

Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates

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Many antibody-drug conjugates (ADCs) are unstable *in vivo* because they are formed from maleimide-containing components conjugated to reactive thiols. These thiosuccinimide linkages undergo two competing reactions in plasma: elimination of the maleimide through a retro-Michael reaction, which results in loss of drug-linker from the ADC, and hydrolysis of the thiosuccinimide ring, which results in a derivative that is resistant to the elimination reaction. In an effort to create linker technologies with improved stability characteristics, we used diaminopropionic acid (DPR) to prepare a drug-linker incorporating a basic amino group adjacent to the maleimide, positioned to provide intramolecular catalysis of thiosuccinimide ring hydrolysis. This basic group induces the thiosuccinimide to undergo rapid hydrolysis at neutral pH and room temperature. Once hydrolyzed, the drug-linker is no longer subject to maleimide elimination reactions, preventing nonspecific deconjugation. *In vivo* studies demonstrate that the increased stability characteristics can lead to improved ADC antitumor activity and reduced neutropenia.

Interest in ADCs for cancer therapy has grown greatly over the past few years^{1–3}, partially as a result of the recent approvals of brentuximab vedotin (Adcetris) for relapsed Hodgkin lymphoma and anaplastic large cell lymphoma⁴, and ado-trastuzumab emtansine (Kadcyla) for patients with HER2⁺ metastatic breast cancer⁵. Both of these therapeutics share common structural features found in most ADCs and in many bioconjugates in general: a thiosuccinimide linkage, formed through the reaction of thiol and alkyl maleimide functionalities on the constituents within the conjugate. This type of chemistry for conjugate formation is widely used, as the reaction between maleimides and thiols is exceedingly rapid under physiological conditions, attains nearly quantitative conjugation without a large excess of either species and can be applied to a vast array of molecules of biological interest^{6,7}. However, it has become apparent that thiosuccinimide formation is reversible, with maleimide elimination occurring slowly under biologically relevant conditions (Fig. 1a)^{8–11}. Nearly all of the ADCs in clinical development^{1,12,13} and the two approved ADCs^{2,4,5} contain alkyl maleimides that can result in measurable drug loss during prolonged circulation^{8,9,14}. The pharmacological consequences of this maleimide elimination from ADCs

are unclear, but may include diminished antitumor activity due to reduced exposure to the antibody-conjugated form of the drug, and greater toxicity arising from nontargeted release of drug-linker. For these reasons, drug-linker elimination is an undesirable feature in ADCs, and much effort is being committed to finding conjugation methodologies that circumvent it^{9,15–17}.

It has long been recognized that the chemistry of maleimides and corresponding thiol adducts is greatly altered by hydrolysis of their five-membered rings¹⁸. Both thiol addition and elimination are attenuated when maleimides and derived thiosuccinimide adducts undergo ring-opening hydrolysis^{9,11,18} (Fig. 1a). This has led to an interest in defining attachment points within proteins that are conducive to spontaneous thiosuccinimide hydrolysis⁹, finding ways to induce hydrolysis after conjugates have formed¹⁹ and developing alternative strategies that circumvent the use of maleimide/thioether conjugation entirely^{8,15,16,20–22}. Although each of these approaches may have benefits, they are either not generally applicable or require abandoning maleimides entirely, thus forfeiting the many practical advantages of thiol-maleimide chemistry. Here, we describe a solution to the instability of alkyl maleimide-derived conjugates through the incorporation of a basic functional group adjacent to the maleimide. With this design, thiosuccinimide ring opening is greatly accelerated by intramolecular base catalysis under gentle conditions, and the resulting adduct is chemically stable (Fig. 1a).

To evaluate the feasibility of this approach, we compared the hydrolysis rates of the anti-CD30 antibody cAC10 conjugated to either *N*-aminoethyl maleimide (1, Fig. 1b; all chemical structures referenced by boldface numbers can be found in Fig. 1b) or to *N*-ethyl maleimide (2) as a control lacking an appended amino group (Fig. 1b). In both cases, conjugates were formed through the reaction of the maleimides with antibody cysteines, which were generated through complete reduction of the interchain disulfides^{23–25}. The conjugates thus formed contained one and three maleimide units per light and heavy chain, respectively. Upon incubation, increased masses of 18 Daltons on the light chain of cAC10-1 and 54 Daltons on the heavy chain were observed, representing the expected mass shifts for the addition of one equivalent of water per conjugation site (Supplementary Fig. 1a). Fitting these hydrolysis kinetics data to a single exponential equation led to estimated hydrolysis half-lives for cAC10-1 of 2.6 h and 2.0 h for the light and heavy chains, respectively (Fig. 2a). Under the same

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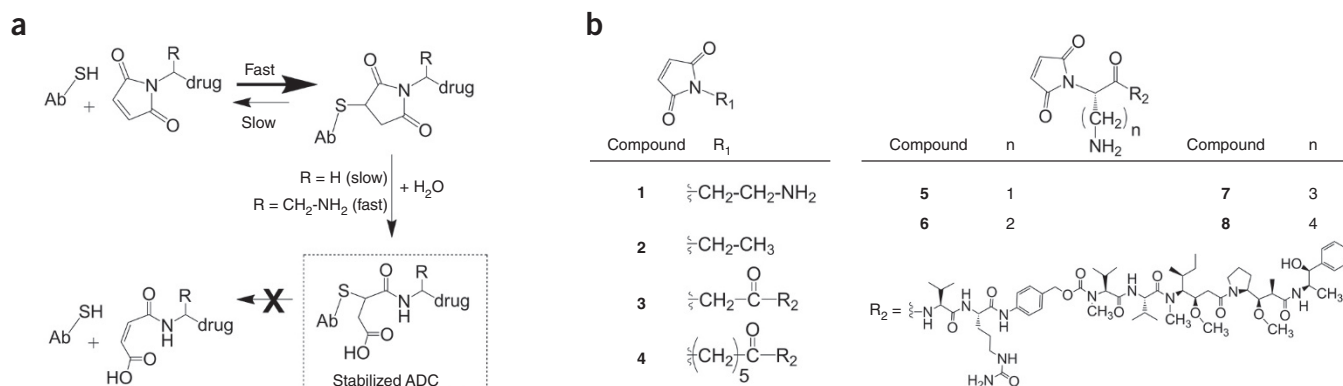


