Supporting Information

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SI Materials and Methods

Modeling of A1 Antibody and Design of Sites for Nonnative Amino Acid Incorporation. Modeling of the 3D structure of the anti-5T4 antibody is done using other antibodies with high sequence homology with the anti-5T4 antibody. This provides comparative modeling to predict the 3D structure of the anti-5T4 Fab.

The selection of a homologous human antibody is completed using the algorithm Psi-Blast against the Protein Database, using the most homologous template considering both heavy and light chains. Three templates are selected, all of which are humanized murine antibodies with sequence identity between 85% and 90%. Protein database codes are as follows: 1T3F, humanized murine anti-IFN-γ Fab (HuZAF); 1B2W, humanized murine anti-γ-IFN antibody; 1L7I, humanized murine anti-ErbB2 antibody.

The sequences are aligned using the Salign algorithm implemented in the comparative modeling software MODELER 9v7 (1). Using the three templates and the sequence alignment described above, 50 models are built and selected based on PDF score and DOPE evaluation. All algorithms are implemented in MODELER 9v7.

The calculation of the solvent accessible surface area (SASA) is implemented in Discovery Studio (Accelrys). All residues with relative SASA more that 40% are considered as solvent accessible. Detection of secondary structure on the model is conducted by the Kabsch and Sander method implemented in Discovery Studio (Accelrys).

Mutation sites are selected and prioritized based on the different criteria: SASA > 40%; no proline or glycine residues; no involvement of the residue in the fold stability; no involvement of the residue in the different interface domains; and side-chain orientation (toward or outward the protein). Although many positions were matching these criteria; we selected only two positions located on the CH1 domain to move forward in this report: HC-A114, HC-S115 (Scheme S1).

Nonnative Amino Acid-Containing Antibody Transient Expression. CHO-S suspension-adapted cells were obtained from Invitrogen. Routine maintenance culture of CHO-S cells was performed in CHO-S FreeStyle chemical defined media (Invitrogen) supplement with 8 mM L-glutamine (Invitrogen/Gibco). Cells were cultured in 125-mL Erlenmeyer flasks (Corning) at 125 rpm on a shaker platform (Bellco) in 8% (vol/vol) CO_2 and 37 °C in a Thermo Fisher incubator (Thermo Fisher). Cells were maintained according to Invitrogen's protocol. Viable cell density and viability were determined using a Beckman Vi-Cell instrument.

FreeStyle MAX transfection reagent was purchased from Invitrogen. The procedure of transfection was performed according to Invitrogen's FreeStyle MAX reagent protocol. Briefly, cells were seeded at 0.5×10^6 cells per mL 1 d before transfection. The cell density was adjusted to 1×10^6 cells per mL right before transfection and *para*-acetylphenylalanine (*p*AF) amino acid added. Purified plasmid DNA was diluted into OptiPRO SFM media and mixed. FreeStyle MAX reagent was also diluted in OptiPRO SFM media and mixed. Following mixing, the diluted FreeStyle MAX reagent was immediately mixed with the diluted DNA solution. The DNA-transfection reagent mixture was incubated at room temperature for 15 min. The mixture was then slowly added into the cell culture. Transfected cells were incubated at 37 °C, 8% (vol/vol) CO_2 on orbital shaker set to 125 rpm. After 7 d, supernatant was collected for purification and productivity of antibody was determined by IgG ELISA.

Drug Linker Syntheses (Scheme S2). ***Synthesis of compound 3.*** Tetraethylene glycol **1** (10 g, 51.5 mmol), *N*-hydroxyphthalimide **2** (8.4 g, 51.15 mmol), and triphenylphosphine (17.6 g, 67 mmol) were dissolved in 300 mL of tetrahydrofuran followed by addition of DIAD (12.8 mL, 61.78 mmol) at 0 °C. The resulting solution was stirred at room temperature overnight, and then concentrated to dryness. The residue was purified by flash column chromatography to give 5.47 g (31%) of compound **3**. 1H NMR (CDCl3): δ 7.84 (2H, q), 7.74 (2H, q), 4.38 (2H, t, J = 4.5), 3.86 (2H, t, J = 4.5), 3.70 (2H, m), 3.68 (2H, m), 3.60 (8H, m).

Synthesis of compound 4. To a solution of compound **3** (200 mg, 0.59 mmol) in 15 mL of dichloromethane was added Dess-Martin Periodinane (300 mg, 0.71 mmol). The reaction mixture was stirred at ambient temperature overnight. The reaction was quenched with the solution of sodium bisulfite in 15 mL of saturated sodium bicarbonate. The mixture was separated. The organic layer was washed with saturated sodium bicarbonate, brine, dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography to give 150 mg (75%) of compound **4**. 1H NMR (CDCl3): δ 9.71 (1H, s), 7.83 (2H, q), 7.74 (2H, q), 4.37 (2H, t, J = 4.5), 4.15 (2H, s), 3.86 (2H, t, J = 4.5), 3.69 (4H, m), 3.67 (2H, m), 3.59 (2H, m).

Synthesis of compound 6. To a solution of monomethyldolastatin hydrochloride **5** previously described (2) (50 mg, 0.062 mmol) in 1 mL of dimethylformamide (DMF) was added compound **4** (63 mg, 0.186 mmol) and 70 μL of acetic acid, followed by addition of 8 mg of sodium cyanoborohydride. The resulting mixture was stirred at ambient temperature for 2 h. The reaction mixture was diluted with water and purified by HPLC to give 60 mg (80%) of compound **6**. MS [electrospray ionization (ESI)] *m/z* 547 [M+2H], 1,092 [M+H].

