjugate was purified by gel filtration or ultrafiltration according to the procedures described above.

Acm deprotection: To a solution containing cAC10-**4**-drug/NEM was added 50 equiv. of aqueous $Hg(OAc)_2$. The resulting solution was vortexed and left at room temperature for 45 min. To the reaction mixture was added an aqueous slurry of Quadrasil MP resin (0.025 mmol/g thiol capacity, 1 equiv. of resin to 1 equiv. mercury acetate added), and the mixture vortexed vigorously for 15 min. At this time, the mixture was centrifuged at 13,200 x *g* for 2 min and the supernatant removed. The conjugate bearing 8 free thiols was either used without subsequent purification, or purified by three rounds of ultrafiltration into PBS-EDTA as described above.

Second conjugation: To a solution containing cAC10-**4**-drug/NEM with 8 free thiols was added 50-100% molar excess of maleimide drug-linker or NEM from a 10 mM DMSO stock. The resulting solution was vortexed and left at room temperature for 15 min. At this time, reverse-phase LC-MS was used to judge reaction progress, and additional drug-linker or NEM was added as needed until all thiols had been alkylated. The conjugate was purified by gel filtration or ultrafiltration according to the procedures described above.

The average drugs per antibody during each step of the conjugation process was within 2.5% of complete drug loading (8 drugs/mAb), as determined by reverse-phase HPLC.

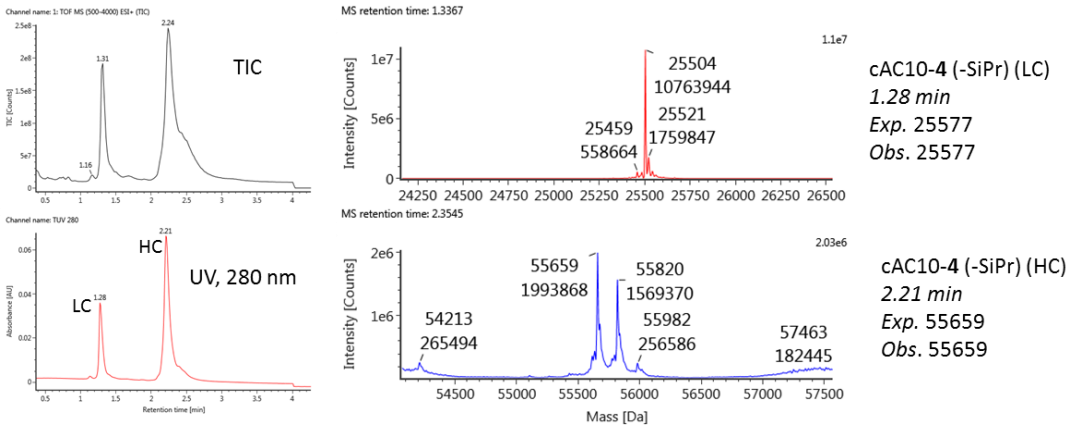


Figure S4. LC-MS characterization after removal of S-(isopropyl) disulfides from cAC10-**3**. Shown are the total ion chromatogram (TIC) and UV chromatogram (280 nm) after reverse-phase separation of light and heavy chain. The deconvoluted mass spectra for light and heavy chain species are shown to the right of the chromatograph, with the expected and observed masses shown. Note that multiple heavy chain mass species are present due to heterogeneity in the N-linked glycan. Only the G0 glycoform mass is noted for the heavy chain. LC = light chain, HC = heavy chain

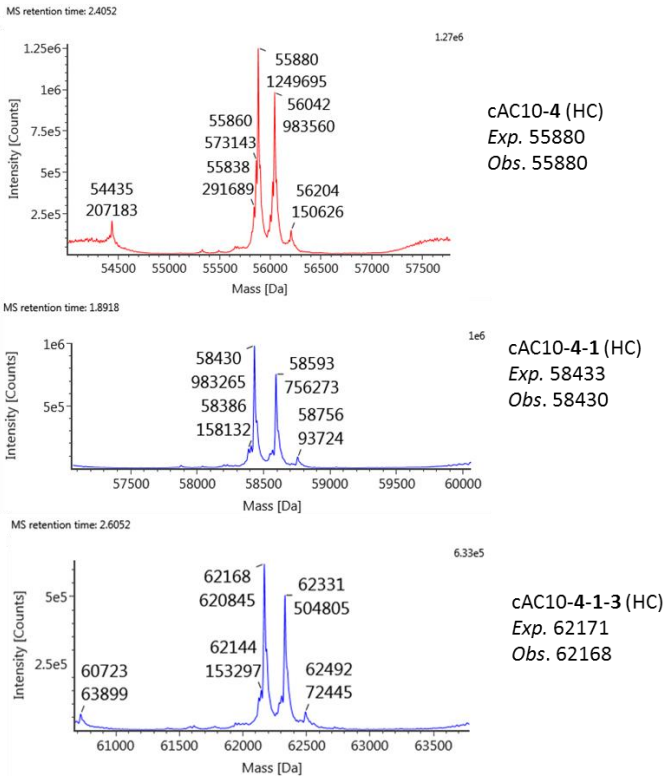


Figure S5. LC-MS characterization of the main heavy chain species during sequential cysteine deprotection and drug-linker conjugation of cAC10-**4**. Shown is the deconvoluted heavy chain mass of drug-carrier conjugate cAC10-**4** before (top) and after –SiPr removal and conjugation of **1** (middle), and –Acm removal and conjugation of **3**. The UV chromatograph for each intermediate in shown in Figure 2 in the main text. Note that multiple heavy chain mass species are present due to heterogeneity in the N-linked glycan. Only the G0 glycoform mass is noted for the heavy chain.