Figure 1 Structures and chemistry of thiol-reactive maleimides. **(a)** Reversible thiol-maleimide conjugation reaction. Succinimide hydrolysis greatly retards fragmentation of the thioether adduct. **(b)** Structures of substituted maleimides described in this study.

conditions, we did not observe any appreciable hydrolysis of cAC10-2 after 24 h (**Supplementary Fig. 1b**). These observations indicated that the presence of the amine on the maleimide N-substituent group of **1** provided a substantial acceleration of the hydrolysis of the thio-succinimide formed upon antibody conjugation. Hydrolysis did not appear to depend greatly on the site of conjugation, as similar rates were obtained on heavy and light chain cysteine residues.

Encouraged by these findings, we sought a maleimide unit possessing a primary amine with the same geometry as **1**, and also a second functionality to serve as a reactive handle for the attachment of a cleavable drug-linker, valine-citrulline-*p*-aminobenzyl-monomethyl auristatin E (val-cit-PAB-MMAE). To achieve this, we prepared a maleimide unit from maleic anhydride and the non-natural amino acid DPR, which has a free carboxylic acid that was coupled to val-cit-PAB-MMAE through a stable amide bond (**Fig. 1b**, compound **5**). As a control that lacks the primary amine, compound **3** was prepared from the commercially available *N*-hydroxysuccinimide ester of *N*-acetyl maleimide. These drug-linkers were conjugated to fully reduced cAC10 along with the previously reported²³ maleimido-caproyl drug-linker **4** to generate ADCs with eight drugs per antibody. The hydrolysis of the light chain of these ADCs was immediately monitored by mass spectrometry under the mild conditions used for conjugation (pH 7.4 and 22 °C), and the resulting data fit to a single exponential equation (**Fig. 2b**). As expected, cAC10-4 did not exhibit any measureable rate of hydrolysis after 24 h. cAC10-3 showed faster hydrolysis with a $t_{1/2}$ of ~40 h, most likely due to the electron-withdrawing influence of the α -amide. In contrast, thiosuccinimide

hydrolysis was profoundly accelerated in cAC10-5, which has both the amide and a pendant primary amine. The $t_{1/2}$ for this reaction was ~25 min. To evaluate the role of amine spacing in self-catalyzed thiosuccinimide hydrolysis, we prepared a series of drug-linkers analogous to **5** with diaminobutyric acid, ornithine and lysine (compounds **6**, **7** and **8**, respectively). When conjugated to cAC10, these drug-linkers yielded ADCs with progressively slower hydrolysis kinetics as the spacing from the thiosuccinimide to the primary amine increased (**Fig. 2b**). These results are consistent with an intramolecular catalysis mechanism in which the proximal amine promotes the attack of the succinimide carbonyl group by water. The geometry of the maleimido-DPR unit is appropriate for rapid and quantitative hydrolysis of thiosuccinimides under very mild conditions, making it ideally suited for applications in bioconjugation.

To confirm that hydrolyzed ADCs made with maleimido-DPR drug-linker **5** exhibit the expected chemical stability characteristics, we performed a series of assays both *in vitro* and *in vivo*. In these studies, conjugates of maleimido-caproyl drug-linker **4** were used as nonhydrolyzed ADC controls. Initially, stability was evaluated in pH 8 buffer at 37 °C containing 10 mM *N*-acetyl cysteine to serve as a scavenger of any maleimide eliminated from the ADC. Over a period of 2 weeks, the drug/antibody ratios were determined using reversed-phase high-performance liquid chromatography (HPLC; **Supplementary Fig. 2a**). Under these conditions, there was no detectable drug loss from cAC10-5, whereas cAC10-4 lost approximately half of its drug component (**Supplementary Fig. 2b**). Stability was next evaluated in rat plasma and *in vivo* in rats that were injected

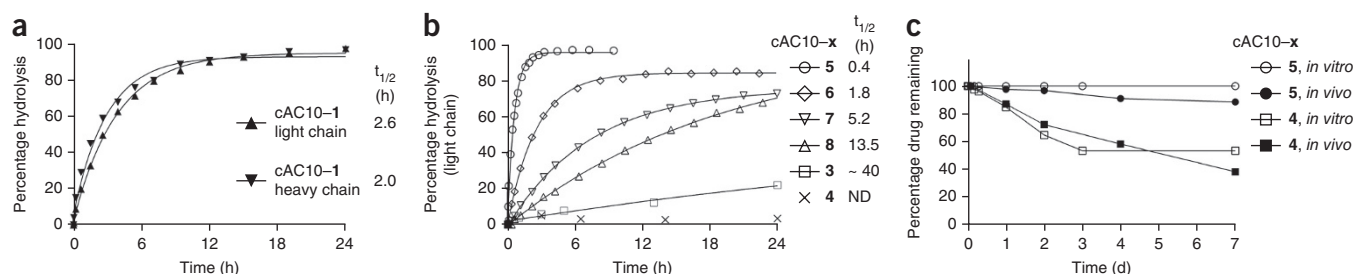
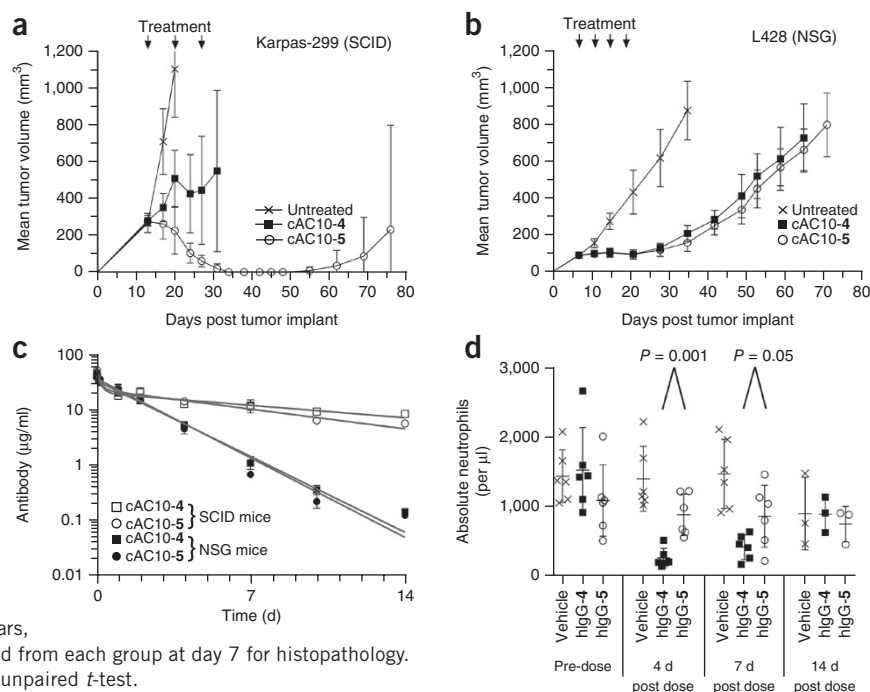


