Supporting Information

Antibody-drug Conjugates Derived From Cytotoxic seco-CBI-Dimer Payloads are Highly Efficacious in Xenograft Models and Form Protein Adducts In Vivo

Dian Su,*† Jinhua Chen,‡ Ely Cosino,† Josefa dela Cruz-Chuh,† Helen Davis,† Geoffrey Del Rosario,† Isabel Figueroa,† Leanne Goon,† Jintang He,† Amrita V. Kamath,† Surinder Kaur,† Katherine R. Kozak,† Jeffrey Lau,† Donna Lee,† M. Violet Lee,† Douglas Leipold,† Luna Liu,† Peter Liu,† Guo-Liang Lu,§ Chris Nelson,† Carl Ng,† Thomas H. Pillow,† Paul Polakis,† Andrew G. Polson,† Rebecca K. Rowntree,† Ola Saad,† Brian Safina,† Nicola Stagg,† Moana Tercel,§ Richard Vandlen,† Breanna S. Vollmar,† John Wai,‡ Tao Wang,‡ BinQing Wei,† Keyang Xu,† Juanjuan Xue,‡ Zijin Xu,‡ Gang Yan,‡ Hui Yao,‡ Shang-Fan Yu,† Donglu Zhang,† Fiona Zhong,† Peter S. Dragovich*†

†Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA
‡ WuXi AppTec Co., Ltd., 288 Fute Zhong Road, Waigaoqiao Free Trade Zone, Shanghai 200131, China
§Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

*E-mail: su.dian@gene.com.
†E-mail: dragovich.peter@gene.com.
# Table of Contents

Title page  S1
Supplementary Figures S1 through S16  S3
Supplementary Tables 1 through 3  S23
MS ionization differences and data interpretation caveats  S26

## Experimental procedures

*In vivo* efficacy and PK studies in mice  S28
*In vivo* stability studies in rats and monkeys  S30
LC-MS F(ab’)2 assay for structural elucidation of TDC biotransformation  S31
LC-MS/MS peptide mapping method for protein identification  S32
Total antibody (Tab) bioanalysis  S33
Cell potency assay of plasma samples with and without adducts  S34
*In vitro* adduct formation in whole blood  S36

## Compound syntheses

Compound 8  S37
Compound 9  S40
Linker-drug 10  S43
Linker-drug 11  S47
Linker-drug 12  S51

Preparation of THIOMAB™ antibody-drug conjugates (TDCs)  S56
Figure S1. Body weight changes associated with TDCs aCD22-LC-K149C-10 and aLy6E-LC-K149C-10 in the WSU-DLCL2 xenograft model.

Legend: red traces: conjugate aCD22-LC-K149C-10, black trace (dotted lines, open symbols): conjugate aLy6E-LC-K149C-10, black trace (solid line, solid symbols): vehicle control. Numbers next to traces indicate doses of each conjugate (in mg/kg) that were administered once IV at the day 0 time point.
Figure S2. *In vivo* stability results obtained for conjugate aLy6E-LC-K149C-10.
Legend. (a) *In vivo* stability results obtained for conjugate aLy6E-LC-K149C-10 by affinity capture LC-MS F(ab’2) analysis of mouse plasma samples 1 day following IV administration. Differences in ionization efficiencies suggest that larger amounts of the protein-adduct species are present relative to TDC aLy6E-LC-K149C-10 than visually depicted (see Supporting Information for more details). (b) LC-MS analysis of LC-CBI-adduct entities derived from reduction of TDC aLy6E-LC-K149C-10 F(ab’2) fragments. (c) Illustration of the expected differences in observed molecular weights of the LC-CBI-adduct entities with GSH and A1M added to the same (Ab in blue) vs different (Ab in green) CBI molecules. Unique fragments that distinguish the two species are highlighted with red frames. HC = heavy chain, LC = light chain.
**Figure S3.** General depiction of minor high molecular weight peaks associated with protein and GSH adducts formed from DAR2 TDCs derived from linker-drug 10.

Legend. The transformations are believed to be antigen-independent and apply to TDCs targeting the CD22 (aCD22-LC-K149C-10), NaPi2b (aNaPi2b-LC-K149C-10), Ly6E (aLy6E-LC-K149C-10), and gD (agD-LC-K149C-10) antigens. The locations of protein and GSH addition products within the unsymmetrical seco-CBI structures are not known with certainty and may be reversed (those depicted are arbitrarily assigned). The location of the singular Cl/phosphate loss depicted in 13Aa-13Da and 14Aa-14Da is similarly arbitrarily assigned.
**Figure S4.** Time-course of aCD22-LC-K149C-10 protein adduct formation in various species.

**Legend.** MS profiles of TDC aCD22-LC-K149C-10 in (a) mouse and (b) rat in vivo and (c) TDC aLy6E-LC-K149C-10 in monkey in vivo determined on days 1, 4 and 7 by affinity capture LC-MS F(ab’2) assay. Differences in ionization efficiencies suggest that larger amounts of the protein-adduct species are present relative to TDCs aCD22-LC-K149C-10 and aLy6E-LC-K149C-10 than visually depicted (see Supporting Information for more details). (d) Zoom-in MS spectra of protein adducts at 7 day post-dose suggesting no significant amount of deconjugation observed at this time point in all the species. * indicates artificial MS peaks generated by the deconvolution algorithm due to low signal-to-noise ratio.
Figure S5. AMBP sequence alignments.

Legend. Uniprot-sequence alignment of AMBP homologs of human (SP|P02760|AMBP_HUMAN), monkey (TR|G7PRL7|G7PRL7_MACFA), rat (SP|Q64240|AMBP_RAT) and mouse (SP|Q07456|AMBP_MOUSE) * suggests identical alignments; . or : indicates incompletely identical alignments. – indicates gaps between species.
**Figure S6.** LC-MS/MS workflow for identification of the plasma protein responsible for adduct formation.
Figure S7. *In vivo* stability results obtained for conjugate aCD22-LC-K149C-11.

**Legend.** *In vivo* stability results obtained for TDC aCD22-LC-K149C-11 by affinity capture LC-MS F(ab’2) analysis of mouse plasma samples 1 day following IV administration. Differences in ionization efficiencies suggest that larger amounts of the protein-adduct species are present relative to TDC aCD22-LC-K149C-11 than visually depicted (see Supporting Information for more details). * Proposed tentatively assigned peaks. Peaks labeled as “15Ab+15Ac” and “16Ab+16Ac” likely result from the combination of two species (-P; -Cl-P) (Supplementary Figure 9) resulting from the unresolved mass difference of 36 Da in the current mass range. The adduct species “c” (-P) and “d” (-2P) were observed with TDC aCD22-LC-K149C-11 but not aCD22-LC-K149C-10, suggesting that the shorter linker in TDC aCD22-LC-K149C-11 might slow the spirocyclization step (Supplementary Fig. S9).
Figure S8. General depiction of protein and GSH adduct formation from DAR2 TDCs derived from linker-drug 11.

Legend. The process is believed to be antigen-independent and applies to TDCs targeting the CD22 (aCD22-LC-K149C-11), NaPi2b (aNaPi2b-LC-K149C-11), and Ly6E (aLy6E-LC-K149C-11) antigens. The locations of protein and GSH addition products within the unsymmetrical seco-CBI structures are not known with certainty and may be reversed (those depicted are arbitrarily assigned).
**Figure S9.** General depiction of minor high molecular weight peaks associated with protein and GSH adducts formed from DAR2 TDCs derived from linker-drug 11.

Legend. The transformations are believed to be antigen-independent and apply to TDCs targeting the CD22 (aCD22-LC-K149C-11), NaPi2b (aNaPi2b-LC-K149C-11), and Ly6E (aLy6E-LC-K149C-11) antigens. The locations of protein and GSH addition products within the unsymmetrical seco-CBI structures are not known with certainty and may be reversed (those depicted are arbitrarily assigned). The location of the singular Cl/phosphate loss depicted in 15Aa-15Ca, 15Ac-15Cc, 16Aa-16Ca, and 16Ac-16Cc is similarly arbitrarily assigned.
**Figure S10.** Time-course of aCD22-LC-K149C-11 protein adduct formation in various species.

**Legend.** MS profiles of conjugate aCD22-LC-K149C-11 in (a) mouse, (b) rat, and (c) monkey in vivo determined on days 1, 4 and 7 by affinity capture LC-MS F(ab’2) assay. Differences in ionization efficiencies suggest that larger amounts of the protein-adduct species are present relative to conjugate aCD22-LC-K149C-11 than visually depicted (see Supporting Information for more details). (d) Zoom-in MS spectra of protein adducts at 7 day post-dose suggesting no significant amount of deconjugation observed at this time point in all the species. * indicates artificial MS peaks generated by the deconvolution algorithm due to low signal-to-noise ratio. # represents incomplete digestion species from IdeS treatment, e.g., TDC-1/2Fc.
Figure S11. *In vitro* cynomolgous monkey whole blood stability of TDC aLy6E-LC-K149C-10 in the presence of high concentration of GSH.

