Glutamic acid-valine-citrulline linkers ensure stability and efficacy of antibody–drug conjugates in mice

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Valine–citrulline linkers are commonly used as enzymatically cleavable linkers for antibody–drug conjugates. While stable in human plasma, these linkers are unstable in mouse plasma due to susceptibility to an extracellular carboxylesterase. This instability often triggers premature release of drugs in mouse circulation, presenting a molecular design challenge. Here, we report that an antibody–drug conjugate with glutamic acid-valine-citrulline linkers is responsive to enzymatic drug release but undergoes almost no premature cleavage in mice. We demonstrate that this construct exhibits greater treatment efficacy in mouse tumor models than does a valine-citrulline-based variant. Notably, our antibody–drug conjugate contains long spacers facilitating the protease access to the linker moiety, indicating that our linker assures high in vivo stability despite a high degree of exposure. This technology could add flexibility to antibody–drug conjugate design and help minimize failure rates in preclinical studies caused by linker instability.
Antibody–drug conjugates (ADCs) are an emerging class of chemotherapy agents with the potential to revolutionize current treatment strategies and regimens for cancers. Indeed, the clinical success of ADCs has been demonstrated with FDA-approved ADCs for the treatment of patients with Hodgkin lymphoma (Adcetris®), acute lymphoblastic lymphoma (Besponsa®), and acute myeloid lymphoma (Miylotarg®) and more than 60 promising ADCs in clinical trials. The striking success has driven scientists and clinicians to further advance this molecular platform for developing effective therapeutics for cancers, microbial infection, and immune modulation.

ADCs consist of potent drugs (payloads) linked to therapeutic monoclonal antibodies (mAbs) through chemical linkers. This molecular format enables pinpoint delivery of highly cytotoxic payloads to target tumor cells, resulting in greater potency, a broader therapeutic window, and more durable treatment effect than is possible with traditional chemotherapy agents. In addition to the choice of the antibody and payload, the ADC linker structure and antibody–payload conjugation modality impact ADC homogeneity, cytotoxic potency, tolerability, and pharmacokinetics (PK). These key parameters critically contribute to overall in vivo therapeutic efficacy. Thus, refining linker and conjugation chemistries is of crucial importance for optimizing the therapeutic potential and safety profiles of ADCs.

Valine–citrulline (VCit) dipeptide linkers connecting a payload with a p-aminobenzyloxycarbonyl (PABC) group are standard cleavable linkers widely used in many successful ADCs including the FDA-approved ADC Adcetris®. VCit linkers are cleaved by cathepsins upon internalization of ADCs by target cancer cells, resulting in traceless release of payloads (Fig. 1a). VCit linkers are stable in cynomolgus monkey and human plasma. However, it has been reported that these linkers can be hydrolyzed in mouse plasma. The extracellular carboxylesterase 1c (Ces1c) has been reported as the enzyme responsible for this hydrolysis, which can result in premature release of toxic payloads in circulation prior to reaching tumors. One approach to circumvent this problem is to use Ces1c-knockout mouse (available from The Jackson Laboratory) or cross the Ces1c null alleles onto an immunocompromised mouse model. However, the use of such genetically engineered mice may hamper smooth implementation of in vivo studies because of long lead time and limited choice of parent genetic background. The linker instability in mouse plasma can also be ameliorated by carefully selecting the linker attachment sites within an antibody and limiting the length of the VCit linker to minimize the exposure of the vulnerable moiety to extracellular enzymes, as demonstrated with several VCit-based ADCs.

However, it has also been confirmed that installation of VCit linkers at exposed conjugation sites and extending the linker structure result in rapid loss of payload in circulation. Almost all initial preclinical studies in drug development are performed using mouse models. Therefore, the instability creates an obstacle for evaluation of the therapeutic potential and safety profiles of VCit-based ADCs. In addition, this issue significantly limits flexibility in the choice of conjugation sites and linker design. Indeed, ADCs constructed using the multi-loading VCit linker recently developed by our group were shown to have instability in mouse plasma and poor treatment efficacy in mouse breast tumor models (described in detail in the Results section).