Figure 2 Hydrolysis rates and plasma stability of conjugates. **(a)** Hydrolysis at pH 7.4, 22 °C of cAC10 light chain and heavy chain conjugated with aminoethyl maleimide **1**. **(b)** Hydrolysis at pH 7.4, 22 °C of cAC10 light chain conjugated with drug linkers **3-8**, illustrating the increase in hydrolysis rate as the primary amine of the linker is positioned closer to the maleimide. Samples were withdrawn from the reaction vial at the indicated time points and the degree of thiosuccinimide hydrolysis determined by LC-MS. Hydrolysis data were fit to a single exponential equation to determine reaction half-lives; cAC10-4 could not be fit as there was no appreciable increase in hydrolysis observed over the course of the experiment. ND, not determined. **(c)** Stability in rat plasma; ADCs were either incubated *in vitro* or dosed intravenously at 10 mg/kg and sampled for 1 week. For *in vivo* stability, each point represents the mean of three animals from each group; error bars, mean $\pm$ s.d.

Figure 3 Antitumor activity, pharmacokinetics and neutropenia in rodent models. ADCs were prepared with drug linkers **4** (closed squares) and **5** (open circles) and dosed in groups of six animals. **(a,b)** The ALCL model Karpas-299 was dosed at 1 mg/kg every 7 d, 3 times, a total of three doses in SCID mice **(a)**; the Hodgkin lymphoma model L428 was dosed at 1 mg/kg every 4 d, 4 times, a total of four doses in NSG mice **(b)**. Plots are terminated when the first animal reaches a tumor volume of 1,000 mm³. **(c)** Pharmacokinetics of the ADCs in non-tumor-bearing SCID mice (used for Karpas-299 xenograft model, open symbols) and NSG mice (used for L428 xenograft model, closed symbols). Data for the SCID mice fit well to a two-compartment model, whereas a single compartment model was sufficient for fitting the NSG data. Points represent the mean value of each group; error bars, mean $\pm$ s.d. **(d)** Neutropenia observed in rats following a 10 mg/kg ADC dose using an hIgG1 isotype antibody. Six animals were dosed with hIgG-4 (filled squares), hIgG-5 (open circles) or vehicle (crosses) and sampled for hematology markers. Error bars, mean $\pm$ s.d. for each group; three animals were removed from each group at day 7 for histopathology. *P* values indicated in the figure represent results of an unpaired *t*-test.



with conjugate. Monitoring drugs per antibody of a heterogeneous ADC *in vivo* can be confounded by the fact that the higher-loaded species are subjected to accelerated clearance²⁶, leading to a decrease in the average drug loading through a mechanism that is unrelated to maleimide elimination. For this reason, ADCs used for the *in vivo* stability experiment were purified by hydrophobic interaction chromatography to yield a uniform product with four drugs per antibody²⁶. Loss of drug over time was determined by reversed-phase liquid chromatography–mass spectroscopy (LC-MS) of an ADC that was affinity purified from the rat plasma matrix. Consistent with the initial assay in buffer, cAC10-5 lost little drug in rat plasma, *in vitro* or *in vivo*, whereas cAC10-4 lost about half of its drug payload in less than 1 week in both settings (Fig. 2c). Notably, the loss of drug from cAC10-4 *in vitro* reached an asymptote after ~3 d, with little further loss beyond that point. Analysis of the MS data of cAC10-4 indicated that the remaining thiosuccinimidyl groups on the antibody had undergone hydrolysis after 3 d (Supplementary Fig. 2c), thus imparting stability to the remaining drug-linker.

The pharmacokinetics of cAC10-4 and cAC10-5 were determined in Sprague-Dawley rats. Using an enzyme-linked immunosorbent assay (ELISA)-based assay to measure total antibody, the two ADCs exhibited superimposable pharmacokinetic profiles (Supplementary Fig. 3a), indicating similar clearance of the cAC10 backbone. A second assay to quantify the MMAE conjugated to antibody in the plasma was also conducted (Supplementary Fig. 3b), revealing that cAC10-5 provided nearly 50% increased exposure over 7 d to antibody-conjugated MMAE compared to cAC10-4. This is consistent with greater retention of the drug-linker on the antibody for the self-stabilized ADC.