Legend. Biotransformations were assigned based on the mass shifts from the DAR2 TDCs. Samples were analyzed by affinity capture LC-MS F(ab’2) assay following *in vitro* incubation in cynomolgous monkey whole blood for 24 h in the presence of A) aLy6E-LC-K149C-10 in the presence of excess added phosphatase and GSH (10 mM) (a) pretreatment of aLy6E-LC-K149C-10 with phosphatase enzyme and subsequent *in vitro* incubation in monkey whole blood in the absence (b) and presence (c) of GSH (10 mM) for 24 h. * indicates artificial MS peaks generated by the deconvolution algorithm due to low signal-to-noise ratio. No protein adducts were observed with the above conjugate in the whole blood experiments in the absence of added phosphatase (data not shown). The assignment of one A1M protein per conjugate is clearly supported by the observed molecular weights of these entities.
Discussion. Incubation of aLy6E-LC-K149C-10 in cynomolgous monkey whole blood in the presence of added phosphatase (Figure 8a), added phosphatase plus high GSH (10 millimolar concentration) (Figure S11a) produced only A1M adducts after 24 hours. Same phenomenon was observed for incubation of pretreatment with phosphatase followed by exposure to monkey whole blood supplemented with high GSH (10 mM) (Figure S11b). In contrast, conversion of the dimeric seco-CBI payloads present in aLy6E-LC-K149C-10 to the corresponding cyclized CBI entities via pretreatment with phosphatase followed by exposure to monkey whole blood supplemented with high GSH (10 mM) generated a mono-GSH adduct in addition to the A1M condensation product (Figure S11c). The former outcomes were qualitatively similar to those observed in monkeys in vivo with the aCD22-LC-K149C-10 conjugate (i.e., no significant amount of GSH adducts formed, c.f., Figure S4c). GSH condensation products resulting from addition of one and two GSH molecules to the phosphatase-activated TDC were also observed during the latter experiment (Figure S11c). Collectively, these data suggest that GSH can compete with the A1M protein for addition to cyclized CBI-containing TDCs but only when the cyclized CBI entities are present in relatively high concentrations (e.g., by pre-forming them via prior phosphatase treatment of the corresponding seco-CBI analogs as in Figure S11c). This hypothesis is consistent with the faster rate of cyclized CBI generation observed in vivo in mice relative to rats and monkeys leading to the formation of greater GSH adduct amounts in the former species (c.f., Figure S4).
**Figure S12.** Graphical depictions of typical Tab assays.

**Legend.** (a) Generic sandwich ELISA, (b) generic capture LC-MS/MS, and (c) direct LC-MS/MS methods. Note that an anti-human antibody was employed in the generic sandwich ELISA approach while protein A was utilized alternatively as the capture reagent in the affinity capture LC-MS/MS technique.
Figure S13. Impact of mAb attachment site on protein adduct formation.

**Legend.** Protein adduct formation observed for LC-K149C, LC-V205C, and HC-A140C TDCs derived from linker-drug 10. TDCs aCD22-LC-K149C-10, aCD22-LC-V205C-10, and aCD22-HC-A140C-10 were assessed at day 1 post-dose in mouse *in vivo*. Biotransformations were assigned based on the mass shifts from the DAR2 TDCs. The assignment of one protein and/or GSH adduct per conjugate is clearly supported by the observed molecular weights of these entities. Differences in ionization efficiencies suggest that larger amounts of the protein-adduct species are present relative to TDCs aCD22-LC-K149C-10, aCD22-LC-V205C-10, and aCD22-HC-A140C-10 than visually depicted (see Supporting Information for more details).

LC = light chain, HC = heavy chain. * indicates artificial MS peaks generated by the deconvolution algorithm due to low signal-to-noise ratio.
Figure S14. Impact of linker length on protein adduct formation (days 4 and 7).

Legend. Protein adduct formation observed for HC-A140C TDCs derived from different linkers (longer cleavable, aCD22-HC-A140C-10 and shorter non-cleavable, aCD22-HC-A140C-11) at days 4 and 7 post-dose in mouse in vivo. Differences in ionization efficiencies suggest that larger amounts of the protein-adduct species are present relative to TDCs aCD22-HC-A140C-10 and aCD22-HC-A140C-11 than visually depicted (see Supporting Information for more details). HC = heavy chain. * indicates artificial MS peaks generated by the deconvolution algorithm due to low signal-to-noise ratio.
Figure S15a. Body weight changes associated with TDCs aCD22-LC-K149C-10, aLy6E-LC-K149C-10, aCD22-LC-V205C-10, and aCD22-HC-A140C-10 in the WSU-DLCL2 xenograft model.

Legend: red traces: TDC aCD22-LC-K149C-10 (LC-K149C), green traces: TDC aCD22-LC-V205C-10 (HC-A140C), blue traces: TDC aCD22-HC-A140C-10 (HC-A140C), black trace (dotted line): TDC aLy6E-LC-K149C-10, black trace (solid line): vehicle control. Numbers next to traces indicate doses of each conjugate (in mg/kg) that were administered once IV at the day 0 time point.
**Figure S15b.** Body weight changes associated with TDCs aCD22-LC-K149C-11, aLy6E-LC-K149C-11, and aCD22-HC-A140C-11 in the WSU-DLCL2 xenograft model.

**Legend:** red traces: TDC aCD22-LC-K149C-11 (LC-K149C), blue traces: TDC aCD22-HC-A140C-11 (HC-A140C), black trace (dotted line): TDC aLy6E-LC-K149C-11, black trace (solid line): vehicle control. Numbers next to traces indicate doses of each conjugate (in mg/kg) that were administered once IV at the day 0 time point.
Figure S16. Body weight changes associated with TDCs aCD22-LC-K149C-10, agD-LC-K149C-10, aCD22-LC-K149C-12, and agD-LC-K149C-12 in the BJAB xenograft model.

Legend: green traces: TDC aCD22-LC-K149C-12, red traces: TDC aCD22-LC-K149C-10, black trace (dotted line, open triangles): TDC agD-LC-K149C-10, black traces (dotted lines, half-filled squares and open crosses): TDC agD-LC-K149C-12, black trace (solid line): vehicle control. Numbers next to traces indicate doses of each conjugate (in mg/kg) that were administered once IV at the day 0 time point.
Figure S17. *In vivo* stability of TDC aCD22-LC-K149C-12.

**Legend.** MS profiles of TDC aCD22-LC-K149C-12 in mouse determined on days 0 (dosing), 3 and 7 by affinity capture LC-MS F(ab’2) assay. Minimal deconjugation (-LD) followed by formation of antibody-Cys adducts (-LD+Cys, -2LD+2Cys) was observed by day 7 post-dose. No significant amounts of A1M protein adducts were observed in these assessments.
Table S1. Antiproliferation activities of TDCs prepared from compounds 10 and 11 (log IC$_{50}$ ± SE log IC$_{50}$).

<table>
<thead>
<tr>
<th>Conjugate Name (antigen-site-linker-drug)</th>
<th>Site$^a$</th>
<th>DAR$^b$</th>
<th>BJAB logIC$_{50}$ (nM)$^c$</th>
<th>WSU-DLCL2 logIC$_{50}$ (nM)$^c$</th>
<th>Jurkat (control) logIC$_{50}$ (nM)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCD22-LC-K149C-10</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>-10 ± 0.11</td>
<td>-11 ± 0.15</td>
<td>-7.1 ± 0.15</td>
</tr>
<tr>
<td>aNaPi2b-LC-K149C-10</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>&gt; -6.9</td>
<td>-7.4 ± 0.075</td>
<td>-7.1 ± 0.14</td>
</tr>
<tr>
<td>aLy6E-LC-K149C-10</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>&gt; -6.9</td>
<td>-7.3 ± 0.085</td>
<td>&gt; -6.9</td>
</tr>
<tr>
<td>agD-LC-K149C-10$^d$</td>
<td>LC-K149C</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCD22-LC-K149C-11</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>-9.6 ± 0.050</td>
<td>-11 ± 0.19</td>
<td>-7.2 ± 0.16</td>
</tr>
<tr>
<td>aNaPi2b-LC-K149C-11</td>
<td>LC-K149C</td>
<td>1.8</td>
<td>-7.2 ± 0.14</td>
<td>-8.2 ± 0.044</td>
<td>-7.8 ± 0.035</td>
</tr>
<tr>
<td>aLy6E-LC-K149C-11</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>-7.2 ± 0.20</td>
<td>-8.04 ± 0.040</td>
<td>-7.8 ± 0.020</td>
</tr>
<tr>
<td>aCD22-LC-V205C-10</td>
<td>LC-V205C</td>
<td>2.0</td>
<td>-10 ± 0.03</td>
<td>-11 ± 0.29</td>
<td>-7.03 ± 0.31</td>
</tr>
<tr>
<td>aCD22-HC-A140C-10</td>
<td>HC-A140C</td>
<td>2.0</td>
<td>-9.6 ± 0.057</td>
<td>-10 ± 0.060</td>
<td>&gt; -6.9</td>
</tr>
<tr>
<td>aCD22-HC-A140C-11</td>
<td>HC-A140C</td>
<td>1.9</td>
<td>-9.8 ± 0.047</td>
<td>-11 ± 0.39</td>
<td>-7.8 ± 0.022</td>
</tr>
<tr>
<td>aCD22-LC-K149C-12</td>
<td>LC-K149C</td>
<td>1.9</td>
<td>-8.01 ± 0.44</td>
<td>-9.6 ± 0.085</td>
<td>&gt; -6.9</td>
</tr>
<tr>
<td>agD-LC-K149C-12$^d$</td>
<td>LC-K149C</td>
<td>1.9</td>
<td>&gt; -6.9</td>
<td>&gt; -6.9</td>
<td>&gt; -6.9</td>
</tr>
</tbody>
</table>

$^a$Site of antibody attachment. $^b$Drug-antibody ratio. $^c$All cell-based assay results are reported as the arithmetic mean of at least three separate runs (n = 3). $^d$gD = Viral antigen (non-targeting control). ND = not determined.
Table S2. Details on peptides identified by LC-MS/MS for the major protein that formed adducts with CBI TDCs in mouse, rat and monkey plasma in vivo. The top protein hits were identified as Uniprot sequence of AMBP homologs of monkey (TR|G7PRL7|G7PRL7_MACFA) rat SP|Q64240|AMBP_RAT) and mouse (SP|Q07456|AMBP_MOUSE), respectively. The amino acids with oxidation or carbamidomethyl modification were shown in lower cases.