Herein, we demonstrate that a glutamic acid–valine–citrulline (EVCit) tripeptide sequence provides exceptionally high long-term stability in mouse and human plasma while retaining the capacity to release the free payload upon cathepsin-mediated cleavage (Fig. 1a). We also demonstrate that an ADC constructed...

Fig. 1 Structures and plasma stability of cathepsin-responsive cleavable peptides. a VCit and EVCit-based ADC linkers. VCit linkers are unstable in mouse plasma due to susceptibility to the extracellular carboxylesterase Ces1c. This instability often triggers premature release of payload in circulation. This study presents that EVCit-based tripeptide sequences with an acidic side chain such as EVCit are responsive to cathepsin-mediated cleavage but highly stable in mouse plasma. b Structures of pyrene-based small-molecule probes containing a VCit (1a), SVCit (1b), EVCit (1c), DVCit (1d), KVcit (1e) or HO-GVCit (1f) cleavable sequence. c Stability of probes (1a–f) in undiluted BALB/c mouse plasma at 37 °C. (1a) blue circle; (1b) orange triangle; (1c) green square; (1d) magenta hexagon; (1e) red inverted triangle; (1f) gray cross. EVCit and DVCit probes (1c, d) showed great plasma stability while highly responsive to cathepsin B-mediated cleavage (see Supplementary Fig. 2). All assays were performed in triplicate. Error bars represent s.e.m. and values in parentheses are 95% confidential intervals.
using the acidic EVCit linker exhibits by far greater in vivo stability and antitumor efficacy in xenograft mouse models bearing human breast cancer than does a VCit-based variant. Our findings indicate that the use of the EVCit linker system could minimize failure rates in preclinical studies using mouse models caused by linker instability, significantly expanding flexibility in designing ADCs. This technology may also provide a broadly applicable solution for enhancing stability and efficacy of other molecular classes of drug conjugates for targeted therapy.

Results

In vitro evaluation of cleavable tripeptide probes. It has been reported that introducing a chemical modification to the N-terminus of the valine residue (P3 position, Fig. 1a) significantly affects the plasma stability of VCit linker-based ADCs23. The authors demonstrated that VCit linkers with a hydrophilic group, such as 2-hydroxyacetamide group, at the P3 position increased ADC stability in mouse plasma without impairing the reactivity to intracellular cathepsin B-mediated cleavage. Based on their findings, we speculated that installation of a highly polar functional group at the P3 position could further enhance resistance to Ces1c-mediated degradation.

To test this hypothesis, we set out to assess a panel of peptide probes containing various amino acids at the P3 position selected to confer plasma stability (Fig. 1b). We first synthesized VCit- and tripeptide probes 1a–f by standard solid- and liquid-phase peptide synthesis and following carbamate formation (Supplementary Fig. 1). These model probes consisted of an XVGit–PABC unit where X is no amino acid (1a), serine (1b), glutamic acid (1c), aspartic acid (1d), lysine (1e), and hydroxyacetyl glycine (1f). Based on a report by Dorywalska and co-workers23, we expected that probes 1b, f would serve as mimics of the hydroxy-functionalized tripeptide ADC linker with increased mouse plasma stability. In particular, the 2-hydroxyacetamide group within probe 1f is the modifier that provided the greatest stability in their report. Thus, we expected that comparing peptide sequences of interest with probe 1f would clearly demonstrate the degree of improvement over the previous linker design. These linkers were covalently linked to 1-pyrenemethylcarbamic acid (PABC) modules containing monomethyl auristatin F (MMAF), polyethylene glycol (PEG) spacer, and dibenzocyclooctyne (DBCO) as a reaction handle for following strain-promoted azide–alkyne click reaction (Supplementary Fig. 4). A DBCO–VCit module was obtained from a commercial source. The number of PEG units in each module was adjusted so that all payload modules had a similar length (PEG4 for the dipeptide VCit and PEG3 for the tripeptides SVCit and EVCit). Each clickable module could be quantitatively conjugated to the common mAb–branched linker conjugate to give highly homogeneous ADCs with an average drug-to-antibody ratio (DAR) of 3.9 (determined by reverse-phase HPLC, Supplementary Fig. 5). We also prepared non-cleavable branched ADC 4 (DAR: 3.9) and an isotype control constructed using the EVCit–MMAF module (5, DAR: 3.9) in the same manner (Supplementary Fig. 6). Size-exclusion chromatography (SEC) analysis revealed that all ADCs produced predominantly in the monomer form (Fig. 2c). We also evaluated long-term stability by incubating each ADC at 37 °C in PBS (pH 7.4) for 28 days. No significant degradation or aggregation was observed by SEC analysis (Supplementary Fig. 7).