The impact of stabilized drug-linkers on antitumor activity was evaluated using cAC10-4 and cAC10-5 in two separate mouse xenograft models selected for high CD30 expression and response to MMAE ADCs. The two model cell lines were implanted into different strains of immunocompromised mice and exhibited substantially different growth rates. In the first model, Karpas-299 (human anaplastic large-cell lymphoma, ALCL) cells were implanted

subcutaneously in female severe combined immunodeficient (SCID) mice, and tumors were allowed to grow a volume of ~250 mm³ before ADC dosing at 1 mg/kg weekly for three doses (Fig. 3a). All six mice treated with cAC10-4 experienced tumor growth delay relative to the untreated group, but none of the responses were durable. In contrast, all of the animals dosed with cAC10-5 had complete responses, with five of six being cured of disease. The results in the Karpas-299 model indicated significantly greater (*P* = 0.0012 through day 31) *in vivo* antitumor activity for the self-stabilized ADC. In the second model, L428 (human Hodgkin lymphoma) cells were implanted subcutaneously in female nonobese diabetic SCID gamma (NSG) mice, and tumors were allowed to grow to a volume of ~100 mm³ before dosing at 1 mg/kg every 4 d for four doses (Fig. 3b). All animals in dose groups of both ADCs experienced significant delay in tumor outgrowth, but all the tumors eventually progressed with no significant differences between the two arms of the experiment.

The model dependence of the relative antitumor activities of these two ADCs prompted us to examine their pharmacokinetic profiles in different mouse strains. As observed in rats (Supplementary Fig. 3a), within a given mouse strain there were no appreciable differences in pharmacokinetics between the two ADCs (Fig. 3c). However, plasma clearance was profoundly dependent on mouse strain, with NSG mice eliminating the conjugates far more quickly than SCID mice. Similar differences between strains were observed when mice were dosed with unconjugated cAC10 (Supplementary Fig. 4), indicating that the differences in pharmacokinetics are unrelated to drug conjugation. As described previously^{8,27}, faster clearance of the antibody is expected to diminish the benefit of enhanced ADC stability. The findings reported here suggest that the relative performance of ADCs in xenograft studies may arise not only from intrinsic differences in the biology of the underlying human cell lines, but also from the disposition of the conjugates by the murine hosts used for model studies.

To gain insights into the impact that stability has on safety, we conducted an exploratory study in rats with ADCs prepared with drug linkers 4 and 5 using a nonbinding humanized IgG1 isotype control antibody. Before dosing (10 mg/kg) and at various time points after

dosing, blood samples were taken for hematology analysis. Neutropenia was observed in the hIgG-4 dose group, but was significantly diminished ($P = 0.001$ at day 4; $P = 0.05$ at day 7) in the animals receiving hIgG-5 (Fig. 3d). Histopathology evaluation revealed correspondingly less bone marrow damage in animals that received hIgG-5 relative to animals that received hIgG-4 (Supplementary Fig. 5). Enhancing ADC stability through self-stabilizing maleimides thus appears to improve tolerability through reduced off-target bone marrow toxicity.

The self-stabilizing maleimido-DPR linker represents a functionality engineered to undergo rapid ring hydrolysis following conjugation to an antibody, with similar kinetics at all of the native IgG1 conjugation sites. The facile nature of the hydrolysis is convenient for ADC manufacture without requiring significant modifications to current methods. The maleimido-DPR unit can be coupled to new or existing cleavable linker-drug combinations, resulting in ADCs that are stable against maleimide elimination in plasma. The pharmacological consequences of this stabilization include model-dependent improvements in preclinical antitumor activity and reduced bone marrow toxicity and associated neutropenia in rats. These self-stabilizing drug-linkers therefore represent a means for the preparation of stable ADCs and may have applications with a diverse array of bioconjugates.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.P.L. and P.D.S. conceived the project rationale and wrote the manuscript; R.P.L. performed hydrolysis rate studies with aminoethyl maleimide; J.R.S. performed hydrolysis rate and stability studies with ADCs; T.D.B. and S.O.D. synthesized the drug-linkers; J.H.H. performed radiolabeling and quantitation for pharmacokinetic studies; M.E.A. performed the xenograft studies and rat pharmacokinetic studies; C.L.B. and S.M.D. performed the rat toxicology studies; C.I.L. prepared ADCs for all *in vivo* studies; F.L. discovered differential antibody pharmacokinetics in SCID and NSG mice.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

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ONLINE METHODS

Materials. *N*-ethyl maleimide and *N*-aminoethyl maleimide (trifluoroacetic acid, TFA, salt) were purchased from Sigma-Aldrich. Chimeric anti-CD30 antibody AC10 was generated as described previously²⁸. Authenticated and mycoplasma-free CD30⁺ cell lines L428 (ACC 197) and Karpas-299 (ACC 31) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Maleimidocaproyl-val-cit-PAB-MMAE (**4**) was prepared as described previously²³.

SCID mice were purchased from Harlan Laboratories (CB-17 SCID mice, product code #182). NSG mice were purchased from Jackson Laboratories (stock #005557). CD IGS Sprague-Dawley rats were purchased from Charles River Laboratories for safety studies, and s.d. Sprague-Dawley rats were purchased from Harlan Laboratories for pharmacokinetic and *in vivo* stability studies. For *in vitro* stability studies, female Sprague-Dawley rat plasma (K2 EDTA anticoagulant) was purchased from Bioreclamation. All animal handling and experimentation were performed under Seattle Genetics Institutional Animal Care and Use Committee guidelines. Seattle Genetics is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Synthesis of drug-linkers. *General.* Unless otherwise noted, materials used for synthesis were obtained from commercial sources in the highest grade purity available and used without further purifications. NMR spectral data were collected on a Varian Mercury 400 MHz spectrometer. Chemical shifts (δ) were reported in p.p.m. and referenced off the deuterium solvent signal. Mass spectra data were obtained on a XEVO TOF MS interfaced to a Waters 2795 HPLC equipped with a C12 Phenomenex Synergi 2.0 $\times$ 150 mm, 4 μ m, 80 Å reversed-phase column. The eluent consisted of a linear gradient of acetonitrile from 5% to 95% in 0.1% aqueous formic acid over 10 min, followed by isocratic 95% acetonitrile for 5 min at flow rate 0.4 ml/min. Preparative HPLC was carried out on a Varian ProStar 210 solvent delivery system configured with a Varian ProStar 330 PDA detector. Products were purified using a C12 Phenomenex Synergi 21.2 $\times$ 250.0 mm, 4 μ m, 80 Å reversed-phase column eluting with 0.05% TFA in a water-acetonitrile gradient, with a flow rate of 15 ml/min, monitoring at 220 nm.