<table>
<thead>
<tr>
<th>CID</th>
<th>AMBP_MOUSE</th>
<th>Sequence</th>
<th># Peptides</th>
<th># Protein Groups</th>
<th>Modifications</th>
<th>XCor</th>
<th>Charge</th>
<th>M+Da</th>
<th>Dn (pm)</th>
<th># Missed Cleavages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MSOTVVEGATTGWNSWSTR</td>
<td>1</td>
<td>1</td>
<td>Met(Oxidation)</td>
<td>7.20</td>
<td>3</td>
<td>2497.30532</td>
<td>3.14</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DSSLQVQGAMNSWNGGMPQK</td>
<td>1</td>
<td>1</td>
<td></td>
<td>6.35</td>
<td>3</td>
<td>3050.56540</td>
<td>1.97</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DSSLQVQGAMNSWNGGMPQK</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>6.06</td>
<td>4</td>
<td>3210.65471</td>
<td>4.75</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSOTVVEGATTGWNSWSTR</td>
<td>1</td>
<td>1</td>
<td></td>
<td>6.01</td>
<td>2</td>
<td>2471.00066</td>
<td>3.06</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DLNLGTGEGWNGGGMPQK</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>5.48</td>
<td>3</td>
<td>2909.45235</td>
<td>2.74</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WNLAOGTVPVMPK</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>5.15</td>
<td>2</td>
<td>3805.86017</td>
<td>3.32</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EQQRQLUGWQNGGGGMPQK</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>4.77</td>
<td>5</td>
<td>3414.26252</td>
<td>1.64</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FQDIQDUGWQNGGGGMPQK</td>
<td>1</td>
<td>1</td>
<td></td>
<td>4.67</td>
<td>4</td>
<td>3072.85094</td>
<td>1.18</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DLNLGTGEGWNGGGMPQK</td>
<td>1</td>
<td>1</td>
<td></td>
<td>4.31</td>
<td>2</td>
<td>2090.05902</td>
<td>0.40</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GWVESTGAPK</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>4.10</td>
<td>2</td>
<td>1354.53408</td>
<td>2.43</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CID</th>
<th>AMBP_RAT</th>
<th>Sequence</th>
<th># Peptides</th>
<th># Protein Groups</th>
<th>Modifications</th>
<th>XCor</th>
<th>Charge</th>
<th>M+Da</th>
<th>Dn (pm)</th>
<th># Missed Cleavages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MSOTVVEGATTGWNSWSTR</td>
<td>1</td>
<td>1</td>
<td>Met(Oxidation)</td>
<td>6.19</td>
<td>4</td>
<td>2713.35013</td>
<td>-0.23</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FUALQVRGNSWVPR</td>
<td>1</td>
<td>1</td>
<td>Met(Oxidation)</td>
<td>5.73</td>
<td>2</td>
<td>2683.05321</td>
<td>1.12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WNLAOGTVPVMPK</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>4.88</td>
<td>2</td>
<td>1706.04033</td>
<td>1.35</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FUALQVRGNSWVPR</td>
<td>1</td>
<td>1</td>
<td></td>
<td>4.00</td>
<td>2</td>
<td>2007.05440</td>
<td>0.74</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GWVESTGAPK</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>3.65</td>
<td>2</td>
<td>1368.66980</td>
<td>0.33</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DLNLGTGEGWNGGGMPQK</td>
<td>1</td>
<td>1</td>
<td></td>
<td>2.91</td>
<td>2</td>
<td>2007.51047</td>
<td>0.78</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CID</th>
<th>GPRLU_MACA</th>
<th>Sequence</th>
<th># Peptides</th>
<th># Protein Groups</th>
<th>Modifications</th>
<th>XCor</th>
<th>Charge</th>
<th>M+Da</th>
<th>Dn (pm)</th>
<th># Missed Cleavages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VAQGVQGEGATFWYPVR</td>
<td>1</td>
<td>1</td>
<td>Met(Oxidation)</td>
<td>5.53</td>
<td>3</td>
<td>2021.00042</td>
<td>-0.27</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MHVTQGEGATFWYPVR</td>
<td>1</td>
<td>1</td>
<td>Met(Oxidation)</td>
<td>5.30</td>
<td>2</td>
<td>2384.18662</td>
<td>7.61</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GQWQGEGATFWYPVR</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>5.00</td>
<td>2</td>
<td>2195.56181</td>
<td>2.57</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VAQGVQGEGATFWYPVR</td>
<td>1</td>
<td>1</td>
<td></td>
<td>4.65</td>
<td>2</td>
<td>2005.03501</td>
<td>2.97</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VAQGVQGEGATFWYPVR</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>4.24</td>
<td>4</td>
<td>2905.05090</td>
<td>3.22</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WNLAOGTVPVK</td>
<td>1</td>
<td>1</td>
<td>C(Conjugated)</td>
<td>3.35</td>
<td>2</td>
<td>1706.66778</td>
<td>-1.09</td>
<td>0</td>
</tr>
</tbody>
</table>
Table S3. Antiproliferation activities of plasma samples obtained from various animals following single IV administrations of TDCs aCD22-LC-K149C-10 and aCD22-LC-K149C-11 (log IC₅₀ ± SE log IC₅₀).

<table>
<thead>
<tr>
<th>Conjugate Name (antigen-site-linker-drug)</th>
<th>Dose (mg/kg)</th>
<th>Species&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Plasma collection day</th>
<th>WSU-DLCL2 cell logIC₅₀ (pM)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCD22-LC-K149C-10</td>
<td>2</td>
<td>mouse</td>
<td>0</td>
<td>-5.5 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>aCD22-LC-K149C-10</td>
<td>2</td>
<td>mouse</td>
<td>7</td>
<td>-4.2 ± 0.036&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>aCD22-LC-K149C-10</td>
<td>5</td>
<td>rat</td>
<td>0</td>
<td>-5.9 ± 0.81</td>
</tr>
<tr>
<td>aCD22-LC-K149C-10</td>
<td>5</td>
<td>rat</td>
<td>7</td>
<td>-4.2 ± 0.062</td>
</tr>
<tr>
<td>aCD22-LC-K149C-11</td>
<td>5</td>
<td>mouse</td>
<td>0</td>
<td>-3.9 ± 0.067</td>
</tr>
<tr>
<td>aCD22-LC-K149C-11</td>
<td>5</td>
<td>mouse</td>
<td>7</td>
<td>-3.4 ± 0.066</td>
</tr>
<tr>
<td>aCD22-LC-K149C-11</td>
<td>3</td>
<td>monkey</td>
<td>0</td>
<td>-3.9 ± 0.076</td>
</tr>
<tr>
<td>aCD22-LC-K149C-11</td>
<td>3</td>
<td>monkey</td>
<td>7</td>
<td>-3.5 ± 0.094</td>
</tr>
</tbody>
</table>

<sup>a</sup>Origin of plasma samples. <sup>b</sup>All cell-based assay results are reported as the arithmetic mean of assessments from three separate animals (n = 3) and are normalized based on TAB exposures. <sup>c</sup>n= 2 animals.
**MS Ionization Differences and Data Interpretation Caveats**

It is important to note that, since the described TDC-A1M adducts appear to be susceptible to ionization suppression, the MS profiles generated using the affinity capture LC-MS F(ab’2) assay are qualitative instead of quantitative in nature. A suppression factor of ~5-fold relative to the parent TDC was estimated by comparing the observed relative ratios (by mass peak area) and theoretical mixed ratios (by mass based on Tab concentration) of DAR2 TDC (15-min post-dose) and TDC-A1M adduct (7-day post-dose) monkey plasma samples (Figure S18). The estimation was done by conducting two separate LC-MS F(ab’2) assay experiments of DAR2 TDC and TDC-A1M adduct (Figure S18a), and a single experiment by mixing the two entities at different ratios (Figure S18b), respectively. Both approaches resulted in a suppression factor of ~5 fold of the TDC-A1M adduct entity compared to the DAR2 TDC species. These outcomes suggested that the ionization suppression was likely due to the hydrophobicity of A1M than the ionization competition in mass spectrometer. As minimal species-different homologies are expected for A1M, the ionization efficiencies of A1M from different species are assumed the same. Thus the MS profiles by LC-MS F(ab’2) were used to estimate the relative biotransformation rate of A1M adduct formation across different species or different TDCs in the same species.
**Figure S18.** Estimation of ionization efficiency of TDC-A1M adduct relative to DAR2 TDC.

Legend. (a) Direct comparison of MS peak area of conjugate aCD22-LC-K149C-11 DAR2 TDC (post dose 15 min) and TDC-A1M adduct (post dose 168 h) in monkey plasma *in vivo*. Plasma samples containing 1.5 µg of Tab in monkey at 3 mg/kg were subjected to affinity capture LC-MS F(ab’2) assay in two separate experiments. (b) Comparison of theoretical mixed ratio (mass) and measured ratios using MS peak area of conjugate aCD22-LC-K149C-11 DAR2 TDC and TDC-A1M adduct in monkey plasma *in vivo*. Plasma samples of DAR2 TDC (post dose 15 min) and TDC-A1M adduct (post dose 168 h, 1.5 µg of Tab) in monkey at 3 mg/kg were at various ratios (0.2:1, 0.5:1, 1:1) were subjected to affinity capture LC-MS F(ab’2) assay in a single experiment. * indicates artificial MS peaks generated by the deconvolution algorithm due to low signal-to-noise ratio.
**Experimental Procedures**

**Reagents**

Lithium heparin treated whole blood and plasma were purchased from BioreclamationIVT (New York, USA). Streptavidin-coated Dynabeads M-280 were purchased from Invitrogen (CA, USA). IdeS, FabRICATOR®, was purchased from Genovis, Inc. (Cambridge, MA, USA). Protein A Magnetic Bead were from Millipore. Capture antibody Monkey absorbed Sheep anti-Human IgG against heavy chain and light chain (H+L) (Binding Site, San Diego, CA, USA), Horse radish peroxidase (HRP) conjugated –monkey absorbed goat anti-human IgG, (H+L) (Bethyl Laboratories, Inc., Montgomery, TX, USA), TMB peroxidase substrate system (KPL Inc., Gaithersburg MD). Other commercial reagents included lambda protein phosphatase (400,000 U/mL) (New England Biolabs, MA, USA), peptide N-glycosidase F (PNGase F) (ProZyme; CA, USA), trypsin gold, mass spectrometry grade (Promega Corporation, Madison, WI, USA), Bond-Breaker™ TCEP, tris(2-carboxyethyl)phosphine solution, neutral pH and IAM, iodoacetamide (Thermo Fisher Scientific, Waltham, MA, USA), and HBS-EP buffer containing 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Polysorbate 20 (GE Healthcare; Little Chalfont, UK). All specific TDC capture reagents, e.g., extracellular domain (ECD), were produced at Genentech (South San Francisco, CA, USA as previously described).\(^{28}\) The detailed preparation of linker-drugs and TDCs is described in the Supporting Information.