To assess how a chemical modification at the P3 position influences ADC hydrophobicity, we performed hydrophobic interaction chromatography (HIC) analysis under physiological conditions (phosphate buffer at pH 7.4, Fig. 2d). Highly polar EVCit ADC 3c was detected earlier in retention time than VCit ADC 3a, whereas the hydroxy group within SVCit ADC 3b marginally affected the ADC hydrophobicity (Fig. 2e). These results demonstrate that constructing ADCs using carboxy-functionalized EVCit linkers can reduce ADC hydrophobicity at physiological pH. This feature is advantageous for the construction of ADCs, especially high-DAR ADCs, because high ADC hydrophobicity is known to trigger aggregation and fast clearance from the body23.

Validation of ADCs in vitro. To investigate how ADC in vitro properties are modulated by a chemical modification to the P3 position, we first evaluated ADCs 3a–c for cathepsin B-mediated cleavage. Each ADC was incubated in the presence of human liver cathepsin B at 37 °C. The half-lives of VCit ADC 3a, SVCit ADC 3b, and EVCit ADC 3c were determined to be 4.6 h, 5.4 h, and
Fig. 2 Construction and characterization of ADCs 3a-c. a Construction of ADCs (3a-c) by MTGase-mediated branched linker conjugation and following strain-promoted azide-alkyne cycloaddition (cyan cylinder: PEG spacer–XVCit–PABC module; yellow spark: MMAF). b Deconvoluted ESI-mass spectra. Top panel: N297A anti-HER2 mAb. Second panel: antibody-branched linker conjugate. Third–fifth panels: highly homogeneous ADCs (3a–c). Asterisk (*) indicates a fragment ion detected in ESI-MS analysis. c SEC traces of ADCs (3a–c). d HIC analysis of ADCs (3a–c) under physiological conditions (phosphate buffer, pH 7.4). e Overlay of the three HIC traces (VCit ADC 3a; blue; SVCit ADC 3b; orange; EVCit ADC 3c; green). DAR, drug-to-antibody ratio; MTGase, microbial transglutaminase; PABC, p-aminobenzoxycarbonyl; PEG, polyethylene glycol.

2.8 h, respectively (Supplementary Fig. 8). This result illustrates that EVCit linkers conjugated to a mAb are more sensitive to cathepsin B-mediated cleavage than VCit and SVCit linkers, which is consistent with the responsiveness of pyrene probes 1a–c (Supplementary Fig. 2b). The ADCs were also tested for responsiveness to cathepsins L and S, which are also responsible for lysosomal cleavage of VCit linkers34 (Supplementary Table 1). Interestingly, VCit ADC 3a was slightly more sensitive to cathepsin L-mediated cleavage than EVGc 3c. Cathepsin S cleaved both linker systems at similar rates. In addition, both sequences were cleaved at almost equivalent rates in a mixture of cathepsins B, L, and S. Thus, while highly responsive to cathepsin B-mediated cleavage, EVGc was not necessarily more sensitive than VCit to cleavage by other cathepsins.