Fluorenylmethyloxycarbonyl chloride (Fmoc)-val-cit-PAB-OCO-*p*NP (**9**). In a 250 ml round bottom flask, Fmoc-val-cit-PABOH²⁹ (5.0 g, 8.31 mmol) and bis-*p*-nitrophenyl carbonate (5.04 g, 16.5 mmol) were dissolved in dimethylformamide (DMF) (45 ml). *N,N*-diisopropylethylamine (DIPEA) (2.89 ml, 16.6 mmol) was added, and the solution was stirred for 2 h under N₂ at room temperature. The reaction mixture was then partially concentrated under reduced pressure and diluted with 130 ml of ethyl acetate/diethyl ether, 1:1, v/v. The solution was cooled to 0 °C and the resulting precipitate was collected by vacuum filtration, yielding 4.5 g of product as an off-white solid. (70%) Reversed-phase HPLC analysis: 98% at 13.96 min. ¹H NMR in DMSO: δ (p.p.m.) 0.85 (dd, 6H), 1.23–1.45 (m, 2H), 1.55–1.67 (m, 2H), 1.94–2.03 (m, 1H), 2.94–3.02 (m, 2H), 3.93 (t, 1H), 4.21–4.33 (m, 3H), 4.41–4.45 (m, 1H), 5.24 (2, 2H), 6.01 (s, 1H), 7.30–7.39 (m, 3H), 7.40–7.48 (m, 5H), 7.56–7.66 (m, 4H), 7.73 (t, 2H), 7.87 (d, 2H), 8.17 (m, 1H), 8.30 (d, 2H), 10.16 (s, 1H). LC-MS (electrospray ionization, ESI) calcd. for (MH)⁺ 767.30; found, *m/z* 767.37.

H-val-cit-PAB-MMAE (**10**). MMAE (5.34g, 6.94 mmol), Fmoc-val-cit-PAB-OCO-*p*NP (**9**, 5.0 g, 6.94 mmol) and HOBt (190 mg, 1.4 mmol) were added to a 250 ml round bottom flask purged with N₂ and dissolved in 15 ml of dimethylacetamide (DMA). DIPEA (2.44 ml, 14 mmol) was then added, and the solution was stirred overnight at room temperature under inert atmosphere. The Fmoc-protected intermediate was isolated by preparative HPLC affording 3.2 g as a white solid. LCMS (ESI) calcd for (MH)⁺ 1345.86; found, *m/z* 1345.89. This material was dissolved in 14 ml of DMF/diethylamine, 1:1, v/v, and stirred for 3 h at room temperature. The reaction mixture was then concentrated to a thick oil under reduced pressure, and the product was precipitated from ether (100 ml) affording 2.0 g (68.5%) of product as an off-white solid. Reversed-phase HPLC analysis: 95% at 10.46 min. ¹H NMR in DMSO: δ (p.p.m.) 0.73–0.93 (m, 24 H), 0.97–1.05 (m, 8 H), 1.31–1.61 (m), 1.70–1.79 (m, 4 H), 1.96–2.1 (m, 4 H), 2.22–2.28 (m, 1 H), 2.43–2.39 (m, 1 H), 2.81–3.07 (m, 8 H), 3.12–3.14 (m), 3.47–3.60 (m), 3.90–4.04 (m, 2 H), 4.23–4.29 (t, 1 H), 4.38–4.49 (m, 2 H), 4.63 (br m, 0.5 H), 4.73 (br m, 0.5 H), 4.96–5.08 (m, 2 H),

5.40 (dd, 1 H), 5.48 (br s, 2 H), 6.06 (br s, 1 H), 7.15–7.19 (m, 1 H), 7.24–7.30 (m, 6 H), 7.57–7.62 (m, 3 H), 7.90–7.95 (m, 1 H), 8.10 (br d, 0.5 H), 8.34 (br t, 0.5 H), 8.55 (br d, 1 H), 10.20 (br m, 1 H). LCMS (ESI) calcd. for (MH)⁺ 1123.71; found, *m/z* 1123.81.

General method for the synthesis of *N*_α-maleimido-*N*-Boc-diamino acids (**11a–d**). *N*_α-Boc diamino acid (1 mmol) and maleic anhydride (98 mg, 1 mmol) were dissolved in acetic acid (1 ml) in a 50 ml round bottom flask, and the solution was stirred at room temperature for 3 h. Solution was then concentrated to an oil under reduced pressure. The maleic acid intermediate was precipitated by adding ~10 ml CH₂Cl₂/hexane, 1:1, v/v, and the precipitate was collected by vacuum filtration. This material was then suspended in toluene (9 ml), followed by the addition of DMA (0.3 ml) and triethylamine (0.42 ml, 3 mmol). The mixture was stirred at 40–60 °C under N₂ until all material was in solution. The flask was then equipped with a condenser and the solution heated to 120 °C and refluxed for 4 h over molecular sieves. The reaction mixture was filtered through a sintered glass funnel and concentrated to near dryness under reduced pressure. The residue was dissolved in ethyl acetate (10 ml), transferred to a separatory funnel and washed with 10% citric acid in water (2 $\times$ 10 ml) and brine (2 $\times$ 10 ml). The organic layer was dried over magnesium sulfate, concentrated under reduced pressure and dried under high vacuum overnight yielding product as a white powder.