**In vivo efficacy and PK studies in mice**

All animal studies were carried out in compliance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Genentech, Inc.
The efficacy of anti-CD22 TDCs was evaluated in a mouse xenograft model of CD22-expressing WSU-DLCL2 or BJAB human non-Hodgkin lymphoma. The WSU-DLCL2 cell line was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany). The BJAB cell line was obtained from the Genentech cell line repository. This cell line was authenticated by short tandem repeat (STR) profiling using the Promega PowerPlex 16 System and compared with external STR profiles of cell lines to determine cell line ancestry. To set up the xenograft model, tumor cells (20 million cells in 0.2 mL Hank’s Balanced Salt Solution; HyClone) were inoculated subcutaneously into the flanks of female C.B-17 SCID mice (Charles Rivers Laboratories). When tumors reached the desired volume (~200 mm$^3$), animals were divided into groups of 5 mice with similar mean tumor size and received a single intravenous injection of TDCs through the tail vein (referred to as Day 0). The treatment information was not blinded during tumor measurement. Tumors were measured in two dimensions (length and width) using calipers and the tumor volume was calculated using the formula: tumor size (mm$^3$) = 0.5 x (length x width x width). Results were plotted as mean tumor volume ± SEM of each group over time.

PK studies were conducted in naive C.B.17 SCID mice. Twenty-four female mice (6-8 weeks old) were obtained from Charles River Laboratories, Inc. (Hollister, CA). Each animal received a single IV dose of seco-CBI-dimer TDCs via tail vein injection (n = 12/group). Blood samples were collected from 3 mice in each dosing group at each of the following time points: 10 minutes, 1 and 6 hours, 1, 2, 3, 7, 10, 14, and 21 days.

Blood samples from mice were collected and used to derive plasma for in vivo TDC stability assessments. All blood samples were collected into tubes containing lithium heparin. Blood samples from mice were collected via retro-orbital bleeds and allowed to sit on wet ice until centrifugation (within 15 minutes of collection). Samples were centrifuged at 10,000 rpm for 5 minutes at 4 °C. Samples were processed to plasma within 1 hour of collection by
centrifugation at 1750 (± 250) x g for 10-15 minutes in a centrifuge set to maintain 2° C to 8° C. All plasma samples were then collected, placed on dry ice, and stored in a freezer set at -70 °C until analysis.

**In vivo stability studies in rats and monkeys**

All animal studies were conducted at Genentech or contract research organization laboratories (details for each study are given below) according to their Standard Operating Procedures and in compliance with applicable regulations concerning the use of laboratory animals. Primary enclosures were as specified in the USDA Animal Welfare Act (9 CFR, Parts 1, 2 and 3) and as described in the Guide for the Care and Use of Laboratory Animals.

Sprague Dawley rat studies were conducted with aCD22-LC-K149C-10 or aCD22-LC-K149C-11 (In Vivo Studies Group, Genentech, CA). Rats were social-housed (2 animals of same sex and same dosing group) in polycarbonate cages via automatic watering system ad libitum. Rats were dosed intravenously on Day 1 with vehicle alone, 5, or 10 mg/kg aCD22-LC-K149C-10 (n = 5 female animals/group). Blood collections were taken on Days 1 (10 minutes post-dose), 4 and 7. A second set of rats were dosed intravenously on Day 1 with vehicle alone, 10, or 15 mg/kg aCD22-LC-K149C-11 (n = 3 female animals or 3 male animals/group). Blood collections were taken on Days 1 (10 minutes post-dose), 4 and 7.

Cynomolgus monkey studies were conducted with aLy6E-LC-K149C-10 and aCD22-LC-K149C-11 (Charles River Laboratories, Reno, NV). Cynomolgus monkeys were social-housed (up to 3 animals of same sex and same dosing group together) in stainless steel cages equipped with a stainless steel mesh floor and an automatic watering valve. Naïve monkeys were dosed intravenously with aLy6E-LC-K149C-10 at a dose level of 4 mg/kg (n = 3 female animals/group). Blood collections were taken on Days 1 (15 minutes post-dose), 4 and 7. A second set of naïve monkeys were dosed intravenously with aCD22-LC-K149C-11 at a dose
level of 3 mg/kg (n = 3 female animal/group). Blood collections were taken on Days 1 (15 minutes post-dose), 4 and 7.

Blood samples from rats and monkeys were collected from animals and used to derive plasma for in vivo TDC stability assessments. All blood samples were collected into tubes containing lithium heparin. Blood samples from rats were collected via retro-orbital bleeds and allowed to sit on wet ice until centrifugation (within 15 minutes of collection). Samples were centrifuged at 10,000 rpm for 5 minutes at 4 °C. Blood samples from monkeys were collected via venipuncture, inverted to ensure mixing with anticoagulant, and chilled on wet ice until centrifugation. Samples were processed to plasma within 1 hour of collection by centrifugation at 1750 (± 250) x g for 10 -15 minutes in a centrifuge set to maintain 2 °C to 8 °C. All plasma samples were then collected, placed on dry ice, and stored in a freezer set at -70 °C until analysis.

**LC-MS F(ab′)2 assay for structural elucidation of TDC biotransformation**

TDC biotransformation characterization and stability assessments were conducted by affinity capture LC-MS F(ab′)2 assay as previously described (28). The resulting F(ab′)2 fragments (~ 100 kDa) from affinity capture were eluted using 50 μL of 30% acetonitrile in water with 1% formic acid. An aliquot of 5 μL of F(ab′)2 elution was subjected to LC-MS analysis. Capillary LC-MS F(ab′)2 was performed on a TripleTOF 5600 mass spectrometer coupled to a Waters nanoACQUITY UPLC system. Online desalting and pre-concentration were conducted on a PS-DVB monolithic column (500-μm i.d. × 50 mm, Thermo Fisher Scientific, Waltham, MA) at 65 °C using a 15 min gradient with mobile phases A, 0.1% formic acid and B, acetonitrile with 0.1% FA at a flow rate of 15 μL/min. Mass spectra were acquired in the intact protein mode, using Analyst® TF 1.6. Deconvolution was performed with
BioAnalyst™ 1.5.1. Relative ratios of individual TDC species were obtained based on their peak areas in the deconvoluted mass spectra.

**LC-MS/MS peptide mapping method for protein identification**

For the purpose of identifying the protein that formed the observed adducts, mouse (day 1, 4 and 7 post-dose), rat (day 12 post-dose) and monkey (day 7 post-dose) plasma samples from *in vivo* studies containing about 2-4 µg of total TDCs were analyzed as follows. To the F(ab’)2 elute from the above affinity capture step, the reducing reagent tris(2-carboxyethyl)phosphine (TCEP) was added to a final concentration of 20 mM. Reduction was conducted at 37 °C for 30 min. The reduced samples were then desalted and concentrated with 50 mM ammonium bicarbonate buffer in an Amicon Ultra-0.5 centrifugal filter unit with ultracele-30 membrane (MilliporeSigma, Burlington, MA, USA) to a final volume of 30 µL. Iodoacetamide (IAM) was then added to the filter to allow cysteine alkylation for 30 min in dark at 37 °C. Buffer exchange with 50 mM ammonium bicarbonate was subsequently conducted to remove the excessive alkylation reagent. The subsequent digestion was done by incubation at 37 °C for 3 h with 1.5 µg of trypsin and 0.3 μL of 100 mM CaCl₂. About 20 µL of the final peptide analytes was transferred from the filter to an injection vial and 5 μL of each sample was subjected to LC-MS/MS analysis as described below.

A Waters nanoAquity HPLC and auto-sampler was used to load samples onto a V/M Symmetry 20 mm C18 trapping column, 180 µm ID packed with 100 Å 5 µm C18 particles (Waters V/M Symmetry C18), and separated across a 100 mm x 100 µm ID analytical column packed with 130 Å 1.7 µm C18 particles (Waters Acquity UPLC Peptide BEH C18). Samples were loaded onto the pre-column in 95% mobile phase A (0.1% formic acid in water), 5% mobile phase B (0.1% formic acid in acetonitrile) for 10 min at a flow rate of 10 µL/ min. To separate the peptides, the gradient (800 nL/min) began with 5% mobile phase B (MPB) held
for 1 min and increased to 85% over the course of 50 min and then held for 4 min. The gradient was dropped back to 5% MPB within 1 minute and allowed to re-equilibrate for 5 min. Eluent was detected on an LTQ Orbitrap Elite Velos (Thermo Fisher Scientific, Bremen, Germany) via an integrated electrospray emitter operating at 1.8 kV. Experiments consisted of full MS analysis in the orbitrap mass analyzer followed by 10 data-dependent CID MS/MS events with a precursor isolation width of 2 Th and mass analysis in the ion trap. For all experiments, an AGC target value of 1,000,000 charges was used for MS1 and 50,000 for MS2. Precursors were dynamically excluded for 180 s with a repeat count of 1 recurring in 30 s, and only peptides with assigned charge states of two or greater were selected for MS/MS interrogation.