Next, we assessed the ADCs for stability in human and mouse plasma. No significant degradation was observed in any of the ADCs after incubation in human plasma at 37 °C for 28 days (Fig. 3a). In contrast, although EVGc ADC 3c showed almost no linker cleavage even after 14-day incubation in undiluted BALB/c mouse plasma, VCit and SVCit ADCs 3a, b lost > 95% and ~70% of the conjugated MMAF after the same period of time (Fig. 3b and Supplementary Fig. 9). This tendency is consistent with what we observed in the small-molecule probes (Fig. 1c). The PEG spacer within the linker scaffold most likely facilitates the enzyme access to the linker–payload moiety. Considering this point, our results indicate that the acidic EVGc linker provides not only reactivity to cathepsin-mediated cleavage but also high stability in plasma despite a high degree of payload exposure.
Cathepsin-cleavable ADCs did not observe significant killing potency in the HER2-positive cell lines, but no cytotoxicity (Fig. 3c, SKBR-3) and negative (MDA-MB-231) breast cancer cell lines cytotoxicity using HER2 positive- (KPL-4, JIMT-1, BT-474, and specific) (Supplementary Table 3). These results suggest that existence of a cleavage mechanism is a key to maximize cell killing potency of ADCs constructed using our branched linker platform.

**Validation of ADCs in vivo.** Finally, we evaluated the ADCs in vivo using mouse models. We first assessed PK profiles of VCit, SVCit, and EVCit ADCs 3a-c using BALB/c mice. Mice were treated with intravenous injection of each ADC or the parental N297A anti-HER2 mAb (3 mg kg⁻¹). Blood was collected periodically via the tail vein. Concentrations of total mAb (both conjugated and unconjugated) and intact ADC (conjugated only) in blood were determined by sandwich enzyme-linked immunosorbent assay (ELISA, Fig. 4a, b, Supplementary Fig. 12, and Supplementary Table 5). All ADCs showed similar clearance rates as that of the parental mAb (t½ of 14.9 days), indicating that installing glutamic acid or serine at the P3 position did not negatively impact the clearance profile (Fig. 4a). As expected, EVCit ADC 3c showed no significant loss of payload caused by cleavage in circulation (t½ of 12.0 days). In contrast, VCit and SVCit ADCs 3a, b quickly lost MMAF (t½ of 2.0 days and 2.4 days, respectively), demonstrating that the VCit and SVCit sequences installed on the mAb–branched linker system were unstable in circulation.

Encouraged by this finding, we tested VCit and EVCit ADCs 3a, c for in vivo treatment efficacy in JIMT-1 and KPL-4 xenograft mouse models (Fig. 4c−f and Supplementary Fig. 13, 14). It has been reported that athymic nude mice quickly clear exogenously introduced IgGs. Therefore, to prevent fast clearance of administered ADCs, tumor-bearing mice were preconditioned by intravenous administration of human IgGs (30 mg kg⁻¹) or vehicle control was injected intravenously into tumor-bearing mice. Tumor volume and body weight were measured every 3 days. No significant toxicity caused by administration of either ADC was observed over the course of study (Supplementary Fig. 14). A single dose of EVCit-based ADC 3c at 3 mg kg⁻¹ was curative and no tumor regrowth was visually observed in either model at the end of study (Fig. 4c−f). Furthermore, ADC 3c was potent even at a lower dose (1 mg kg⁻¹) in the JIMT-1 model and all five mice that received this treatment survived over the course of study (Fig. 4c, e). In contrast, VCit ADC 3a exhibited only partial inhibition of tumor growth despite the high in vitro cell
ADCs in both models). A single dose of VCit ADC (vehicle control (gray inversed triangle) was administered to mice when a mean tumor volume reached ~100 mm³ (indicated with a black arrow). Error bars represent s.e.m.

JIMT-1 (magenta, ▲) and KPL-4 (green square) ADCs (conjugated only, blue circle), EVCit ADC (3 mg kg⁻¹, green square; 1 mg kg⁻¹, magenta triangle), or vehicle control (gray inversed triangle) was administered to mice when a mean tumor volume reached ~100 mm³ (indicated with a black arrow). Error bars represent s.e.m.