Maleimido-Dpr(Boc)-OH (**11a**). Obtained from *N*_β-Boc-L-2,3-diaminopropionic acid in 72% yield. ¹H NMR (DMSO): δ 1.29 (s, 9 H), 3.41 (m, 1 H), 3.52 (m, 1 H), 4.57 (dd, 1 H), 6.97 (t, 1 H), 7.07 (s, 2 H). LCMS (ESI) calcd. for (M+Na)⁺ 307.09; found, *m/z* 307.17.

Maleimido-Dab(Boc)-OH (**11b**). Obtained from *N*_β-Boc-L-2,4-diaminobutyric acid in 71% yield. ¹H NMR (DMSO): δ 1.35 (s, 9 H), 2.15 (m, 2 H), 2.91 (m, 1 H), 4.58 (dd, 1 H), 6.818 (t, 1 H), 7.13 (s, 2 H). LCMS (ESI) calcd. for (M+Na)⁺ 321.11; found, *m/z* 321.20.

Maleimido-Orn(Boc)-OH (**11c**). Obtained from *N*_δ-Boc-L-ornithine in 57% yield. ¹H NMR (DMSO) δ 1.25 (m, 2 H), 1.36 (s, 9 H), 2.87 (m, 2 H), 4.56 (dd, 1 H), 6.80 (t, 1 H), 7.10 (s, 2 H). LCMS (ESI) calcd. for (M+Na)⁺ 335.12; found, *m/z* 335.21.

Maleimido-Lys(Boc)-OH (**11d**). Obtained from *N*_ε-Boc-L-lysine in 80% yield. ¹H NMR (DMSO): δ 1.42 (s, 9 H), 1.78 (m, 2 H), 1.93 (m, 2 H), 3.14 (t, 2 H), 4.48 (q, 1 H), 7.12 (s, 2 H). LCMS (ESI) calcd. for (M+Na)⁺ 349.14; found, *m/z* 349.12.

General method for the synthesis of *N*_α-maleimido-diamino acid-val-cit-PAB-MMAE (**5–8**). In a 50 ml round bottom flask *N*-maleimido-*N*-Boc-diamino acid (**12**, 0.089 mmol), H-val-cit-PAB-MMAE (**10**, 100 mg, 0.089 mmol) and HATU (41 mg, 0.107 mmol) were dissolved in DMF (2 ml). DIPEA (34 μ l) was added and the solution was stirred for 1 h at room temperature under inert atmosphere. The Boc-protected linker intermediate was isolated by preparative HPLC and lyophilized affording material as a white powder. Material then was dissolved in 10% TFA in CH₂Cl₂ (2 ml) and stirred for 1 h at room temperature. The reaction mixture was concentrated to dryness, reconstituted in DMSO (1 ml) and purified by preparative HPLC generating final drug-linker compounds as a white powder.

Maleimido-DPR-val-cit-PAB-MMAE (**5**). Obtained from **12a** in 48% yield. Reversed-phase HPLC analysis: 99% at 10.46 min. ¹H NMR (DMSO-*d*₆): δ 0.71–0.89 (m, 24 H), 0.95–1.05 (m, 8 H), 1.31–1.59 (m), 1.62–1.80 (m, 4 H), 1.89–2.14 (m, 4 H), 2.23–2.29 (dd, 1 H), 2.39–2.43 (d, 1 H), 2.84–2.88 (d, 3 H), 2.96–3.06 (m), 3.11 (s), 3.12–3.14 (dd), 3.30–3.32 (d), 3.46–3.60 (m), 3.76–3.79 (d), 3.94–4.04 (m), 4.14–4.18 (t), 4.23–4.29 (t), 4.35–4.48 (m), 4.63 (br m, 0.5 H), 4.73 (br m, 0.5 H), 4.78–4.82 (dd, 2 H), 4.95–5.08 (m, 2 H), 6.02 (br s, 1 H), 7.10 (s, 2 H), 7.15–7.19 (m, 1 H), 7.24–7.32 (m, 6 H), 7.55–7.61 (m, 3 H), 7.88 (m), 8.05 (br m, 0.5 H), 8.23–8.30 (m), 8.46 (d, 1 H), 10.02 (br s, 1 H). LCMS (ESI) calcd. for C₆₅H₁₀₀N₁₂O₁₅ (MH)⁺ 1289.75; found, *m/z* 1,289.87.

Maleimido-Dab-val-cit-PAB-MMAE (**6**). Obtained from **12b** in 9% yield. Reversed-phase HPLC analysis: 97% at 10.41 min. ¹H NMR (DMSO-*d*₆): δ (p.p.m.) 0.75–0.89 (m, 24 H), 0.97–1.05 (m, 8 H), 1.24–1.63 (m), 1.63–1.87 (m), 1.89–2.15 (m), 2.23–2.29 (dd, 1 H), 2.39–2.43 (d, 1 H), 2.76–2.80 (m, 2 H), 2.84–2.88 (d, 3 H), 2.93–3.06 (m), 3.11 (s), 3.12–3.14 (dd), 3.30–3.32 (d), 3.46–3.60 (m), 3.76–3.80 (dd), 3.94–4.04 (m), 4.14–4.19 (t), 4.23–4.29 (t), 4.33–4.43 (m), 4.44–4.53 (m), 4.63 (br m), 4.74 (br m), 4.95–5.11 (m, 2 H), 6.02 (br s, 1 H), 7.08 (s, 2 H), 7.14–7.19 (m, 1 H), 7.24–7.32 (m, 6 H), 7.56–7.58

(m, 2 H), 7.63 (br m, 3 H), 7.88–7.90 (d, 0.5 H), 8.01–8.03 (d, 1 H), 8.19–8.21 (d, 1 H), 8.27 (br m), 10.00 (br s, 1 H). LCMS (ESI) calcd. for $C_{66}H_{102}N_{12}O_{15}$ (MH)⁺ 1303.77; found, m/z 1,303.85.