Spectral reduction, spectral searching and matching were all carried out in Proteome Discoverer (1.4.0.288). Spectra were searched against a concatenated target-decoy database (www.uniprot.org) with fully tryptic enzyme specificity, allowing up to three missed cleavages. Static modifications of carbamidomethylation (+57 Da) on cysteine residues and variable modification of oxidation on methionine residues (+16 Da) were specified. A mass tolerance of +/- 10 ppm was used for precursors, while a mass tolerance of +/- 0.8 Da was used for fragment with ion trap detection. Identifications were filtered to a FDR of 1%. Proteins were reduced for parsimony and filtered to 1% FDR as well.

**Total antibody (Tab) Bioanalysis**

**Enzyme-Linked Immunosorbent Assay (ELISA).** A generic ELISA was used to analyze Tab concentrations and has been described previously.\(^\text{44}\) Briefly, Nunc\textsuperscript{®} MaxiSorp\textsuperscript{TM} 384-well plates (Nalge Nunc International, Rochester, NY) were coated with 25 μL of 0.5 μg/mL capture reagent sheep anti-human IgG antibody (H+L) diluted in coat buffer (0.05 M carbonate/bicarbonate buffer pH 9.6) and incubated overnight at 4°C. Following blocking, plasma samples diluted in sample diluent (PBS/0.5% BSA/0.05% Tween 20/5mM
EDTA/0.25% CHAPS/ 0.35M NaCl/15 ppm proclin, pH 7.4) were added to the wells and incubated for 2 hours at RT. After washing 6 times, a detection antibody, HRP-conjugated goat anti-human antibody (H+L), diluted to 80 ng/mL in assay buffer (PBS/0.5% BSA/15 ppm proclin/0.05% Tween 20, pH7.4) was added to the wells and incubated on a shaker for 1 hour at 4°C. The plates were washed 6 times with wash buffer and developed using TMB peroxidase substrate for 15 minutes followed by 1 M Phosphoric acid to stop the reaction. Absorbance was measured at 450 nm against a reference wavelength of 620 nm. The concentration of the samples was extrapolated from a 4-parameter fit of the standard curve.

**LC-MS/MS.** Protein A capture LC-MS/MS was conducted as an alternative to ELISA. Briefly, TDC-containing mouse sera (10 μL aliquots) were enriched by an affinity capture approach with protein A magnetic beads (25 μL). The captured CBI TDCs molecules were subjected to “on-bead” proteolysis with trypsin following standard protein denaturation, reduction, and alkylation processing steps (65). An LC-MS/MS approach with direct digestion was performed by subjecting TDC-containing mouse sera (10 μL aliquots) to trypsin digestion following the same standard protein denaturation, reduction, and alkylation processing steps as above. For both protein A capture and direct LC-MS/MS, the signature peptide characteristic to Fc portion of human IgG1 produced by this procedure was then quantified by LC-MS/MS as a surrogate for Tab measurement.

**Cell potency assay of plasma samples with and without adducts**

On Day 0, BJAB, WSU-DLCL2, and Jurkat cell lines, were seeded at 4000 cells per well in 40 μL RPMI-1640 culture media supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μM cystine, and 0.015 g/L L-methionine in 384-well flat clear bottom white polystyrene tissue culture-treated microplates (Corning, NY). TDCs, 2 mg/mL in 20 mM Histidine Acetate, 240 mM Sucrose, 0.02% PS20 pH 5.5 buffer, were transferred to cells
seeded in 384-well plates using ECHO acoustic liquid handling technology (Labcyte Inc, Sunnyvale, CA) to create a 10-point dose-response curve in triplicate starting from 20 μg/mL with 1:3x serial dilution. One set of wells containing cells that received buffer alone were treated as neutral controls, and another set of wells without cells that received buffer alone were treated as inhibitor controls. Cells were cultured in a humidified incubator set at 37 °C and maintaining an atmosphere of 5% CO₂. On Day 4, cells were equilibrated to room temperature, then 40 μL/well Cell Titer Glo II® reagent (Promega; Madison, WI) was added, plates shaken for 10 min, then incubated for 30 min at RT in the dark. Luminescence was read using an EnVision 2101 Multilabel Reader (PerkinElmer, Waltham, MA). Luminescence intensity data were normalized to control wells using Genedata software [Genedata (USA), Inc.; San Francisco, CA]. Normalized luminescence intensity data were analyzed using GraphPad Prism 6 (GraphPad Software, Inc.; La Jolla, CA), and IC₅₀ values were calculated using a four parameter sigmoidal fit. The data were plotted as the mean of luminescence for each set of replicates with standard error bars. The antiproliferation activity of compounds 8 and 9 were determined in an 10-point dose response study starting from 50 nM down to 0.19 pM in 1:4 fold serial dilutions with final 0.25% DMSO in culture medium.

For the anti-proliferation of in vivo biological samples, WSU-DLCL2 and Jurkat cells were seeded at 4000 cells per well on Day 0 in 20 μL RPMI-1640 culture media supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Individual mouse, rat, and cynomolgous monkey plasma samples (n=3 per time points) were diluted 1:10 in an 11-point dose response curve in 1:3 fold serial dilutions. Then, 20 μL of the diluted plasma samples were slowly added on seeded cells to make final 1:2x dilutions. IC₅₀ values were determined by taking the Tab obtained from LC-MS/MS analysis as the top concentration divided by 20 from the sample dilution for each animal per time point.
Luminescence intensity data were normalized to control wells in a manner similar to that described above for the TDCs.

**In vitro adduct formation in whole blood**

An appropriate amount of TDC stock solution (typically 1-10 mg/mL) was added to monkey and human whole blood, followed by addition of 10 µL of 10×MnCl₂ and 3 µL of Lambda protein phosphatase provided by the vendor. The resultant 100 µL of whole blood sample with 50 µg/mL of TDC was incubated at 37 °C for 24 hours with gentle shaking. Typically, 20 µL of the whole blood sample was submitted to LC-MS F(ab’)2 assay for biotransformation assessment as described above.
Compound Synthesis

LC-MS conditions: mobile phase: 1.5 mL / 4 L TFA in water (solvent A) and 0.75 mL / 4 L TFA in acetonitrile (solvent B), using the elution gradient 5%-95% (solvent B) over 0.8 minutes and holding at 95% for 0.4 minutes at a flow rate of 1.5 mL/min; column: MK RP18e 25-2 mm; wavelength: UV 220 nm and 254 nm; column temperature: 50 °C; MS ionization: ESI.

Compound 8.

To a solution of compound A (1.2 g, 4.27 mmol) in 1,4-dioxane (2.0 mL) was added compound B (2.94 g, 34.18 mmol) and (iPr)_2NEt (2.76 g, 21.36 mmol) and Pd(tBu_3P)_2 (437 mg, 0.85 mmol). The reaction was stirred at 120 °C for 1.0 h under microwave irradiation. The reaction mixture was then concentrated, diluted with water (60 mL) and extracted with EtOAc (100 mL x 3). The organic layers were combined, dried over Na_2SO_4, and concentrated. The residue was purified by chromatography on silica (solvent gradient: 0-20% EtOAc in petroleum ether) to afford compound C (1.2 g, 96%) as a yellow solid. ^1H NMR (400 MHz,
CDCl$_3$ δ 8.17 (s, 1H), 8.10 (d, $J = 16$ Hz, 2H), 7.77 (d, $J = 8.0$ Hz, 2H), 7.72-7.67 (m, 2H), 6.57 (d, $J = 16.4$ Hz, 1H), 6.42 (d, $J = 16$ Hz, 1H), 3.85 (s, 6H).

To a mixture of compound C (1.20 g, 4.12 mmol) in EtOH (40 mL) and water (30 mL) was added iron powder (1.15 g, 20.6 mmol) and NH$_4$Cl (2.2 g, 41.2 mmol). The reaction mixture was stirred at 80 °C for 2 h then was filtered through a pad of celite. The filtrate was diluted with water (80 mL) and the resulting mixture was extracted with EtOAc (80 mL x 2). The combined organic layers were washed with brine (50 mL), dried over Na$_2$SO$_4$, and filtered. The filtrate was concentrated to afford compound D (1.00 g, 93%) as a yellow solid, which was used directly in the next step. LCMS: RT = 0.70 min, m/z = 261.8 [M+1]$^+$.}

(iPr)$_2$NEt (890 mg, 6.89 mmol) was added to a mixture of compound E (869 mg, 4.59 mmol) in CH$_2$Cl$_2$ (20 mL) at 18 °C. HATU (1746 mg, 4.59 mmol) was added, and the resulting mixture was stirred at 18 °C for 10 min. Compound D (300 mg, 1.15 mmol) was then added, and the mixture was stirred at 18 °C for 8 h. After concentrating the reaction contents under reduced pressure, the residue was diluted with water (30 mL) and the resulting mixture was extracted with EtOAc (50 mL x 2). The combined organic layers were washed with brine (10 mL), dried over Na$_2$SO$_4$ and filtered. The filtrate was concentrated and the residue was purified by chromatography on silica (solvent gradient: 0-7% CH$_3$OH in CH$_2$Cl$_2$) to provide compound F (800 mg, 80%) as a yellow solid. LCMS: RT = 0.72 min, m/z = 455.1 [M+23]$^+$.}

LiOH (221 mg, 9.25 mmol) was added to a solution of compound F (800 mg, 1.85 mmol) in THF (20 mL) and water (10 mL) and the resulting mixture was stirred at 18 °C for 8 h. After dilution with water (20 mL) and acidification to pH = 5 with HCl (2.0 M), the mixture was extracted with EtOAc (40 mLx2). The combined organic layers were washed with brine (10 mL), dried over Na$_2$SO$_4$ and filtered. The filtrate was concentrated to give compound G
(610 mg, 76%) as a yellow solid, which was used directly in the next step. LCMS: RT = 0.73 min, m/z = 427.1 [M+23]⁺.