Changes in the percentages of surviving mice over time in the JIMT-1 (▲) and KPL-4 (□) xenograft tumor models (female NCr nude mice, n = 3 for vehicle in the KPL-4 model; n = 5 for vehicle in the JIMT-1 model and ADCs in both models). A single dose of VCit ADC (3a, 3 mg kg⁻¹, blue circle), EVCit ADC (3c, 3 mg kg⁻¹, green square; 1 mg kg⁻¹, magenta triangle), or vehicle control (gray inversed triangle) was administered to mice when a mean tumor volume reached ~100 mm³ (indicated with a black arrow). Error bars represent s.e.m.

Antitumor activity of anti-HER2 ADCs (3a–c) in the JIMT-1 (c) and KPL-4 (d) xenograft tumor models (female NCr nude mice, n = 3 for vehicle in the KPL-4 model; n = 5 for vehicle in the JIMT-1 model and ADCs in both models). A single dose of VCit ADC (3a, 3 mg kg⁻¹, blue circle), EVCit ADC (3c, 3 mg kg⁻¹, green square; 1 mg kg⁻¹, magenta triangle), or vehicle control (gray inversed triangle) was administered to mice when a mean tumor volume reached ~100 mm³ (indicated with a black arrow). Error bars represent s.e.m.

**Discussion**

We have shown that VCit-containing acidic tripeptides with high polarity, in particular an EVCit tripeptide sequence, have significantly enhanced stability in mouse and human plasma while remaining susceptible to intracellular cathepsin-mediated proteolytic cleavage. Notably, the small molecule-based stability assay clearly demonstrates that a carboxylic acid side chain at the P₃ position provides much greater stabilization effect than does a 2-hydroxacetamide group, the modifier that reportedly conferred the VCit sequence with the highest stability in mouse plasma. These features make the EVCit sequence ideal cleavable ADC linker design for increasing the hydrophilicity under physiological conditions, maximizing the therapeutic potential, and minimizing the risk of systemic toxicity in mouse models caused by premature payload release. Indeed, a homogeneous anti-HER2 ADC constructed using an EVCit–PABC linker along with our branched linker technology exhibited higher hydrophilicity and by far greater long-term in vivo stability than did ADCs equipped with a conventional VCit or SVCit, an analogue of the hydroxy-functionalized tripeptide ADC linker that reportedly exhibited increased stability in mouse plasma. In addition, although treatment with a VCit-based anti-HER2 ADC showed poor therapeutic effect, treatment with the stable EVCit ADC led to complete remission in two xenograft mouse models of HER2-positive breast cancer. Both ADCs contain long PEG spacers within the linker scaffold fully exposing the cleavable peptide moieties. Thus, the EVCit linker system most likely provides greater resistance to Ces1c-mediated degradation in mouse models even with a high degree of exposure. Although the EVCit sequence is promising in its present form, future structure–activity relationship studies on the interaction between this peptide sequence and the mouse Ces1c will provide in-depth insights into the observed stabilizing effect. Such understanding may enable us to design further improved ADC linkers.
The use of EVCit or similar peptide linkers (e.g., EVA, DVCit, DVA) could serve as a simple but powerful solution to salvage many types of ADCs previously abandoned due to linker instability in mouse models. The high polarity of the EVCit linker could also help mitigate the aggregation and fast clearance issues associated with hydrophobic high-DAR ADCs. In addition, EVCit linker may be preferentially chosen over non-cleavable linkers in the future design of various ADCs. Non-cleavable linkers are designed to withstand proteolytic degradation in circulation. They have been successfully used for constructing potent ADCs along with MMAF, monomethyl auristatin D (MMAID), and emtansine (DM1). However, the use of non-cleavable linkers reportedly attenuates or nullifies ADC potency of some payload molecules including doxorubicin, monomethyl auristatin E (MMAE), a hydrophilic auristatin derivative, and a pyrrolobenzodiazepine dimer (PBD). Attenuation of ADC potency arises because non-cleavable linkers lack a defined cleavage mechanism; after intracellular protein degradation, final active metabolites retain the linker component. It has been demonstrated that this drawback can be circumvented in some cases by fine-tuning chemical structures of the linker and payload. However, the success of such efforts depends on the choice of the linker installation sites, conjugation modality, and payload. Indeed, as we have demonstrated in this and previous reports, our branched linker technology requires both adequate spacers and cleavable mechanisms within the linker scaffold for maximal ADC potency. These components are critical to alleviate the structural congestion and to ensure rapid payload release in an active form from each linker arm.