Synthesis of compound 7. Compound **6** (60 mg, 0.05 mmol) was dissolved in 1 mL of DMF. A volume of 32 μL of hydrazine was added. The resulting solution was stirred at ambient temperature for 1 h. The reaction was quenched with 1 M hydrochloride solution. The reaction mixture was purified by HPLC to give 33 mg (55%) of compound **7**. MS (ESI) *m/z* 482 [M+2H], 962 [M+H].

Site-Specific Antibody Drug Conjugate Purification and Conjugation. Cell culture media was loaded directly onto a MabSelect SuRE (GE Healthcare) column equilibrated in 1× PBS, pH 7.4 (Gibco). Monoclonal antibody (mAb) was eluted from protein A with 0.1 M sodium citrate, pH 3.5. pH neutralized with 1:5 1 M Tris, pH 8.

Protein A pool was diluted to <6 mS/cm, and pH adjusted to 5 with glacial acetic acid and loaded onto an SP HP (GE Healthcare) column equilibrated in 20 mM sodium acetate, pH 5. mAb eluted with a 0–100% linear gradient over 50 CV. Eluent buffer contained the following: 20 mM sodium acetate, 1 M NaCl, pH 5.

Main elution peak was pooled, concentrated, and buffer exchanged into 20 mM sodium acetate, pH 4, using Amicon Ultra 10K mwco centrifugal filter devices (Millipore).

mAb was conjugated to drug/linker under the following conditions: 10 mg mAb/mL, 10:1 drug/mAb molar ratio, 1% acetic hydrazide. Reaction incubated at 28 °C for 40–60 h.

After incubation, conjugation reaction was diluted into 20 mM Tris, 0.75 M ammonium sulfate, pH 7, and loaded onto a Phenyl HP column (GE Healthcare) equilibrated in the same buffer. mAb was eluted from the column with a 0–100% linear gradient over 50 CV. Eluent buffer contained the following: 20 mM Tris, 20% isopropanol, pH 7.

Hydrophobic interaction chromatography (HIC) fractions were pooled, and concentrated and buffer exchanged (>100×)

into 1× PBS, pH 7.4 (Gibco). Final material was sterile filtered using a 0.22-μm PES syringe filter.

Peptide Mapping. WT A1, A1 HC-S115*p*AF, and A1 HC-S115*p*AF-NC-D1 were reduced in 6 M guanidine-HCl, 0.1 M Tris, pH 8.0, 0.05 M DTT for 1 h at 37 °C. Reduced samples were alkylated with 0.1 M iodoacetamide at room temperature in the dark for 40 min followed by quenching with 0.1 M DTT. Samples were buffer exchanged into 50 mM Tris, 5 mM calcium chloride, pH 7.5, followed by trypsin addition at 1:20 (trypsin:protein), and incubated for 18 h at 37 °C.

Peptide-mapping samples were analyzed on an Agilent 1260 HPLC in tandem with an Agilent 6510 Q-ToF. Samples were loaded onto a HyPurity C18 1.0 × 150-mm column with 100% mobile phase A (0.1% formic acid in 98% HPLC H_2O/2% acetonitrile) and eluted with a gradient of 0.5%/min with mobile phase B (0.1% formic acid in acetonitrile) over 68 min. The flow rate was 0.07 mL/min and column temperature was set to 40 °C.

Mass spectra were collected in positive-ion mode with gas temperatures at 325 °C, drying gas at 8 L/min, and nebulizer pressure at 25 psig. The capillary voltage was set to 3,500 V with the fragmentor voltage set to 175 V. Acquisition was set to auto MS/MS with a collision energy slope of 3.5 V/100 Da and an offset of 2.5 V.

Plasma Stability Analysis by Liquid Chromatography with Tandem Mass Spectrometry. A1 HC-A114*p*AF and A1 HC-S115*p*AF conjugated with either HC-D1 or SHC-D1 were incubated at 37 °C in NU/NU female mouse plasma (BioReclamation) at 0.2 mg/mL. Aliquots of 50 μL were pulled in triplicate for each time point, 0, 4, 8, 24, 48, and 72 h, and stored at less than −65 °C until analysis. Samples were treated with 200 μL of 1.25 μM internal standard [monomethyl auristatin D (MMAD)] in ethanol. A standard curve from 1 to 500 nM of MMAD was also prepared with 1 μM internal standard. Samples were incubated at room temperature for 1 h with shaking at 1,400 rpm followed by centrifugation at 19,300 × *g* for 30 min. The supernatants were removed and diluted with HPLC water and applied onto a Waters XBridge C-18 5-μm 2.1 × 50-mm column with an Agilent 1260 HPLC. The column was equilibrated in 80% mobile phase A (15 mM ammonium acetate in HPLC H_2O) and 20% mobile phase B (0.1% formic acid in acetonitrile) with an increase to 90% over 5 min with a flow rate of 0.5 mL/min. The column was regenerated with 90% mobile phase B for 1 min with reequilibration for 3 min.

Mass spectra were collected on an Agilent 6510 Q-ToF in positive-ion mode with capillary voltage at 3,500 V, capillary temperature at 325 °C, drying gas at 10 L/min, nebulizer pressure at 30 psig, and fragmentor voltage at 200 V. Collision voltages were set to 30 V for MMAD (*m*/*z* 771.5) and internal standard (*m*/*z* 732.5) with quantitation of daughter ions *m*/*z* 205.07 and 170.10, respectively.