A mixture of compound G (40.0 mg, 0.090 mmol), compound H (prepared as described in: Angew. Chem. Int. Ed. 2010, 49, 7336 or US 2015/209445; 69.2 mg, 0.30 mmol), EDCI (106 mg, 0.550 mmol) and TsOH (1.59 mg, 0.010 mmol) in dimethylacetamide (7 mL) stirred at 20 °C. After 5 h, additional portions of TsOH (1.59 mg, 0.010 mmol) and EDCI (53 mg, 0.275 mmol) were added and stirring was continued at 20 °C for an additional 5 h. The mixture was diluted with water (10 mL) and subsequently extracted with EtOAc (20 mL x 2). The combined organic layers were washed with brine (10 mL), dried over Na₂SO₄ and filtered. The filtrate was concentrated and the residue was washed with MTBE to give compound I (50 mg, 55%) as a gray solid. LCMS: RT = 1.01 min, m/z = 735.1 [M+1-100]⁺.

HCl in EtOAc (4.0 M, 5.0 mL, 20 mmol) was added to solution of compound I (50.0 mg, 0.060 mmol) in CHCl₂ (10 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h then was concentrated. The residue was washed with CH₃OH (10 mL x 2) to give compound 8 (HCl salt, 14 mg, 29%) as a brown solid. LCMS: RT = 0.76 min, m/z = 735.2 [M+1]+; ¹H NMR (400 MHz, DMSO-d₆) δ 10.42 (br, 5H), 8.12-8.10 (m, 4H), 7.87-7.81 (m, 5H), 7.68 (d, J = 16 Hz, 1H), 7.54-7.50 (m, 2H), 7.37-7.26 (m, 5H), 4.56-4.49 (m, 4H), 4.26 (brs, 2H), 4.02-3.99 (m, 2H), 3.87-3.84 (m, 2H), 3.13 (t, J = 6.8 Hz, 2H), 2.83 (t, J = 6.8 Hz, 2H).
Compound 9.

To a solution of J (2.50 g, 13.3 mmol) in dry THF (12 mL) at -30 to -40 °C in a dry ice-CH$_3$CN bath was added boron trifluoride diethyl etherate (BF$_3$·Et$_2$O, 4.92 mL, 39.9 mmol) dropwise under N$_2$. After the mixture was stirred at -30 °C for 10 min, tBuONO (2.39 mL, 19.9 mmol) was added dropwise. The reaction mixture was allowed to warm up to room temperature and was stirred for 1.5 h to give a suspension. Petroleum ether (50 mL) was added to give more precipitate. The supernatant was removed by decantation, and the remaining solid was washed with petroleum ether to afford a white solid. This solid was dissolved in dry CH$_3$CN (20 mL) and cooled in an ice bath.KI (11.00 g, 66.3 mmol) and I$_2$ (6.00 g, 23.64 mmol) were added. The reaction mixture was stirred at room temperature for 4 h whereupon saturated Na$_2$S$_2$O$_3$ solution (50 mL) was added to quench the reaction. The mixture was extracted with ethyl acetate three times. The combined organic extracts were washed with water followed by brine, dried over anhydrous Na$_2$SO$_4$, and filtered through a pad of Celite. The filtrate was concentrated and the residue was purified by silica gel column chromatography using a mixture of ethyl acetate and petroleum ether (v/v 1:9) as eluent to give K as a pale
yellow solid (2.83 g, 71%); $^1$H NMR (CDCl$_3$) $\delta$ 7.50 (d, J = 8.5 Hz, 1H), 7.16 (d, J = 2.2 Hz, 1H), 6.84 (dd, J = 2.2, 8.5 Hz, 1H), 5.39 (s, 1H).

A mixture of K (500 mg, 1.67 mmol), tert-butyl acrylate (0.73 mL, 5.02 mmol), palladium (II) acetate (7.5 mg, 0.033 mmol) and tri-ortho-tolyl phosphine (41 mg, 0.13 mmol) in redistilled triethylamine (5 mL) was heated at reflux overnight under N$_2$ to give a dark grey suspension. After cooling to room temperature, all volatile components were subsequently pumped off. The resultant residue was dissolved in ethyl acetate and the precipitate was filtered off. The filtrate was evaporated and the residue obtained was purified by column chromatography using a mixture of ethyl acetate and petroleum ether (v/v 1:6) as eluent to give L (160 mg, 28%) as an off-white solid. $^1$H NMR (DMSO-d$_6$) $\delta$ 10.43 (s, 1H), 7.75 (d, J = 16.4 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.45 (d, J = 16.0 Hz, 1H), 7.18 (d, J = 8.0 Hz, 1H), 7.07 (d, J = 0.8 Hz, 1H), 6.56 (d, J = 16.4 Hz, 1H), 6.41 (d, J = 15.6 Hz, 1H), 1.48 (s, 9H), 1.48 (s, 9H).

HRMS (ESI) found m/z 369.1687 (M + Na). C$_{20}$H$_{26}$NaO$_5$ requires 369.1672.

To a solution of L (160 mg, 0.46 mmol) in DMF (2 mL) was added K$_2$CO$_3$ (254 mg, 1.84 mmol) and CH$_3$I (0.28 mL, 4.50 mmol). The mixture was stirred at room temperature overnight and the precipitate was filtered off. The resultant filtrate was washed with water followed by brine, dried over anhydrous Na$_2$SO$_4$, and filtered through a pad of Celite. The solvent was removed and the resultant residue was purified by silica gel column chromatography using a mixture of ethyl acetate and petroleum ether (v/v 1:10) as eluent to give M as a colorless oil (72 mg, 43%); $^1$H NMR (CDCl$_3$) $\delta$ 7.87 (d, J = 16.2 Hz, 1H), 7.54 (d, J = 15.9 Hz, 1H), 7.49 (d, J = 8.0 Hz, 1H), 7.10 (dd, J = 1.3, 8.0 Hz, 1H), 7.00 (d, J = 1.2 Hz, 1H), 6.47 (d, J = 16.1 Hz, 1H), 6.38 (d, J = 15.9 Hz, 1H), 3.91 (s, 3H), 1.539 (s, 9H), 1.53 (s, 9H). HRMS (ESI) found m/z 383.1838 (M + Na). C$_{21}$H$_{28}$NaO$_5$ requires 383.1829.

To a solution of M (70 mg, 0.19 mmol) in CH$_2$Cl$_2$ (2 mL) at room temperature was added TFA (1 mL, 12.98 mmol). The mixture was stirred for 2.5 h at room temperature to give
a white suspension. All volatile components were subsequently pumped off, and the resultant residue was triturated with CH₂Cl₂ and ethyl acetate to give N as a white solid (41 mg, 85%).

\(^1\)H NMR (DMSO) δ 12.41 (br s, 2H), 7.80 (d, J = 16.2 Hz, 1H), 7.72 (d, J = 8.0 Hz, 1H), 7.58 (d, J = 16.0 Hz, 1H), 7.41 (d, J = 1.2 Hz, 1H), 7.30 (dd, J = 1.0, 8.1 Hz, 1H), 6.47 (d, J = 16.0 Hz, 1H), 6.38 (d, J = 16.1 Hz, 1H), 3.92 (s, 3H). HRMS (ESI) found m/z 271.0573 (M + Na). C₁₃H₁₂NaO₅ requires 271.0577.

A mixture of O (prepared as described in: J. Med. Chem. 2003, 46, 2132 or J. Med. Chem. 2012, 55, 766; 161 mg, 0.48 mmol), N (40 mg, 0.16 mmol), EDCI hydrochloride (185 mg, 0.97 mmol) and toluenesulfonic acid (2.8 mg, 0.016 mmol) in dimethylacetamide (1 mL) was stirred at room temperature overnight. All the volatile components were pumped off and the resultant residue was triturated with methanol to give a yellow solid. This material was dissolved in THF and precipitated by the addition of methanol to afford 9 as a yellow solid (45 mg, 41%, HPLC purity 98%); \(^1\)H NMR (DMSO-d₆) δ 10.43 (s, 2H), 8.12-8.10 (m, 4H), 8.00-7.95 (m, 2H), 7.85-7.80 (m, 2H), 7.72 (d, J = 15.4 Hz, 1H), 7.54-7.50 (m, 4H), 7.37-7.26 (m, 4H), 4.57-4.45 (m, 4H), 4.28-4.22 (m, 2H), 4.00-3.99 (m, 5H), 3.90-3.83 (m, 2H). HRMS (ESI) found m/z 701.1596 (M + Na). C₃₉H₃₂Cl₂N₂NaO₅ requires 701.1580.
Compound 10.

Key Intermediate T

Final Assembly
To the mixture of compound A (1.5 g, 5.34 mmol) and compound P (2.74 g, 21.36 mmol) in 1,4-dioxane (1.0 mL) was added DIEA (3.45 g, 26.7 mmol) and Pd(t-Bu3P)2 (545.8 mg, 1.07 mmol). The reaction mixture was stirred at 120 °C for 1.0 h under microwave irradiation whereupon TLC (10% EtOAc in petroleum ether, Rf = 0.4) showed the reaction had gone to completion. The mixture was poured into EtOAc (200 mL) and was washed with brine (50 mL x 3). The organic phase was dried over Na2SO4, concentrated and the residue was purified by chromatography on silica (solvent gradient: 0-20% EtOAc in petroleum ether) to afford the desired product Q (1.13 g, 56%) as a brown solid. 1H NMR (400 MHz, CDCl3) δ 8.14 (s, 1H), 7.99 (d, J = 16 Hz, 1H), 7.74 (dd, J = 8.0, 2.0 Hz, 1H), 7.67 (d, J = 8.4 Hz, 1H), 7.58 (d, J = 16.4 Hz, 1H), 6.50 (d, J = 16.0 Hz, 1H), 6.35 (d, J = 16.0 Hz, 1H), 1.56 (s, 18H).