In summary, our findings support the conclusion that the EVCit linker technology is a significant contribution to efforts for developing next-generation ADCs and other drug conjugates. This technology will allow for flexible molecular design by minimizing challenges of linker instability and poor potency in preclinical studies. With further validation and optimization, this linker technology will benefit a diverse array of conjugation methods and linker systems developed to date, including conventional couplings at lysine or cysteine residues, site-specific conjugations at soluble accessible moieties (e.g., conjugation at the C-terminus of the antibody heavy chain) and branched ADC linkers for heterologous payload loading. Furthermore, the long half-life of the acidic tripeptide linkers, as seen for EVCit and DVCit probes, may be useful for constructing small molecule-based drug conjugates for targeted therapy.

**Methods**

**Compounds.** Synthesis details and characterization data of all compounds in this study are described in the Supplementary Method section and Supplementary Fig. 15–38.

**Cathespin B-mediated cleavage assay using pyrene probes.** Each test compound (10 mM in DMSO, 2 µL) was mixed with 97 µL of MES buffer (25 mM MES-Na, 1 mM DTT, pH 5.0) and 1 µL of 1-pyrenemethylamine (10 mM in DMSO, internal standard) and incubated at 37 °C for 10 min. Pre-warmed human liver cathespin B (20 ng/mL, EMD Millipore) in 100 µL MES buffer was added to the mixture, followed by incubation at 37 °C. Aliquots (10 µL) were collected at each time point (0, 1, 6, 24, 48, and 96 h) and 40 µL of cold acetonitrile containing 1% formic acid was added to precipitate proteins. Supernatant of each sample was obtained and analyzed for quantification by analytical HPLC as described above. All assays were performed in triplicate.

**MTGase-mediated antibody-linker conjugation.** See the Supplementary Information for the preparation of human mAbs with a N297A mutation. Anti-HER2 IgG1 with a N297A mutation (291 µg in PBS, 11.7 ng/mL, 3.43 mg antibody) was incubated with branched linker 1 (18.3 µL of 100 mM stock in water, 80 equiv) and Activa T (77 µL of 40% solution in PBS, Ajinomoto, purchased from Modernist Pantry) at room temperature for 16–20 h. The reaction was monitored using an Agilent G1946D LC/ESI-MS system equipped with a MabPac RP column (3 × 50 mm, 4 µm, Thermo Scientific). Elution conditions were as follows: mobile phase A = water (0.1% formic acid); mobile phase B = acetonitrile (0.1% formic acid); gradient over 6.8 min from A:B = 75:25 to 1:99; flow rate = 0.4 mL/min. The conjugated antibody was purified by SEC (Superdex 200 increase 10/300 GL, GE Healthcare, solvent: PBS, flow rate = 0.6 mL/min) to afford an antibody-linker conjugate (3.15 mg, 92% yield determined by bicinchoninic acid assay).

**Strain-promoted azide–alkyne cycloaddition for payload installation.** DBO–VCR–PARC–MMAF (12.6 µL of 4 mM stock solution in DMSO, 1.5 equivalent per azide group) was added to a solution of the mAb-linker conjugate in PBS (303.0 µL, 4.0 mg/mL), and the mixture was incubated at room temperature for 1 h. The reaction was monitored using an Agilent G1946D LC/ESI-MS system equipped with a MabPac RP column and the crude products were purified by SEC (Superdex 200 increase 10/300 GL, GE Healthcare, solvent: PBS, flow rate = 0.6 mL/min) to afford a payload conjugate (1 mg/mL).