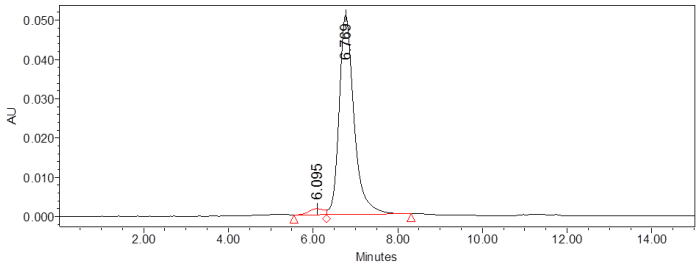


Figure S6. SEC characterization of the multi-drug ADC cAC10-**1-3** prepared using drug carrier **4**. The conjugate was 98 % monomeric (6.77 min).

Analytical characterization of conjugates and conjugate intermediates:

cAC10-**4**:
Light chain: t_R = 1.41 min; expected mass: 25577, observed 25577
Heavy chain: t_R = 2.34 min; expected mass: 55880 (heavy chain + 3 carriers), observed 55880

cAC10-**4**(-SiPr):
Light chain: t_R = 1.38 min; expected mass: 25503, observed 25504
Heavy chain: t_R = 2.34 min; expected mass: 55658 (heavy chain - 3 -SiPr), observed 55659

cAC10-4-**1**:
Light chain: t_R = 1.28 min; expected mass: 26429, observed 26427
Heavy chain: t_R = 1.84 min; expected mass: 58434 (heavy chain +3 drugs), observed 58430

cAC10-4-**2**:
Light chain: t_R = 1.49 min; expected mass: 26834, observed 26382
Heavy chain: t_R = 2.09 min; expected mass: 59651 (heavy chain +3 drugs), observed 59646

cAC10-4-**NEM**:
Light chain: t_R = 0.98 min; expected mass: 25629, observed 25627
Heavy chain: t_R = 1.38 min; expected mass: 56034 (heavy chain +3 NEM), observed 56031

cAC10-4-**1**-**3**:
Light chain: t_R = 1.83 min; expected mass: 27675, observed 27673
Heavy chain: t_R = 2.56 min; expected mass: 62172 (heavy chain +3 drugs), observed 62170
SEC t_R = 6.77 min, 98.0 % monomeric

cAC10-4-**2**-**3**:
Light chain: t_R = 2.04 min; expected mass: 28080, observed 28081
Heavy chain: t_R = 2.76 min; expected mass: 63389 (heavy chain +3 drugs), observed 63389
SEC t_R = 6.69 min, 95.0 % monomeric

cAC10-4-**1**-**NEM**:
Light chain: t_R = 1.33 min; expected mass: 26483, observed 26482
Heavy chain: t_R = 1.90 min; expected mass: 58596 (heavy chain +3 NEM), observed 58598
SEC t_R = 6.91 min, 97.5 % monomeric

cAC10-4-**2**-**NEM**:
Light chain: t_R = 1.53 min; expected mass: 26888, observed 26889
Heavy chain: t_R = 2.14 min; expected mass: 59813 (heavy chain +3 NEM), observed 59815

SEC t_R = 6.74 min, 97.4 % monomeric

cAC10-4-NEM-**3**:
Light chain: t_R = 1.55 min; expected mass: 26875, observed 26875
Heavy chain: t_R = 2.19 min; expected mass: 59772 (heavy chain +3 drugs), observed 59772
SEC t_R = 7.03 min, 97.8 % monomeric

Cell binding analysis

Binding of antibody or ADC to cell-surface CD30 was assessed by flow cytometry on CD30(+) L540cy Hodgkin lymphoma cells. Cells (2 x 10^5) were combined with 4-fold serial dilutions of each antibody treatment in flow buffer (PBS, 2 % fetal bovine serum, 0.2 % NaN_3) in a total volume of 100 µL. The cells were incubated on ice for 30 min, and then washed twice with ice-cold flow buffer. At this time a FITC-labeled goat anti-human Fc secondary antibody (109-095-098, Jackson ImmunoResearch) was added at the recommended dilution in a total volume of 100 µL flow buffer. The cells were incubated on ice for 30 min, and then washed twice with ice-cold flow buffer. Labeled cells were examined by flow cytometry on an Attune NxT flow cytometer (Thermo Fisher Scientific). Data was analyzed using FlowJo software and plotted using GraphPad Prism 6. Binding constants were determined by nonlinear regression using a one site binding model.

