Supporting Information:

Improving the Serum Stability of Site-Specific Antibody Conjugates with Sulfone Linkers

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EXPERIMENTAL PROCEDURES

THIOMAB Cloning, Expression, and Purification

To generate antibody expression vectors, the trastuzumab heavy and light chain sequences were modified to contain an upstream murine light chain secretory leader sequence (GeneArt) and ligated into the HindIII and XhoI restriction sites of pTT5. Site-specific Cys substitutions were introduced by overlap PCR and ligated into the HindIII and XhoI restriction sites of pTT5, and Cys mutations were numbered to be consistent with that previously reported (HC-A114C, LC-V205C, Fc-S396C). DNA sequencing confirmed vector identity, and Maxiprep was used to purify DNA for transfections (Invitrogen, PureLink HiPure Plasmid Filter Maxiprep Kit).

Human embryonic kidney (HEK) 293T cells (ATCC) were maintained in DMEM (Dulbecco’s Modified Eagle’s Medium with GlutaMAX; Gibco) containing 10% (vol/vol) Fetal Bovine Serum (FBS; Gibco) and 1% (vol/vol) Antibiotic-Antimycotic (Anti-Anti; Gibco) in a humidified 5% CO₂ atmosphere at 37 °C. For transient transfections, 4.5 x 10⁶ HEK293T cells were seeded onto poly-L-lysine hydrobromide (Sigma-Aldrich) coated T75 flasks and cultured for 24 to 48 h with DMEM containing 10% FBS in the absence of Anti-Anti. At ~90% confluence, 25 µg of each heavy and light chain expression vectors were transfected by Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. For 5 to 7 days, culture supernatants were collected and filtered using 0.22 µm Steriflip units (Millipore).

Antibodies were purified by a 1 mL HiTrap Protein A column (GE Healthcare) using an AKTA FPLC (GE Healthcare). Briefly, 20 column volumes (CV; 1 mL/min) of PBS (pH 7.4) were used to equilibrate the column, and supernatant was flowed over the column and washed with 10 to 20 CV of PBS. Antibodies were eluted with ~10 CV of 100 mM acetic acid (pH 2.9),
and fractions (1.5 mL) were neutralized with 150 µL 1 M Tris-HCl (pH 9.0). The column was washed with 10 CV of 1 M acetic acid (pH 2.4), 10 CV of PBS (pH 7.4), and 10 CV of 20% ethanol. Pooled fractions were buffer exchanged into PBS (pH 7.4) and concentrated with a Vivaspin 20 10K MWCO PES centrifugal concentrator (Sartorius Stedim Biotech), which was then syringe filtered using a 0.2 µm HT Tuffryn Membrane Acrodisc filter (Pall Life Sciences). All antibodies were determined to be >95% pure by SDS-PAGE.

**THIOMAB Conjugation Reactions and Plasma Stability**

Conjugation reactions were carried out using 1 mg/mL antibody and 2 equivalents per labeling site of fluorescein-attached maleimide or sulfone linker in PBS (pH 7.4) at room temperature. Mild reduction was performed to free engineered Cys sulfhydryl groups blocked with either Cys or glutathione followed by diafiltration and oxidation prior to conjugation as described. Reactions were allowed to proceed for 1, 2, 4, and 8 h. Unreacted compound was immediately removed by size exclusion column chromatography using a Micro Spin 6 column (Bio-Rad). Gel-loading solution containing 250 µM tris(2-carboxyethyl)phosphine (TCEP) was added to each sample, and labeling was analyzed by SDS-PAGE using 10% NuPAGE Bis-Tris Gels (Life Technologies). Fluorescence and Coomassie stained gels were detected/quantified using a GelDoc XR Imaging System (Bio-Rad). Dye to antibody ratios were determined by absorbance measurements as described. Conjugate plasma stability was assessed in triplicate as previously described. In brief, fluorescently labeled THIOMABs were diluted into human plasma and incubated at 37 °C for the duration of the time course study. Time points were removed and stored at -80 °C, and then SDS-PAGE was performed as above.
Analysis of HER2 Binding by Flow Cytometry

BT-474 cells were maintained in RPMI media 1640 with GlutaMAX (Gibco) containing 10% FBS and 1% Anti-Anti. 2 x 10^5 BT-474 cells were transferred to 1.5 mL Eppendorf tubes, and trastuzumab or THIOMAB conjugate was added at the indicated concentrations. Cells were incubated on ice for 1 h and washed three times with 500 μL FACS buffer (PBS, 1% BSA, 0.01% NaN3, pH 7.4). AP-conjugated AffiPure F(ab’2) Fragment Goat Anti-Human IgG + IgM (H+L) secondary antibody (Jackson ImmunoResearch) was diluted 1:1,000 in 100 μL FACS buffer and added to each sample for 1 h on ice. Samples were washed three times with 500 μL FACS buffer and transferred to filter-top FACS tubes. Fluorescence was measured by flow cytometry (BD LSRII; BD Biosciences). For each sample, 30,000 live events were collected, and data were analyzed using FlowJo software (Tree Star, Inc.). All samples were tested in triplicate.