Preparation of Conventional and Site-Specific Cysteine Conjugates. For comparison purposes, we sought to evaluate cysteine-linked anti-Her2 conjugates bearing the NC-D1 linker-payload in both conventional (hinge region disulfide) and site-specific (THIOMAB) formats. To accomplish this, we attached the NC-D1 linker-payload through an oxime linkage to a spacer element that was appropriately functionalized for conjugations to cysteine. We chose to use 1-(4-acetylbenzyl)-1*H*-pyrrole-2,5-dione as the spacer element to closely emulate the oxime linkage present in the *p*AF-based oxime conjugates while also providing the maleimide handle for conjugation to cysteine residues.

Both the conventional and site-specific cysteine-linked comparator conjugates were prepared by first forming the oxime linkage between the maleimide-containing spacer element and NC-D1 and then conjugating the resulting maleimide-derivatized NC-D1 oximes (mNC-D1) to anti-Her2 antibody Her and its mutant Her HC-A114C. The desired oxime was formed by stirring a solution of NC-D1 with a slight excess of 1-(4-acetylbenzyl)-1*H*-pyrrole-2,5-dione in tetrahydrofuran at 32 °C for 3 h, and purified by chromatography over silica gel. The conventional cysteine conjugate was prepared by reducing the disulfide linkages in Her antibody with tris(2-carboxyethyl)phosphine (TCEP), followed by conjugation of the mNC-D1 and purification by size exclusion chromatography. The final conjugate product had a loading of 4.5 as determined by mass spectroscopy (MS). The THIOMAB conjugate was prepared by first treating Her HC-A114C with cysteine, removing the resulting disulfide adducts by diafiltration, allowing for reformation of interchain disulfides by air oxidation, then conjugation to the mNC-D1, and purification by size exclusion chromatography. The final conjugate product had a loading of 2.3 as determined by MS.

In addition, A1 mc-D1 was prepared via partial reduction of the mAb with TCEP followed by reaction of reduced cysteine residue with linker payload mc-D1, similar to the method described above for Her mAb cysteine conjugation. The A1 mc-D1 conjugate had a loading of 4.3 as determined by MS.

Cell Lines. MDAMB468 (ATCC HTB-132), HCC1954 (CRL2338), and Raji (CCL-86) cell lines were obtained from the American Type Culture Collection (ATCC). MDAMB435/5T4 transfected cells were prepared as described previously (3). MDAMB361DYT2 cells were obtained from Dr. D. Yang (Georgetown University, Washington, DC). The cell lines were determined to be Mycoplasma free by a PCR Mycoplasma detection assay (ATCC). The cell lines MDAMB468, HCC1954, and MDAMB361DYT2 cells were maintained in MEM medium supplemented with 10% (vol/vol) FBS. The MDAMB435/5T4 was maintained in the same media above supplemented with 1.5 mg/mL of selection antibiotic G418. Raji cells were maintained in RPMI medium 1640 supplemented with 10% vol/vol FBS. Before using Raji, viable cells were isolated by density-gradient centrifugation (30 min at 1,000 × *g*) using Lymphoprep (Nycomed).

Binding and Internalization. In vitro binding of A1 unconjugated antibody or antibody drug conjugates (ADCs) was evaluated on 5T4 (+++) MDAMB435-tranfectant cell lines and anti-Her2 antibody and its drug conjugates on NCI-N87 cells. A 96-well cell-ELISA–based method was developed where an HRP-conjugated secondary antibody was used to detect bound anti-5T4 or anti-her2 primary antibody. Data are expressed as relative luminescence units (RLU) following subtraction of the background (secondary antibody-alone control).

A 96-well cell-ELISA–based method was developed to evaluate internalization of the mAbs or ADCs. Internalization at 37 °C was calculated as the percentage loss of cell surface-bound signal at 4 °C.

In Vitro Cell Growth Inhibition Assays. The effect of antibodies or conjugates on cell viability was assessed following exposure to various treatments. Cytotoxicity in vitro was assessed using a cell viability indicator (MTS; Promega). Cells were seeded in 96-well microtiter plates at a density of 5,000–10,000 cells per well and exposed to various concentrations of drugs. IC_{50} values (concentrations that inhibited cell growth by 50%) were calculated and reported as the concentration of Ab (in nanograms per milliliter) by logistic nonlinear regression following determination of the number of viable cells after 96 h of drug exposure.

In Vivo Efficacy Studies. Female nu/nu (nude) or SCID Berge mice, 6–8 wk of age, were obtained from Charles River Laboratories or Taconic. All procedures using mice were approved by the Pfizer Institutional Animal Care and Use Committees according to established guidelines. Mice were injected s.c. with tumor cells (10×10^6 MDAMB361DYT2 tumor cells in 50% Matrigel in

irradiated nude mice, 7×10^6 MDAMB435/5T4 in nude mice, or 10×10^6 HCC1954 cell in 50% Matrigel in SCID mice), and animals with staged tumors were administered i.v. with saline (vehicle), or conjugate at different dose levels, q4d × 4 starting on day 1 after randomization. All ADCs were dosed based on Ab content. Tumors were measured at least once a week and their size (in cubic millimeters ± SEM) was calculated as $mm^3 = 0.5 \times$ (tumor $width^2$) × (tumor length) calculated.