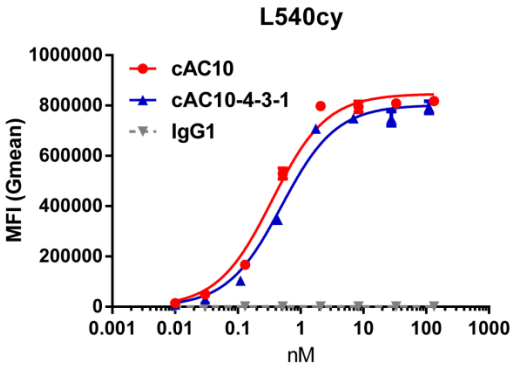


Figure S7. Saturation binding of cAC10 antibody, cAC10-**4**-(**3**-**1**) ADC, or non-binding IgG1 isotype control on CD30(+) L540cy cells. The calculated K_D values for cAC10 naked antibody and cAC10-4-3-1 were 0.35 nM and 0.50 nM, respectively. Neither cAC10 or cAC10-**4**-(**3**-**1**) bound to CD30(-) U-266 cells (not shown).

***in vitro* cytotoxicity experiments**

In vitro potency was assessed on multiple cancer cell lines: L540cy (Hodgkin lymphoma) and DEL, and DEL-BVR[6] (anaplastic large cell lymphomas), U-266 (multiple myeloma). L540cy and DEL were obtained from DSMZ and U-266 was obtained from ATCC. Authenticity of cell lines was confirmed using the Cell Check 16 panel (IDEXX Bioresearch). U-266 cells stably expressing firefly luciferase were generated using in vivo ready lentiviral particles from GenTarget, Inc. (San Diego, CA). U-266 cells were grown to 1 x 10^6

cells/mL (>90% viable) and transduced with lentiviral particles for 72 hours in RPMI 1640 media + 10% FBS. Cells were placed under selection in neomycin and stable clones were produced by dilution cloning into 96 well plates. A stable U-266luc cell line was selected using the Bright-Glo Luciferase Assay System (Promega) using an EnVision Multilabel Plate Reader (Perkin Elmer). For all cytotoxicity experiments, cells were cultured in log-phase growth, then seeded for 24 hours in 96-well plates containing 150 µL RPMI-1640 supplemented with 10-20 % FBS. Serial dilutions of ADCs in cell culture media were prepared at 4x working concentrations, and 50 µL of each dilution was added to the 96-well plates. Following addition of ADCs, cells were incubated for 4 days at 37 °C. After 96 hours, growth inhibition was assessed by CellTiter-Glo (Promega) and luminescence was measured on an Envision plate reader. The IC_{50} value determined in triplicate is defined here as the concentration that results in half maximal growth inhibition over the course of the titration curve. For the *in vitro* bystander assay, L540cy and luciferase(+) U-266 cells (U-266luc) were seeded in 96-well plates at a 1:1 ratio. Test article dilutions were added to the cells as outlined above. After 96 hours, growth inhibition of the U-266luc cells was assessed by BrightGlo and luminescence was measured on an Envision plate reader.

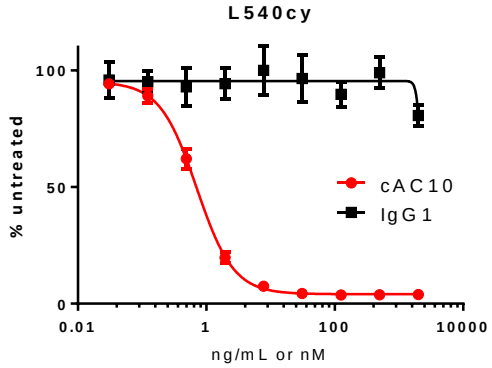


Figure S8. An isotype control ADC (IgG1-**4**-**1**-**3**) was inactive on CD30(+) L540cy cells whereas cAC10-**4**-**1**-**3** was highly active.

***in vivo* xenograft experiments**

All experiments were conducted in concordance with the Institutional Animal Care and Use Committee in a facility fully accredited by the Association and Accreditation of Laboratory Animal Care. Therapy experiments were conducted using DEL-BVR, Karpas 299, or Karpas-35R cells that were implanted subcutaneously into severe combined immunodeficiency (SCID) mice (Harlan, Indianapolis, IN). The admixed tumor model was implanted with a mixture containing 2.5 million Karpas 299 and 2.5 Karpas-35R cells.[7] Upon tumor engraftment, mice were randomized to study groups when the average tumor volume reached approximately 100 mm^3. The ADCs were dosed once by intraperitoneal injection. Animals were euthanized when tumor volumes reached 1000 mm^3. Tumor volume was calculated with the formula (volume = ½ x length x width x width). Mice showing durable regressions were terminated around day 40-65 after implant.

References:

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