**HIC analysis.** Each ADC (1 mg/mL, 10 µL in PBS) was analyzed using an Agilent 1100 HPLC system equipped with a MabPac HIC-Butyl column (4.6 × 100 mm, 5 µm, Thermo Scientific). Elution conditions were as follows: mobile phase A = 50 mM sodium phosphate containing ammonium sulfate (1.5 M) and 5% isopropanol (pH 7.4); mobile phase B = 50 mM sodium phosphate containing 20% isopropanol (pH 7.4); gradient over 30 min from A:B = 99:1 to 1:99; flow rate = 0.5 mL/min.

**Long-term stability test.** Each ADC (1 mg/mL, 100 µL in PBS) was incubated at 37 °C. Aliquots (10 µL) were taken at each time point (7, 14, and 28 days) and immediately stored at −80 °C until use. Samples were analyzed using an Agilent 1100 HPLC system equipped with a MabPac SEC-1 analytical column (4.0 × 300 mm, 5 µm, Thermo Scientific). Elution conditions were as follows: flow rate = 0.2 mL/min; solvent = PBS.

**Antibodies for ELISA.** All antibodies used in the ELISA assays in this study were purchased from commercial vendors as follows: Rabbit anti-MMAF antibody (LEV-PAF1) from Levena Biopharma, goat anti-human IgG Fab–HRP conjugate (109-035-097), goat anti-human IgG Fc antibody (109-005-098), and donkey anti-human IgG–HRP conjugate (709-035-149) from Jackson ImmunoResearch, and goat anti-rabbit IgG–HRP conjugate (32260) from Thermo Scientific.

**Plasma stability test using ADCs.** [1] Stability in mouse plasma. Each ADC (100 µg/mL, 1.2 µL in PBS) was added to undiluted BALB/c mouse plasma (118.8 µL) to a final concentration of 1 µg/mL. After incubation at 37 °C for varying time, aliquots (15 µL each) were taken and stored at −80 °C until use. Samples were analyzed by sandwich ELISA assay. A high-binding 96-well plate (Corning) was coated with rabbit anti-MMAF antibody (100 ng per well). After overnight coating at 4 °C, the plate was blocked with 100 µL of 2% BSA in PBS containing 0.05% Tween 20 (PBS-T) with agitation at room temperature for 1 h. Subsequently, the solution was removed and each ADC sample (100 µL in PBS-T containing 1% BSA) was added to each well, and the plate was incubated at room temperature for 2 h. After each well was washed three times with 100 µL of PBS-T, 100 µL of goat anti-human IgG Fab–HRP conjugate (1:10,000) was added. After being incubated at room temperature for 1 h, the plate was washed three times with 100 µL of PBS-T and 100 µL of 3.3, 3.5′-tetracemethylbenzidine (TMB) substrate (0.1 mg/mL) in phosphate–citrate buffer/30% H2O2 (1:0.0003 volume to volume, pH 5) was added. The reaction was developed for 10–30 min, 25 µL of 3N HCl was added to each well, and then the absorbance at 450 nm was recorded using a plate reader (Biotek Cytlation 5). Concentrations were calculated based on a standard curve. [2] Stability in human plasma. Assays were performed in the same manner using human HER2 (100 ng per well, ACROBiosystems) for plate coating, rabbit anti-MMAF antibody (100 ng) and goat anti-rabbit IgG–HRP conjugate (1:400) as secondaries and tertiary detection antibodies, respectively. All assays were performed in triplicate.