Pharmacokinetic Studies in Mouse. Female nu/nu (nude) mice, 6–8 wk of age, were obtained from Charles River Laboratories or Taconic. All procedures using mice were approved by the Pfizer Institutional Animal Care and Use Committees according to established guidelines. Mouse blood (10 μL) was collected from mice ($n = 4$ or 5) for up to 336 h after a single 3 mg/kg dose of antibody or ADC. Quantitation of the humanized A1 antibody (huA1) concentrations in mouse plasma was achieved using an ELISA. Briefly, the capture protein was 5T4 (Pfizer) and the detection antibody was biotinylated goat anti-human κ-chain IgG (Southern Biotech) and streptavidin-HRP conjugate (Jackson ImmunoResearch). Optical density was measured on a spectrophotometer (Molecular Devices). The ADC was detected by ELISA with 5T4 as the capture antigen and a biotinylated anti-MMAD antibody (Ambrx) and HRP-streptavidin conjugate (Jackson ImmunoResearch) for detection. The pharmacokinetic parameters were determined using a TK analysis module within Watson LIMS (version 7.2; Thermo) using a standard noncompartmental model.

Statistical Analysis. In vivo treatment effects were compared via analysis of variance (ANOVA) at each of the observation time points separately. The treatment pairwise comparisons were considered statistically significant if the P values were less than 0.005 over a range of observation time points. For PK, the data were analyzed via an unpaired Student's t test and considered statistically significant if the P values were less than 0.05.

Rat Toxicology Study of Her HC-A114*p*AF-NC-D1. The safety profile of Her HC-A114*p*AF-NC-D1 was evaluated in male Sprague–Dawley rats (Charles River Laboratories). Procedures were performed with the approval of the Animal Use and Care Committee of Charles River Laboratories, Peidmont Research Center. Rats were housed individually in cages under a 12-h light/dark photoperiod and allowed ad libitum access to water and standard rodent chow for the duration of the study. Rats were administered a single i.v. dose of vehicle control (50 mM histidine, 100 mM NaCl, 5% trehalose, pH 6.0) or 20, 60, or 90 mg/kg of Her HC-A114*p*AF-NC-D1 via tail vein injection ($n = 5$ animals per group). Toxicologic evaluation included biweekly body weights, clinical observations, standard hematology, and clinical chemistry (study days 5 and 14) and histopathology analysis. At necropsy, selected tissues and organs were collected and subsequently processed routinely to H&E slides and evaluated by a board-certified pathologist (Seventh Wave Laboratories). Hematology and clinical chemistry data were evaluated at Ambrx using Graph Pad Prism 5. Group means were compared by a one-way ANOVA with a post hoc analysis. Differences were considered significant at $P < 0.05$, dose of 20, 60, or 90 mg/kg Her HC-A114*p*AF-NC-D1 or vehicle control.

Toxicokinetic Analysis. To assess systemic exposure during the rat toxicology study, three groups of five male Sprague–Dawley rats were administered 20, 60, or 90 mg/kg Her HC-A114*p*AF-NC-D1, once, via i.v. injection on day 1. Test article was formulated in 50 mM histidine, 100 mM NaCl, 5% trehalose, pH 6.0. Blood samples were collected at 6, 24, 96, 168, 240, and 336 h post dose and processed to serum. Serum concentrations were measured using a sandwich electrochemiluminescence assay (ECLA), which was developed at Ambrx using Meso Scale Discovery (MSD) high bind plates. To assay for total antibody (with or without drug), test article was captured with recombinant human ErbB2/HER2 Fc chimera (R&D Systems) and detected with biotinylated goat anti-human κ polyclonal antibody (SouthernBiotech). To assay for intact site-specific antibody drug conjugate (NDC), capture and detection used native and biotinylated rabbit antibody to the small molecule toxin. Pharmacokinetic parameters were estimated using WinNonlin (Pharsight, version 5.1). Noncompartmental analysis for individual animal data with linear-up/log-down trapezoidal integration was used, and concentration data were uniformly weighted.

1. Šali A, Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* 234(3):779–815.
2. Miyazaki K, et al. (1995) Synthesis and antitumor activity of novel dolastatin 10 analogs. *Chem Pharm Bull (Tokyo)* 43(10):1706–1718.
3. Damelin M, et al. (2011) Delineation of a cellular hierarchy in lung cancer reveals an oncofetal antigen expressed on tumor-initiating cells. *Cancer Res* 71(12):4236–4246.