Maleimido-Orn-val-cit-PAB-MMAE (7). Obtained from **12c** in 11% yield. Reversed-phase HPLC analysis: 98% at 10.42 min. ¹H NMR (DMSO-*d*₆): δ (p.p.m.) 0.72–0.87 (m, 24 H), 0.95–1.03 (m, 8 H), 1.24–1.62 (m), 1.64–1.84 (m), 1.89–2.15 (m), 2.21–2.27 (dd, 1 H), 2.37–2.41 (d, 1 H), 2.70–2.79 (m, 2 H), 2.83–2.86 (d, 3 H), 2.91–3.04 (m), 3.10 (s), 3.16–3.23 (dd), 3.28–3.31 (d), 3.41–3.60 (m), 3.75–3.77 (dd), 3.94–4.04 (m), 4.14–4.18 (t), 4.21–4.27 (t), 4.32–4.42 (m), 4.46–4.52 (m), 4.62 (br m), 4.73 (br m), 4.93–5.10 (m), 6.01 (br s, 1 H), 7.06 (s, 2 H), 7.15–7.17 (m, 1 H), 7.22–7.30 (m, 6 H), 7.54–7.56 (m, 2 H), 7.72 (br m, 3 H), 7.86–7.88 (d, 0.5 H), 7.99–8.01 (d, 1 H), 8.03 (br m, 0.5 H), 8.17–8.19 (d, 1 H), 8.27 (br m, 0.5 H), 9.99 (br s, 1 H). LCMS (ESI) calcd. for $C_{67}H_{104}N_{12}O_{15}$ (MH)⁺ 1317.78; found, m/z 1,318.05.

Maleimido-Lys-val-cit-PAB-MMAE (8). Obtained from **12d** in 39% yield. Reversed-phase HPLC analysis: 98% at 10.45 min. ¹H NMR (DMSO-*d*₆): δ 0.73–0.90 (m, 24 H), 0.95–1.03 (m, 8 H), 1.14–1.22 (m), 1.30–1.59 (m), 1.62–1.84 (m), 2.91–2.13 (m), 2.23–2.29 (dd, 1 H), 2.37–2.41 (d, 1 H), 2.70–2.75 (m, 2 H), 2.83–2.87 (d, 3 H), 2.91–3.04 (m), 3.10 (s), 3.16–3.23 (dd), 3.28–3.31 (d), 3.46 (br m), 3.50–3.60 (m), 3.75–3.80 (dd), 3.90–4.00 (m), 4.13–4.17 (t), 4.21–4.27 (t), 4.32–4.35 (m), 4.45–4.51 (m), 4.61 (br m, 1 H), 4.71 (br m, 1 H), 4.94–5.11 (m, 2 H), 5.99 (br s, 1 H), 7.04 (s, 2 H), 7.14–7.18 (m, 1 H), 7.22–7.30 (m, 6 H), 7.54–7.62 (m, 5 H), 7.86–7.88 (d, 0.5 H), 7.96–7.99 (d, 1 H), 8.02 (br m, 0.5 H), 8.16–8.17 (d, 1 H), 8.25 (br m, 0.5 H), 9.99 (br s, 1 H). LCMS (ESI) calcd. for $C_{68}H_{106}N_{12}O_{15}$ (MH)⁺ 1331.79; found, m/z 1,332.02.

Maleimidoacetyl-val-cit-PAB-MMAE (3). In a 10 ml round bottom flask *N*-(α -maleimidoacetoxy) succinimide ester (11.2 mg, 0.045 mmol) and Val-Cit-PAB-MMAE (**10**, 50 mg, 0.045 mmol) were dissolved in DMA (1 ml). DIPEA (15.5 μ l, 0.089 mmol) was added, and the solution was stirred for 1 h at room temperature. The reaction mixture was diluted with DMSO (1 ml) and the product was isolated by preparative HPLC and lyophilized affording 40 mg (71%) of material as a white powder. Reversed-phase HPLC analysis: 99% at 12.24 min. ¹H NMR (DMSO-*d*₆): δ (p.p.m.) 0.73–0.89 (m, 24 H), 0.97–1.05 (m, 8 H), 1.23–1.61 (m), 1.64–1.89 (m), 1.95–2.2 (m, 3 H), 2.06–2.14 (m, 2 H), 2.22–2.29 (dd, 1 H), 2.39–2.43 (d, 1 H), 2.85–2.88 (d), 2.93–3.07 (m), 3.11 (s), 3.17–3.24 (dd), 3.30–3.32 (d), 3.48 (br m), 3.55–3.60 (m), 3.76–3.79 (dd), 3.91–4.05 (m), 4.13 (s, 2 H), 4.23–4.29 (m), 4.61 (br m), 4.73 (br m), 4.95–5.08 (m), 5.99 (br s, 1 H), 7.08 (s, 2 H), 7.13–7.20 (m, 1 H), 7.24–7.31 (m, 6 H), 7.56–7.62 (m, 3 H), 7.87–7.89 (d, 0.5 H), 8.05 (br m, 0.5 H), 8.23–8.29 (m, 3 H), 10.00 (br s, 1 H). LCMS (ESI) calcd. for $C_{64}H_{97}N_{11}O_{15}$ (MH)⁺ 1,260.72; found, m/z 1,260.70.

Preparation of conjugates. ADCs were prepared by reduction of antibody interchain disulfides followed by addition of a 25% excess of maleimide, as described previously^{25,30}. The drug/antibody ratio of ADCs generated by this method is dictated by the extent of disulfide reduction. For conjugates used to determine hydrolysis rates and *in vitro* stability, full reduction to eight thiols per antibody was carried out with excess Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), which was removed by passage over a PD-10 size exclusion column (GE Healthcare) before addition of maleimide, to produce conjugates with eight drugs (or model maleimides). Conjugates used for *in vivo* activity and toxicology studies were partially reduced by addition of 2.2 equivalents of TCEP, then conjugated to achieve an average of four drugs per antibody. All ADCs were purified by previously described methods²⁵. The ADCs used for *in vivo* stability were further purified by hydrophobic interaction chromatography to yield ADCs uniformly loaded with four drugs per antibody. This additional step was conducted to eliminate the role of variable pharmacokinetics across differentially loaded ADC species, which would complicate the interpretation of the *in vivo* drug loading data²⁶.