To the mixture of compound Q (0.50 g, 1.33 mmol) in ethanol (20 mL) and water (20 mL) was added iron (0.37 g, 6.66 mmol) and NH4Cl (0.71 g, 13.3 mmol). The reaction mixture was stirred at 80 °C for 2 h whereupon TLC (33% EtOAc in petroleum ether, Rf = 0.3) showed starting material was consumed. The mixture was filtered through a pad of Celite, and the filtrate was diluted with water (80 mL) and extracted with EtOAc (80 mL × 2). The combined organic layers were washed with brine (50 mL), dried over Na2SO4, and filtered. The filtrate was concentrated, and the residue was purified by flash column chromatography (0-10% EtOAc in petroleum ether), to give compound R (0.16 g, 35%) as a brown solid. 1H NMR (400 MHz, CDCl3) δ 7.68 (d, J = 16 Hz, 1H), 7.46 (d, J = 16 Hz, 1H), 7.37 (d, J = 8 Hz, 1H), 6.92 (d, J = 8.0 Hz, 1H), 6.81 (s, 1H), 6.32 (d, J = 16 Hz, 2H), 1.53 (s, 18H).

A mixture of TsOH (1.93 g, 10.13 mmol), EDCI (7.77 g, 40.53 mmol), compound R (7.0 g, 20.26 mmol) and Fmoc-beta-Ala-OH (S, 9.46 g, 30.4 mmol) in DMA (20 mL) was stirred at 20 °C for 15 h. TLC (25% EtOAc in petroleum ether, Rf = 0.5) showed starting
material was consumed and a new spot was generated. The mixture was concentrated, and the residue was purified by flash column chromatography (0-20% EtOAc in petroleum ether) to give compound T (6.8 g, 52%) as a yellow solid. LCMS: RT = 1.05 min, m/z = 661.1 [M+23]+.

$^1$HNMR (400 MHz, CDCl$_3$)  δ 8.02 (s, 1H), 7.87 (s,1H), 7.70-7.68 (m, 2H), 7.64-7.58 (m, 5H), 7.57-7.37 (m, 2H), 7.35 (s,1H), 6.41 (d, J = 16 Hz, 1H), 6.35 (d, J = 16 Hz, 1H), 5.83 (brs, 1H), 4.43-4.41 (m, 2H), 4.21-4.19 (m, 1H), 3.60 (br, 2H), 2.69 (br, 2H), 1.54 (s, 9H), 1.51 (s, 9H).

A solution of compound T (0.80 g, 1.25 mmol) and piperidine (213 mg, 2.5 mmol) in DMF (10 mL) was stirred at 25 °C for 1 h. The mixture was concentrated to give compound U (400 mg, 77%) as a yellow solid, which was used directly in the next step. LCMS: RT = 0.69 min, m/z = 439.1 [M+23]+.

To a solution of compound V (400 mg, 0.96 mmol) and compound U (617 mg, 1.25 mmol) in DMF (8.0 mL) at 25 °C was added DIEA (0.33 mL, 1.92 mmol). The mixture was stirred at 25 °C for 12 h. The mixture was then concentrated and the residue was purified by flash chromatography on silica gel eluting with 10-50% CH$_3$OH in CH$_2$Cl$_2$ to give compound W (200 mg, 28%) as a white solid. LCMS: RT = 0.80 min, m/z = 574.1 [M+23]+.

To a solution of compound W (200 mg, 0.27 mmol) and compound X (prepared as described in WO 2016/205176; 283 mg, 0.70 mmol) in DMF (10.0 mL) was added DIEA (135 mg, 1.05 mmol). The mixture was stirred at 25 °C for 12 h then was concentrated. The residue was purified by flash chromatography on silica gel eluting with 0-10% CH$_3$OH in CH$_2$Cl$_2$ to afford compound Y (220 mg, 71%) as a colorless oil. LCMS: RT = 0.92 min, m/z = 864.5 [M+1]+.

To a solution of compound Y (220 mg, 0.25 mmol) in CH$_2$Cl$_2$ (3.0 mL) was added TFA (3.0 mL, 2.55 mmol) at 25 °C. The mixture was stirred at 25 °C for 1 h then was concentrated to give compound Z (180 mg, 94%) as a white solid. LCMS: RT = 0.72 min, m/z = 752.3
[M+1]+; ¹H NMR (400 MHz, DMSO-d₆) δ 9.93 (s, 2H), 8.13 (s, 1H), 7.82-7.46 (m, 8H), 6.98 (s, 2H), 6.54 (d, J = 16.0 Hz, 2H), 5.98 (brs, 1H), 5.41 (brs, 2H), 4.22 (brs, 1H), 3.03-2.90 (m, 6H), 2.67-2.50 (m, 4H), 2.36 (brs, 4H), 1.69 (brs, 3H), 1.46-1.33 (m, 7H), 1.23-1.16 (m, 2H).

To a solution of Compound Z (110 mg, 0.15 mmol) and compound AA (prepared as described in WO 2016/205176; 187 mg, 0.45 mmol) in DMF (5.0 mL) was added EDCI (278 mg, 1.45 mmol), TsOH (92 mg, 0.48 mmol) and 4A molecular sieves. The mixture was stirred at 25 °C for 12 h then was purified by prep-HPLC (acetonitrile 62–80% / 0.1% NH₄HCO₃ in water) to give the compound BB (10 mg, 4.4%) as a yellow solid.

To a solution of compound BB (10.0 mg, 0.010 mmol) in CH₂Cl₂ (1.0 mL) was added TFA (0.5 mL, 0.07 mmol) at 0 °C. The mixture was stirred at 0 °C for 30 min, then was concentrated to give compound 10 (3.8 mg, 42%) as a yellow solid. LCMS: RT = 1.66 min, m/z = 670.7 [M/2+1]+; HRMS (30-100_1_4MIN_CD): 1340.3751 [M-1]-.
**Compound 11.**

*Key Intermediate JJ*

A mixture of compound CC (2.0 g, 7.94 mmol), compound P (5.1 g, 39.8 mmol), Pd(t-Bu₃P)₂ (0.200 g, 0.40 mmol) and DIPEA (5.13 g, 39.7 mmol) in 1,4-dioxane (8.0 mL) was
stirred under microwave irradiation for 1 h at 120 °C. The reaction mixture was cooled to 25 °C, filtered, and the filtrate was diluted with EtOAc (50 mL). The organic layer was washed with 1.0 M citric acid (20 mL × 3) and brine (20 mL) then was dried (Na₂SO₄), filtered, and concentrated. The residue was purified by column chromatography on silica gel (petroleum ether) to afford compound DD (2.1 g, 76%) as a yellow solid. The reaction was repeated four times to generate 9.0 g of compound DD in total. ¹H NMR (400 MHz, DMSO- d₆) δ 10.43 (s, 1H), 7.75 (d, J = 16.4 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.45 (d, J = 16.0 Hz, 1H), 7.18 (d, J = 8.0 Hz, 1H), 7.07 (d, J = 0.8 Hz, 1H), 6.56 (d, J = 16.4 Hz, 1H), 6.41 (d, J = 15.6 Hz, 1H), 1.48 (d, J = 2.0 Hz, 18H).

To a stirred solution of compound DD (9.0 g, 26 mmol) in dry THF (600 mL) was added compound EE (9.36 g, 28.6 mmol), Ph₃P (10.22 g, 39.0 mmol), and DIAD (7.88 g, 39.0 mmol) at 0 °C. The mixture was stirred at 0 °C for 1 h and then at 25 °C for an additional 15 h. The mixture was subsequently concentrated and the residue was purified by column chromatography (petroleum ether:EtOAc = 3:1) to give compound FF (21.0 g, 123%) as a white solid. LCMS : (5-95 AB, 1.5 min), 1.047 min, MS = 678.1 [M+23].

To a mixture of compound FF (20.0 g, 30.5 mmol) in DMF (200 mL) was added piperidine (12.5 g, 147 mmol). The mixture was stirred at 25 °C for 2 h whereupon TLC (10% CH₃OH in CH₂Cl₂, Rf = 0.4) indicated the reaction was completed. The reaction mixture was diluted with EtOAc (80 mL), and the mixture was washed with water (30 mL x 3). The organic layer was dried over Na₂SO₄, filtered, and concentrated in vacuo. The residue was purified column chromatography (10% CH₃OH in CH₂Cl₂) to give compound GG (8.5 g, 64%) as a brown oil.

To a stirred solution of compound GG (7.5 g, 17.3 mmol) in acetonitrile (160 mL) was added triethylamine (5.25 g, 51.9 mmol) and compound HH (4.02 g, 26.0 mmol). The mixture
was stirred at 80 °C for 2 h whereupon LCMS \{(5-95/1.5min): RT (220/254 nm) = 1.03 min, [M+Na]+536.1\} showed 76% of desired product. The mixture was cooled to 25 °C and concentrated to give crude product. This material was purified by flash chromatography on silica (20 % EtOAc in petroleum ether) to give compound II (6.50 g, 73%) as a light yellow oil. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.86 (s, 1H), 7.82 (s, 1H), 7.54-7.47 (m, 2H), 7.26 (s, 2H), 7.09 (d, $J = 8.0$ Hz, 1H), 7.03-6.98 (m, 1H), 6.69 (s, 2H), 6.53-6.31 (m, 2H), 4.18-4.13 (m, 2H), 3.90-3.86 (m, 2H), 3.78-3.71 (m, 4H), 2.04 (s, 1H), 1.53 (d, $J = 2.9$ Hz, 18H), 1.28-1.23 (m, 1H).