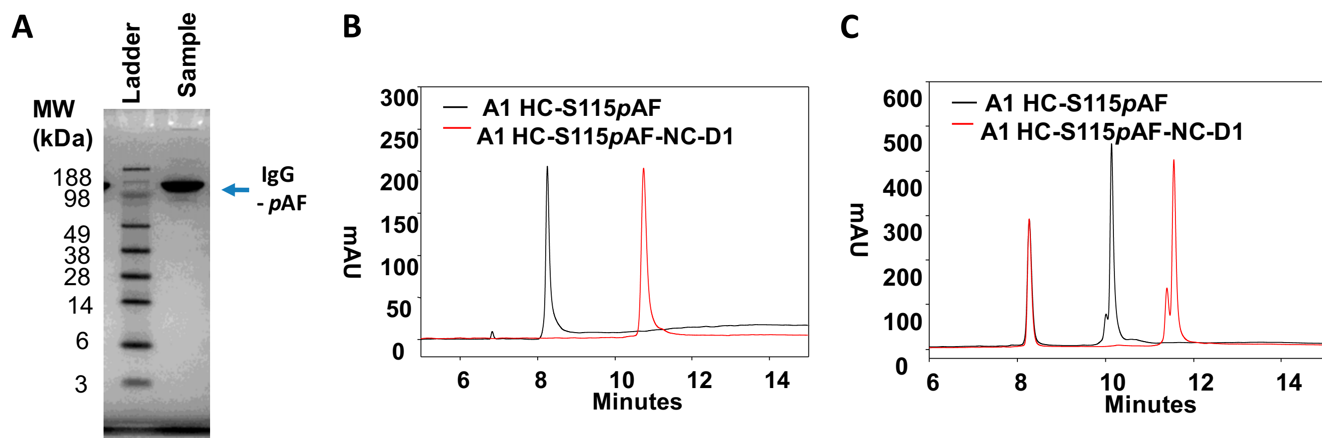


Fig. S1. SDS PAGE and RP-HPLC analysis of purified nonnative amino acid containing antibody and its drug conjugate. (*A*) The 4–12% Bis-Tris Coomassie SDS/PAGE analysis of post-protein A purification of a fed-batch shake flask culture. (*B*) RP-HPLC 214nm profile of A1 HC-S115*p*AF-NC-D1 shows a shift in retention time correlating to >95% di-conjugated A1 HC-S115*p*AF. (*C*) RP-HPLC 214nm profile of the reduced A1 HC-S115*p*AF-NC-D1 shows a shift in retention time of the heavy chain only correlating to toxin-linker conjugation. The light chain peak at 8.2 min was determined to be unmodified during conjugation.

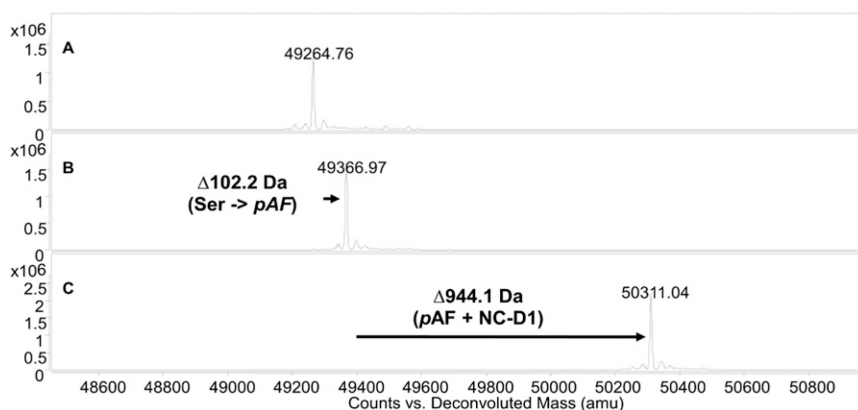


Fig. S2. Intact MS analysis of (*A*) deglycosylated WT A1 heavy chain. (*B*) Deglycosylated A1 HC-S115*p*AF heavy-chain mass shift correlates to *p*AF incorporation for a serine residue (102.1 Da). (*C*) Deglycosylated A1 HC-S115*p*AF-NC-D1 heavy-chain mass shift is in agreement with a single toxin-linker conjugation (944.3 Da) to each heavy chain.