Monitoring hydrolysis by ESI-MS. For hydrolysis rate studies, the pH of the reaction solution was confirmed following maleimide addition and the conjugates were immediately transferred to HPLC vials and placed in a temperature-controlled autosampler. LC-MS injections were then made directly from these vials at various time points. The mass data were collected with a Waters QTOF API-US and deconvoluted with the MaxEnt algorithm of MassLynx software. The peak intensity values returned by this algorithm for hydrolyzed

and nonhydrolyzed peaks were used to determine the extent of the hydrolysis reaction at each time point. These values were then plotted against time and fitted with a single exponential equation using Prism 5.0 (GraphPad Software, San Diego, CA) to determine reaction rate constants, that were then converted to reaction half-times by the standard transformation $t_{1/2} = \ln(2)/k$.

Stability studies. *Buffer.* ADCs with eight drugs per antibody were spiked into pH 8 buffer containing 150 mM Tris, 1 mM EDTA, 10 mM *N*-acetyl cysteine to a concentration of 2.5 mg/ml, sterile filtered using a 0.2 μ m filter and incubated at 37 °C. At each time point, a 250 μ g aliquot was removed and frozen at –80 °C. The incubation mixture was assayed for thiol concentration using Ellman's reagent at each time point, and additional *N*-acetyl cysteine added to maintain a 10 mM concentration of reduced thiol. After the final time point, all samples were processed by full reduction with 10 mM DTT at 37 °C for 10 min before analysis by LC-MS. ADC separation was achieved with an Aeris XB-C8 column (100 $\times$ 2.1 mm, 3.6 μ m, Phenomenex) using a Waters 2690 HPLC coupled to a Waters 2487 UV detector and Q-TOF API-US mass spectrometer. The light and heavy chains of the ADC were resolved over a 24-min gradient, allowing for integration of each peak (detected by UV) to obtain a drug/antibody ratio as previously described²¹. The deconvoluted masses of each peak were used to confirm the assignments and the extent of thiosuccinimide hydrolysis.

Rat plasma. ADCs with eight drugs per antibody were spiked into citrated rat plasma (Bioreclamation) to a concentration of 250 μ g/ml and sterile filtered using a 0.2 μ m filter. Aliquots of 50 μ l were prepared in sterile tubes and incubated at 37 °C. At the indicated time points, individual tubes were removed from the incubator and frozen at –80 °C. After the final time point, all samples were processed by adding 25 μ l IgSelect affinity resin (GE Healthcare) to each tube and rotating 2 h at 4 °C. The slurry was transferred to a 96-well filter plate (Seahorse Bioscience), washed with PBS pH 7.4 (Gibco) and eluted with IgG Elution Buffer (Pierce) into a 96-well collection plate containing 10 μ l 1M Tris, pH 8. Samples were reduced with 10 mM DTT at 37 °C for 10 min before analysis by LC-MS. ADCs were then analyzed as described above. For *in vivo* stability studies, ADCs were dosed at 10 mg/kg in 7- to 8-week-old female s.d. Sprague-Dawley rats (15 per dose group, randomly assigned), and at each time point a terminal bleed was taken from three animals of each group and processed to plasma. The plasma samples were transferred to 15-ml centrifuge tubes and diluted 1:1 v/v with PBS pH 7.4 (Gibco). A 125- μ l aliquot of IgSelect affinity resin (GE Healthcare) was added to each sample and rotated overnight at 4 °C. Samples were transferred to a 96-well filter plate (Seahorse Bioscience), processed and analyzed as described above.

Xenograft studies. As a model of ALCL, Karpas-299 cells were implanted subcutaneously in female SCID mice (ages 4–6 weeks). Once tumors had grown to ~250 mm³, ADCs were dosed intraperitoneally (i.p.) at 1 mg/kg on a q7dx3 schedule. As a model of Hodgkin lymphoma, L428 cells were implanted subcutaneously in female NSG mice (ages 4–6 weeks). Once tumors had grown to ~100 mm³, ADCs were dosed i.p. at 1 mg/kg on a q4dx4 schedule. At these doses, cAC10-4 was not expected to be curative based on prior experience with these models, thus allowing for observable improvements in the activity of cAC10-5. In both studies, six mice were dosed with each ADC or left untreated; animals were assigned to dose groups to minimize differences in initial tumor volumes between groups. Investigators were not blinded to the dosing groups.

Mouse pharmacokinetics. Pharmacokinetics experiments in mice were done using cAC10 antibody radiolabeled with *N*-succinimidyl propionate, (propionate-2,3-³H)- (Moravsek Biochemicals, Cat. No.: MT 919).

Radiolabeled cAC10, cAC10-4 and cAC10-5 were injected at 3 mg/kg in female SCID and NSG mice (4–6 weeks old at dosing, three mice per dose group, randomly assigned) via the tail vein. Blood was drawn into K₂ EDTA tubes via the saphenous vein at various time points and processed to plasma. Plasma samples were added to Ecosint-A liquid scintillation cocktail (National Diagnostics) and the total radioactivity was measured by liquid scintillation counting. The resulting disintegrations per minute values were converted to μ Ci, and the specific activity of the radiolabeled test articles was used to calculate the concentration of antibody at each time point.

Safety studies. ADCs or vehicle were dosed intravenously in 12- to 14-week-old female CD 1GS rats (Charles River Laboratories) at 10 mg/kg (six rats per dose group, randomly assigned). This dose level was selected on the basis of prior experience with ADCs using drug-linker **4**. Prior to dosing and at post-dose days 4, 7 and 14, serum samples were taken for hematology analysis (Idexx). At day 7, 3 animals from each group were euthanized to collect tissues for histopathology analysis.

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