Examination of HER2 Binding by ELISA

ELISA plates (Costar 3690) were coated with 25 ng (25 μL) of HER2-Fc (R&D Systems) in PBS by incubating at 4 °C overnight. Plates were washed twice with PBST (0.1% Tween 20), blocked with 5% BSA in PBST for 1 h at 37 °C, and then washed 4 times with PBST. Next, antibody was added at 50 ng/mL in PBST with 1% BSA and incubated at 37 °C for 90 minutes. Plates were washed 8 times with PBST. Goat Anti-Human Kappa AP conjugate (Bethyl Laboratories) was used as secondary antibody at a 1:1,000 dilution, which was then incubated at 37 °C for 1 hour and washed 4 times with PBST. Detection was performed at 405 nm after the addition of AP substrate (Sigma 104 phosphatase substrate).
SELENOMAB Conjugation Reactions and Plasma Stability

The cloning, expression, and purification of R11-scFv-Fc-Sec and R11-scFv-Fc-stop antibody were performed as previously described. Conjugation reaction were carried out using 4 µM (~0.5 mg/mL) R11-scFv-Fc-Sec or the negative control R11-scFv-Fc-stop and 40 µM fluorescein-attached maleimide or sulfone linker in 100 mM sodium acetate (pH 5.2) buffer in the presence of 20 µM dithiothreitol (DTT). The reactions were allowed to proceed for 1 h at room temperature. Unconjugated compounds were removed by 30-kDa cutoff centrifugal filter device (Millipore) and buffer-exchanged with PBS. The labeling was analyzed by SDS-PAGE under non-reducing conditions. Conjugate plasma stability was carried out as described for THIOMABs.

Analysis of ROR1 Binding by Flow Cytometry

Human mantle cell lymphoma HBL-2 and chronic lymphocytic leukemia MEC-1 cells maintained in 10% (v/v) FBS in RPMI 1640 medium supplemented with penicillin-streptomycin (all Life Technologies) were collected by centrifugation, resuspended in FACS buffer, and 2 x 10^5 cells were transferred to 1.5 mL Eppendorf tubes. The cells were first blocked with 10 µg polyclonal human IgG (Thermo Scientific) for 30 min on ice and then incubated with 3 µg R11-scFv-Fc-Sec or R11-scFv-Fc-stop conjugate for 30 min on ice. Samples were washed three times with 500 µL FACS buffer and transferred to FACS tubes. Fluorescence was measured on an LSR II Flow Cytometer (Becton-Dickinson). All samples were tested in triplicate.
**Figure S1.** Comparison of THIOMAB labeling using phenyloxadiazole (ODA), benzothiazole (BTZ), and phenyltetrazole (PTZ) fluorescein-linked compounds. (A-B) Efficient labeling of LC-V205C and Fc-S396C THIOMABs was only observed with the ODA linker. (C) pH dependence (7.4, 8, or 9) of Fc-S396C labeling. Increasing the nucleophilicity of the engineered Cys residues did not significantly improve conjugation yields for the BTZ and PTZ linkers. (D) Site-specific labeling of THIOMABs with ODA fluorescein-linked compound. Fluorescent (left) and Coomassie stained (right) gel images. Heavy (HC) and light (LC) chains are denoted.
Figure S2. Flow cytometry histogram of the dose-dependence for HER2 antigen binding by trastuzumab on BT-474 cells.

Figure S3. Trastuzumab and THIOMAB conjugate binding to HER2-Fc by ELISA. Assays were performed with sub-saturating antibody concentrations (50 ng/mL) in triplicate.
Figure S4. R11-scFv-Fc-Sec conjugate stability in human plasma at 0, 4, 8, 12, 24, 48, and 72 h. (A) Fluorescent (top) and Coomassie stained (bottom) SDS-PAGE gels are shown. Antibody and albumin bands are indicated by arrows. Molecular weights from a prestained protein ladder are shown on the left. (B) Summary of maleimide and phenyloxadiazole sulfone conjugate stability.
**Figure S5.** Time course for HC-A114C labeling with maleimide or phenyloxadiazole sulfone fluorescent compound at T = 1, 2, 4, or 8 h. Fluorescent (top) and Coomassie stained (bottom) gels are shown with heavy and light chains indicated.

**Figure S6.** Kinetically controlled dual labeling of LC-V205C/Fe-S396C THIOMAB. A two-step conjugation strategy was used to first label Fc-S396C using the phenyloxadiazole sulfone linker followed by maleimide or phenyloxadiazole sulfone LC-V205C conjugation. Sequential treatments are indicated by the above bars, and reaction times are denoted. Fluorescent (top) and Coomassie stained (bottom) gels are shown with heavy and light chains indicated.
Table S1. Summary of dye-to-antibody ratios (DAR) for THIOMABs labeled with maleimide or phenyloxadiazole sulfone linkers.

| THIOMAB   | DAR\textsuperscript{Mal} | DAR\textsuperscript{Sulfone} |
|-----------|--------------------------|------------------------------|
| LC-V205C  | 1.7                      | 1.8                          |
| Fc-S396C  | 1.6                      | 1.9                          |
| HC-A114C  | 1.9                      | 1.8                          |
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