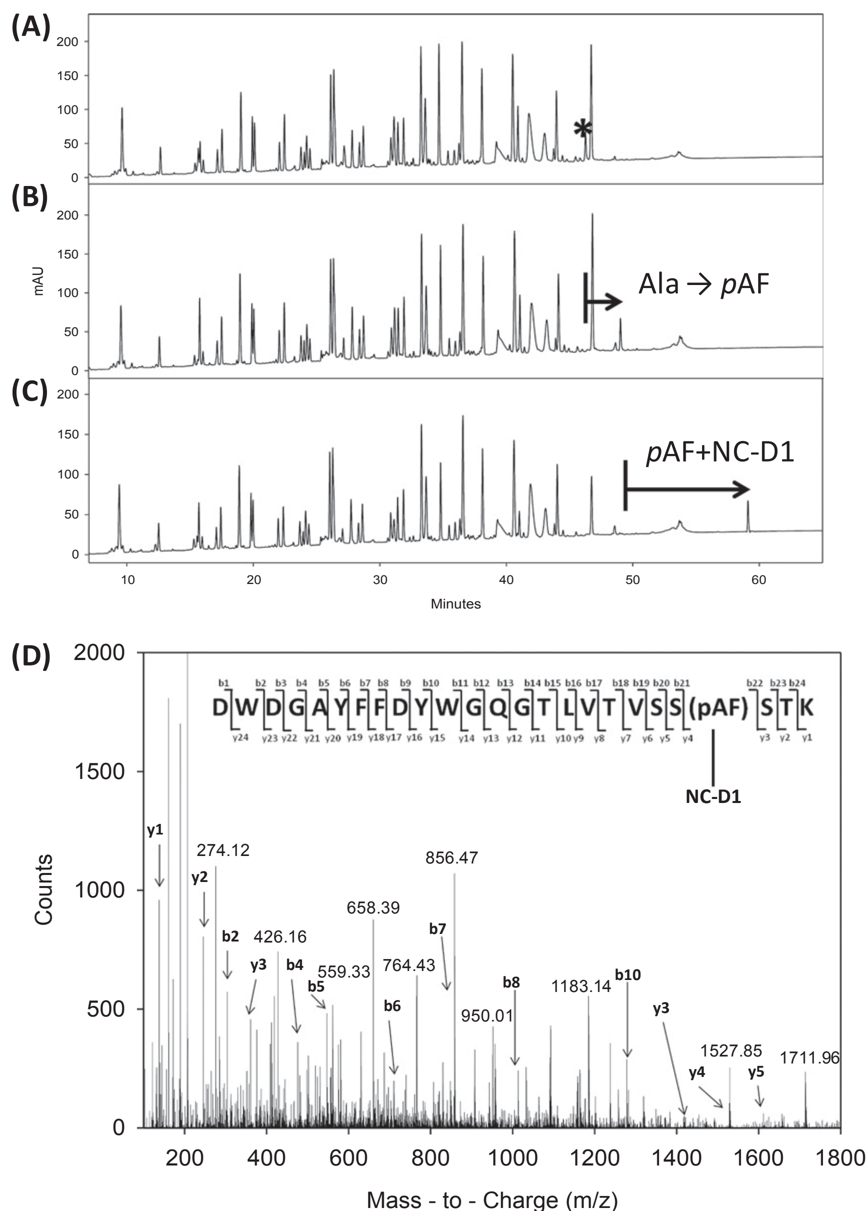


Fig. S3. Peptide mapping and MS/MS analysis of deglycosylated heavy chain of WT A1 and HC-S115*p*AF ± NC-D1: (*A*) The WT T11 peptide was identified with an asterisk (*) at 46.3 min in the WT A1 trypsin digest. (*B*) The 214-nm trace of the A1 HC-S115*p*AF digest is comparable to WT; however, the WT T11 peptide peak was not detectable and a new peak was detected at 50.2 min. (*C*) The A1 HC-S115*p*AF NC-D1 trypsin digest shows the *p*AF-containing peptide shifted to 60.2 min, suggesting conjugation of NC-D1. (*D*) MS/MS of T11 peptide of A1 HC-S115*p*AF +NC-D1. The MS (MH^{3+} = 12,872.97 Da) of the T11 peptide confirms incorporation of the *p*AF-NC-D1 at S site. The y-ion masses after y3 reflect the appropriate mass shift for *p*AF-NC-D1 modification at site S115.

Fig. S4. *p*AF site-specific conjugated antibodies retain binding and internalization compared with their unmodified Abs. (*A*) Binding of A1 Ab (open column), A1 mc-D1 (column filled with vertical lines), and A1 HC-S115*p*AF-NC-D1 (filled column) to MDAMB435/5T4 at an antibody concentration of 4 μg/mL. (*B*) Internalization of A1 Ab (filled circles), and A1 HC-S115*p*AF-NC-D1 (filled triangles) over time on MDAMB468 cells. (*C*) Binding of anti-Her2 Ab (open column), Her mNC-D1 (column filled with diagonal lines), and Her HC-A114*p*AF-NC-D1 (filled column) and Her HC-A114C-mNC-D1 (column filled with vertical lines) to N87 cells at an antibody concentration of 10 μg/mL. (*D*) Internalization of Her Ab, Her mNC-D1, Her HC-A114C-mNC-D1, and Her HC-HA114*p*AF-NC-D1 over time on N87 cells.

Fig. S5. In vitro cytotoxicity of A1 mc-D1 (*A*) and A1 HC-S115*p*AF-NC-D1 (*B*) on several 5T4-positive and -negative cell lines and in vitro cytotoxicity of Her mNC-D1 (*C*), Her HC-A114*p*AF-mNC-D1 (*D*), and Her HC-A114C-mNC-D1 (*E*) on several Her2-positive and -negative cell lines.

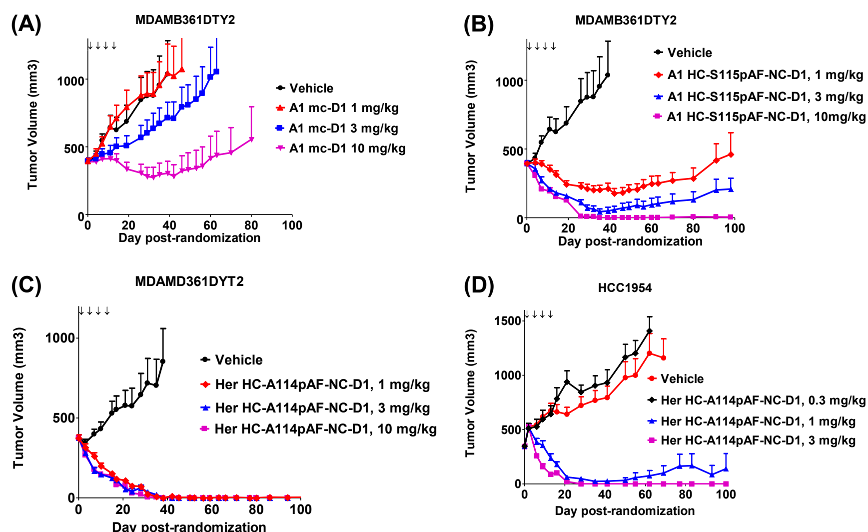


Fig. S6. Nonnative amino acid site-specific conjugates display superior in vivo efficacy compared with conventional and site-specific cysteine conjugates. Female nu/nu mice were s.c. implanted with tumor cells and staged at ~0.1–0.4 g (n = 8–10 mice per group). After randomization, the mice were treated i.v. with conjugates of 1, 3, or 10 mg/kg or 0.3, 1, or 10 mg/kg on days 1, 5, 9, and 13. The mean tumor volumes ± SEM (error bar above in the figures) are plotted over time. (*A*) In vivo efficacy determination of A1 mc-D1 in 5T4-positive tumor models of MDAMB361DYT2 at 1, 3, and 10 mg/kg on days 1, 5, 9, and 13. (*B*) In vivo efficacy determination of A1HC-S115*p*AF-NC-D1 in 5T4-positive tumor models of MDAMB435/5T4 and MDAMB361DYT2 at 1, 3, and 10 mg/kg on days 1, 5, 9, and 13. (*C* and *D*) In vivo efficacy determination of Her HC-A114*p*AF-NC-D1 in her2-positive tumor models of MDAMB361DYT2 and HCC1945 at 0.3, 1, and 3 mg/kg or 1, 3, and 10 mg/kg, respectively, on days 1, 5, 9, and 13.