**Human cathespin cleavage assay for ADCs.** Each ADC (1 mg/mL) in 30 µL of MES buffer (10 mM MES-Na, 40 µM DTT, pH 5.0) was incubated at 37 °C for...
10 min. To the solution was added pre-warmed human cathepsin B (20 ng μL−1), cathepsin L (20 ng μL−1), cathepsin S (2 ng μL−1), all cathepsins from EMD Millipore. MDA-MB-231 cells were treated with 100 μL of blocking buffer (0.2% BSA in PBS) with agitation at room temperature for 2 h. After the blocking buffer was discarded, serially diluted samples in 100 μL PBS containing 0.1% BSA were added and the plate was incubated overnight at 4 °C with agitation. The buffer was discarded and the cells were washed three times with 100 μL of PBS containing 0.25% Tween 20. Cells were then incubated with 100 μL of donkey anti-human IgG-Fc–HRP conjugate (diluted 1:10000 in PBS containing 0.1% BSA) at room temperature for 1 h. The plate was washed three times with PBS containing 0.25% Tween 20, and 100 μL of TMB substrate (0.1 mg mL−1) in phosphate–citrate buffer/30% H2O2 (1:10000 volume to volume, pH 5) was added. After color was developed for 10–30 min, 25 μL of 3N HCl was added to each well and then the absorbance at 450 nm was recorded using a plate reader (Biotek Cytation 5). Concentrations were calculated based on a standard curve. EC50 values were then calculated using Graph Pad Prism 7 software. All assays were performed in triplicate.

Cell viability assay. Cells were seeded in a culture-treated 96-well plate (10,000 cells per well in 100 μL culture medium) and incubated at 37 °C under 5% CO2 for 24 h. All cell samples (50 µL) were added to each well and the plate was incubated at 37 °C for 72 h (KPL-4 and SKBR-3) or 96 h (JIMT-1, MDA-MB-231, and BT-474). After the old medium was replaced with 100 μL fresh medium, 20 μL of a mixture of WST-8 (1.5 mg mL−1, Cayman chemical) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS, 100 μM, Cayman Chemical) was added to each well, the plate was placed in the incubator at 37 °C for 2 h. After gently agitating the plate, the absorbance at 460 nm was recorded using a plate reader. EC50 values were calculated using Graph Pad Prism 7 software. All assays were performed in triplicate.

Animal experiments. All procedures were approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston and performed in accordance with the institutional guidelines for animal care and use.

In vivo pharmacokinetics study. Female BALB/c mice (6–8 weeks old, n = 3 per group. The Jackson Laboratory) were injected intravenously with each mAb or ADC sample (100 μL) at a dose of 3 mg kg−1. After injection, blood (5–10 μL) was drawn through the tail vein at each time point (15 min, 6 h, 1 day, 2 days, 4 days, 9 days, and 14 days) and processed with 5 mM EDTA in PBS. Plasma samples were stored at −80 °C until use. All mice were humanely euthanized after last blood collection. Plasma samples were analyzed by sandwich ELISA. For determination of the total antibody concentration (both conjugated and unconjugated), goat anti-human IgG Fc antibody (500 ng per well) and goat anti-human IgG Fab–HRP conjugate (1:50000) were used for plate coating and detection, respectively. For determination of ADC concentration (conjugated only), rabbit anti-MMAF antibody (100 ng per well) and goat anti-human IgG Fab–HRP conjugate (1:100000) were used in the same manner. Assays were performed in the same manner as described above (see the section of the plasma stability test for ADCs). Concentrations were calculated based on a standard curve. Half-life of the elimination phase t1/2 was estimated using method based on AUC0−t/2 area under the curve (AUC0−14 days, h×μg mL−1) of each sample was calculated using GraphPad Prism 7 software (Supplementary Table 5).
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Author contributions

Y.A. and C.M.Y. contributed equally to the work. K.T. conceived the project rationale and overall strategy. C.M.Y. performed the xenograft studies. C.M.Y. performed the cell killing assays. Y.A. and K.T. performed the pharmacokinetic and PK outcomes. C.M.Y. prepared the probes, linkers, and payload components and performed the manuscript. W.X., X.G., N.Z., and Z.A. produced mutated monoclonal antibodies. Y. A. and C.M.Y. prepared the probes, linkers, and payload components and performed the in vitro assays using the probes. Y.A. constructed and characterized all ADCs. Y.A. and C.M.Y. performed the cell killing assays. Y.A. and K.T. performed the pharmacokinetic studies. C.M.Y. performed the xenograft studies.

Additional information

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Competing interests: Y.A., C.M.Y., N.Z., Z.A., and K.T. are named inventors on a pending patent application relating to the work filed by The University of Texas Health Science Center at Houston. The remaining authors declare no competing interests.

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