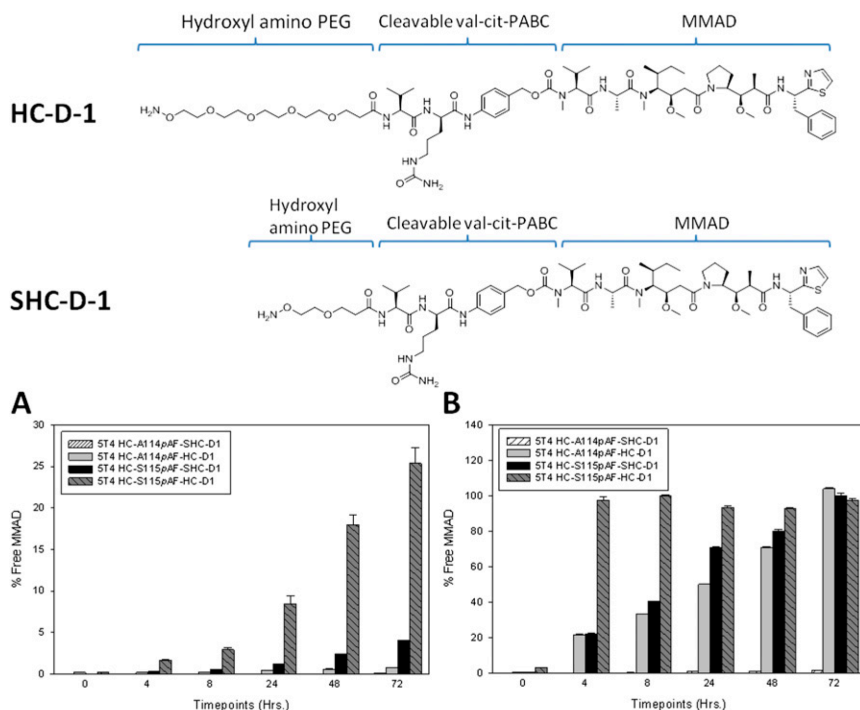
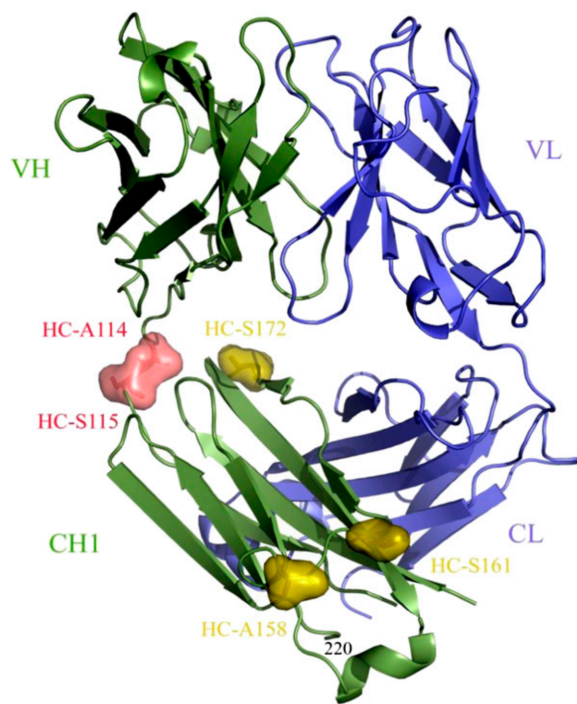


Fig. S7. Plasma stability studies of Val-Cit-PABC cleavable linkers (HC-D1 and SHC-D1) on different sites of NDC in (*A*) rat plasma and (*B*) mouse plasma. A1 variants with *p*AF incorporated at site HC-A114*p*AF or HC-S115*p*AF were conjugated with HC-D1 or SHC-D1 and incubated in rat or mouse plasma for a duration of 72 h at 37 °C. Time points were analyzed in triplicate by liquid chromatography with tandem mass spectrometry for released MMAD. The Val–Cit–PABC linker was much more susceptible to cleavage in the mouse plasma matrix compared with rat plasma. The stability of HC-D1 and SHC-D1 were significantly improved when conjugated to site HC-A114*p*AF vs. site HC-S115*p*AF. It was also observed that SHC-D1 had greater stability than HC-D1, which was much more apparent when conjugated to site HC-S115*p*AF. In total, 25.4% of MMAD was released with HC-D1 at site HC-S115*p*AF after 72 h compared with 4.0% free MMAD with SHC-D1. Error bars indicate +SD.

Fig. S8. Mean body weight change in percentage (*A*) and mean body weight (in grams) (*B*) from study day 1 of rats treated with a single i.v. dose of 20, 60, or 90 mg/kg Her HC-A114*p*AF-NC-D1 or vehicle control.

Fig. S9. Toxicokinetics of Her NDC (Her HC-A114*p*AF-NC-D1) in Sprague–Dawley rats. Rats ($n = 5$ per group) were dosed with a single i.v. injection of 20, 60, or 90 mg/kg, and blood was sampled to 14 d. Total antibody (Ab) and intact NDC serum concentrations were determined. The systemic exposure for both Ab and NDC increased in a dose-dependent manner.



Scheme S1. Modeling of A1 antibody and design of sites for nonnative amino acid incorporation.

Scheme S2. Synthesis of NC-D1.

Table S1. In vitro potency (IC_{50} ng/mL) of ADCs on 5T4-positive and -negative cell lines

Target	ADC or NDC	Drug load*	Antigen-positive cell line		Antigen-negative cell line	Specificity[†]
			MDA MB435/5T4 (3+)	MDA MB468 (2+)	Raji (–)	
5T4	A1 mc-D1[‡]	4.0	28 ± 1	10,564 ± 2,343	>45,000	>1,607
	A1 HC-S115 *p*AF-NC-D1	2.0	44 ± 16[§]	4,673 ± 972	33,777 ± 13,477	768

*Drug load: drug-to-antibody ratio.
[†]Specificity: ratio of the IC_{50} values of antigen-negative cells to antigen-positive cells (MDA MB435/5T4 as positive cell line).
[‡]Conventional conjugation.
[§]Mean ± SD.

Table S2. In vitro potency (IC_{50} ng/mL) of ADCs on Her2-positive and -negative cell lines

Target	ADC or NDC	Drug load*	Antigen-positive cell line				Antigen-negative cell line	Specificity[†]
			HCC1954 (3+)	N87 (3+)	BT474-M1 (3+)	MDA MB 361-DYT2 (2+)	MDA MB 468 (–)	
Her2	Her mNC-D1[‡]	4.5	9.5 ± 2.3	40 ± 24	394 ± 122	570 ± 218	5,381 ± 3,567	480
	Her HC-A114*p*AF-NC-D1	2.0	7.7 ± 1	23 ± 11	34 ± 13	69 ± 48	40,792 ± 5,951	5,912
	Her HC-A114C-mNC-D1	2.3	18 ± 2.5	87 ± 54	64 ± 16	1,552 ± 144	4,617 ± 910	280

*Drug load: drug-to-antibody ratio.
[†]Specificity: ratio of the IC_{50} values of antigen-negative cells to antigen-positive cells (HCC1954 as positive cell line).
[‡]Conventional conjugation.

Table S3. Selected clinical chemistry values for rats treated with a single i.v. injection of Her HC-A114*p*AF-NC-D1 or vehicle control on study day 5 and study day 14

	BUN, mg/dL		Creatinine, mg/dL		TP, g/L		ALP, U/L		ALT, U/L		AST, U/L	
	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14
Vehicle	29.4 ± 7.8	20.0 ± 3.8	0.9 ± 0.2	0.5 ± 0.1	9.8 ± 1.2	7.6 ± 0.9	285 ± 62.8	224.6 ± 75.3	79.8 ± 17.6	62.4 ± 7.9	339 ± 113.1	163.2 ± 42.4
20 mg/kg	27.0 ± 0	17.0 ± 0.7	0.8 ± 0.1	0.5 ± 0.1	8.9 ± 0.5	7.2 ± 0.3	269.4 ± 46.1	192.8 ± 23.4	80.4 ± 7.5	70.2 ± 20.9	286.2 ± 60.0	141.2 ± 12.5
60 mg/kg	30.0 ± 3	17.2 ± 2.8	0.8 ± 0.2	0.4 ± 0.0	9.9 ± 0.7	7.6 ± 0.3	439.5 ± 44.5*	272.8 ± 51.0	76 ± 46.3	106.6 ± 77.4	328.25 ± 372	243.2 ± 228.4
90 mg/kg	16 ± 13.9	17.2 ± 0.8	0.9 ± 0	0.4 ± 0.0	8.6 ± 0.6	7.8 ± 0.3	355 ± 88.5	267.6 ± 86.0	92 ± 15.1	95 ± 22.7	298.2 ± 36.3	194 ± 47.4

ALP, alkaline phosphatase; ALT, alanine amino transferase; AST, aspartate amino transferase; BUN, blood urea nitrogen; TP, total protein.

*Statistically significant from vehicle control ($P < 0.05$).

Table S4. Hematology values for rats treated with a single i.v. injection of Her HC-A114*p*AF-NC-D1 or vehicle control on study day 5 and study day 14

Experiments	WBCs, $10^3/\mu L$		Neutrophils, $10^3/\mu L$		Platelets, $10^3/\mu L$	
	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14
Vehicle	12.3 ± 1.5	13.2 ± 1.9	1.5 ± 1.1	1.4 ± 0.3	1,289.5 ± 207	1,506.4 ± 513.2
20 mg/kg	18.3 ± 2.9	15.7 ± 4.1	1.8 ± 0.2	2.6 ± 1.1	1,156.7 ± 85.3	954.4 ± 239.2
60 mg/kg	16.6 ± 3.8	14.9 ± 11.4	2.4 ± 0.7	2.2 ± 1.7	1,247 ± 204.9	887.0 ± 563.9
90 mg/kg	13.2 ± 2.6	ND	4.4 ± 1.1*	ND	1,030.8 ± 103.3	ND

ND, not determined.
*Statistically significant from vehicle control ($P < 0.05$).

Table S5. Histopathology findings of rats treated with a single i.v. injection of Her HC-A114*p*AF-NC-D1

Tissues	Histopathology finding	Dose levels		
		20 mg/kg	60 mg/kg	90 mg/kg
Kidney	Tubular basophilia (minimal)	1/5	1/5	
	Inflammation (minimal)		2/5	1/5
	Congestion (mild to moderate)		2/5	4/5
Liver	Congestion (mild to moderate)	2/5	5/5	5/5
	Degeneration/necrosis (minimal to mild)		1/5	2/5
	Disorganized hepatic plates (minimal to mild)			2/5
Lungs	Congestion (minimal to moderate)	5/5	5/5	5/5
	Edema (minimal to moderate)	1/5	4/5	5/5
	Interstitial infiltrate, macrophages (minimal to moderate)	3/5	5/5	5/5
Spleen	Decreased lymphocytes (minimal)		1/5	3/5
Testes	Spermatogenic degeneration (mild to marked)	1/5	5/5	5/5
Thymus	Inflammation (minimal to marked)		1/5	5/5
	Decreased lymphocytes (minimal to moderate)		2/5	4/5