To a solution of compound II (6.50 g, 12.7 mmol) in CH$_2$Cl$_2$ (60 mL) was added TFA (15 mL). The mixture was stirred at 20 °C for 1 h whereupon LCMS \{(5-95/1.5min): RT (220/254 nm) = 0.73 min, [M+Na]+424.0\} showed 99% of desired product. After the mixture was concentrated to give the crude product, MTBE (80 mL) was added. The mixture was stirred for 0.5 h and the resulting solid was collected by filtration, washed with MTBE (30 mL x 2) and dried to give compound JJ (4.30 g, 84%) as a yellow solid. $^1$H NMR (400 MHz, DMSO-$_d_6$) δ 12.40 (br d, $J = 4.3$ Hz, 2H), 7.81-7.64 (m, 2H), 7.55 (d, $J = 15.8$ Hz, 1H), 7.38 (s, 1H), 7.27 (d, $J = 8.2$ Hz, 1H), 6.94 (s, 2H), 6.68-6.52 (m, 2H), 4.22-4.18 (m, 2H), 3.79-3.74 (m, 2H), 3.59 (s, 4H).

To a solution of compound JJ (335 mg, 0.83 mmol) and compound AA (prepared as described in WO 2016/205176; 782 mg, 1.78 mmol) in pyridine (18 mL) was added T3P (5.5 mL, 4.17 mmol). The mixture was stirred at 0 °C for 1 h whereupon LCMS \{(5-95AB/1.5min): RT = 1.16 min, [M+1]+992.1\} showed 88% of desired product. The reaction was quenched with ice-water (30 mL) and extracted with EtOAc (50 mL x 3). The combined organic layers were washed with brine (30 mL x 2) then were dried over Na$_2$SO$_4$, filtered and concentrated. The crude product thus obtained was treated with hexane/MTBE (50 mL, 10:1) to give compound KK (1.21 g, 56%) as a yellow solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 8.71 (br s, 1H),
8.24 (br d, J = 8.4 Hz, 2H), 7.95 (br d, J = 16.1 Hz, 1H), 7.82 (br d, J = 15.0 Hz, 1H), 7.71 (br d, J = 8.4 Hz, 2H), 7.52 (br t, J = 7.6 Hz, 2H), 7.43-7.35 (m, 2H), 7.11 (br d, J = 1.1 Hz, 1H), 6.92 (br d, J = 14.8 Hz, 1H), 6.66 (s, 2H), 4.53 (br d, J = 10.4 Hz, 2H), 4.47-4.35 (m, 2H), 4.26 (br s, 2H), 4.13 (br d, J = 6.6 Hz, 2H), 4.01-3.92 (m, 4H), 3.77 (br s, 4H), 3.55-3.44 (m, 2H), 1.63-1.51 (m, 36H).

To a solution of compound KK (720 mg, 0.59 mmol) in CH₂Cl₂ (18 mL) was added a TFA solution (20% in CH₂Cl₂, 18 mL) at 0 °C. The mixture was stirred at 25 °C for 1 h, then all the volatile components were removed under vacuum. The residue was triturated with EtOAc (30 mL) to give the compound 11 (567 mg, 97%) as a yellow solid. ¹H NMR (400 MHz, DMSO-d₆) δ 8.59-8.57 (m, 2H), 8.10 (br d, J = 8.0 Hz, 2H), 7.92-7.83 (m, 4H), 7.69 (d, J = 15.6 Hz, 1H), 7.57-7.40 (m, 6H), 7.32 (br s, 2H), 6.95 (s, 2H), 4.74-4.53 (m, 4H), 4.47-4.33 (m, 4H), 3.97-3.85 (m, 6H), 3.65-3.63 (m, 4H).

¹H NMR (400 MHz, DMSO-d6) δ 8.59-8.57 (m, 2H), 8.10 (d, J = 8.0 Hz, 2H), 7.92-7.83 (m, 4H), 7.69 (d, J = 15.6 Hz, 1H), 7.57-7.40 (m, 6H), 7.32 (br s, 2H), 6.95 (s, 2H), 4.74-4.53 (m, 4H), 4.47-4.33 (m, 4H), 3.97-3.85 (m, 6H), 3.65-3.63 (m, 4H).
To a stirred solution of compound **LL** (20.0 g, 99.9 mmol) in EtOH (20 mL) and water (5.0 mL) was added aqueous 85% KOH (5.6 g, 99.9 mmol) in water (5 mL) at 18 °C. The reaction mixture was stirred at 80 °C for 4 h whereupon LCMS {((5-95AB/1.5 min): RT = 0.69 min, [M+H]+172.7} showed 53% of desired product. The mixture was concentrated and the residue was partitioned between EtOAc (100 mL) and H₂O (130 mL). The aqueous phase was acidified with HCl (2.0 M) until pH = 3 and then extracted with EtOAc (120 mL x 2). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated to give compound **MM** (13 g, 76%) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ = 8.53 (brs, 1H), 4.27-4.16 (m, 2H), 2.59-2.55 (m, 4H), 2.03-1.94 (m, 2H), 1.27-1.23 (m, 3H).

To a solution of compound **MM** (12.0 g, 69.7 mmol) and N-hydroxsuccinimide (8.42 g, 73.2 mmol) in THF (200 mL) was added DCC (15.1 g, 73.2 mmol) at 18 °C. The reaction
mixture was stirred at 18 °C for 12 h whereupon TLC (50% EtOAc in petroleum ether, Rf = 0.6) showed that the starting material was consumed. The resulting solid was filtered off, and the filtrate was concentrated to give compound NN (14 g, 75%) as a colorless oil. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 4.27-4.16 (m, 2H), 2.90-2.56 (m, 8H), 2.08-1.84 (m, 2H), 1.32-1.25 (m, 3H).

NaHCO\(_3\) (13.0 g, 154 mmol) and compound NN (13.8 g, 51.4 mmol) were added sequentially to compound OO (9.0 g, 51.4 mmol) in a mixture of 1,2-dimethoxyethane (200 mL) and water (200 mL) at 18 °C. The reaction mixture was allowed to stir at 18 °C for 10 h whereupon TLC (EtOAc, Rf = 0.4) showed that the reaction had gone to completion. The mixture was concentrated to dryness, and the residue was partitioned between EtOAc (300 mL) and H\(_2\)O (200 mL). The aqueous phase was acidified with HCl (1.0 M) to pH = 3 and then concentrated to give the compound PP (12 g, 71%) as a colorless oil.

To a solution of compound PP (3.0 g, 9.11 mmol) and DCC (1.86 g, 9.03 mmol) in THF (25 mL) and DMF (25 mL) was added HOSu (0.95g, 8.26 mmol) at 18 °C. The resulting mixture was stirred at 18 °C for 12 h then was filtered. The filtrate was concentrated to give compound QQ (3.5 g, 90%) as a colorless oil. LCMS: RT = 0.65 min, m/z = 427.1 [M+1]+.

To a solution of compound U (250 mg, 0.60 mmol) in dry DMF (10 mL) was added Et\(_3\)N (182 mg, 1.8 mmol) and compound QQ (512 mg, 1.2 mmol). The mixture was stirred at 18 °C for 3 h, then water (30 mL) was added and the mixture was extracted with EtOAc (30 mL \(\times\) 3). The combined organic layers were washed with brine (30 mL \(\times\) 3), dried over Na\(_2\)SO\(_4\), filtered, and concentrated to give the crude product. This material was washed with petroleum ether (30 mL \(\times\) 4) and MTBE (45 mL \(\times\) 2) and dried to give compound RR (260 mg, 60%) as a yellow solid. LCMS: RT = 0.92 min, m/z = 728.3 [M+1]+.

*Key Intermediate WW*
To a solution of compound SS (4.00 g, 10.25 mmol) in THF (50 mL) was added benzylamine (1.21 g, 11.3 mmol) at 25 °C. The reaction mixture was stirred at 25 °C for 18 h then was concentrated. The residue was dissolved in EtOAc (80 mL) and was washed with HCl (2.0 M, 20 mL). The organic layer was dried over Na$_2$SO$_4$, filtered, and was concentrated. The residue was purified by flash column chromatography (eluting with 0~50% of EtOAc in petroleum ether) to give compound TT (1.60 g, 445%) as a colorless oil. $^1$H NMR (400 MHz, CDCl$_3$) δ 5.75 (brs, 2H), 5.54–5.38 (m, 2H), 5.18–5.16 (m, 1H), 4.45–4.41 (m, 2H), 4.12–4.03 (m, 2H), 2.14–1.98 (m, 12H).

To a solution of compound TT (1.60 g, 4.59 mmol) and CCl$_3$CN (3.32 g, 23.0 mmol) in CH$_2$Cl$_2$ (25 mL) was added DBU (140 mg, 0.92 mmol) dropwise at 0 °C. The resulting mixture was stirred at 0 °C for 2 h then was diluted with CH$_2$Cl$_2$ (50 mL) and washed with water (30 mL) and brine (30 mL). The organic layer was dried over Na$_2$SO$_4$, filtered, and was concentrated. The residue was purified by a flash column chromatography (eluting with 0~50% of EtOAc in petroleum) to give compound UU (2.00 g, 88%) as a white solid. $^1$H NMR (400 MHz, CDCl$_3$) δ 8.67 (s, 1H), 6.60 (d, $J = 3.6$ Hz, 1H), 5.57 (d, $J = 4.0$ Hz, 1H), 5.46–5.36 (m, 2H), 4.43–4.41 (m, 1H), 4.17–4.10 (m, 2H), 2.18 (s, 3H), 2.05–2.03 (m, 9H).

A solution of BF$_3$-Et$_2$O (53.1 mg, 0.37 mmol) in CH$_2$Cl$_2$ (2 mL) was added to a mixture of compound VV (prepared as described in WO 2016/205176; 250 mg, 0.75 mmol), compound TT (480 mg, 0.97 mmol) and 4A molecular sieves in CH$_2$Cl$_2$ (60 mL) at -20 °C. The reaction mixture was stirred at -20 °C for 1 h. An additional portion of BF$_3$-Et$_2$O (319 mg, 2.25 mmol) in CH$_2$Cl$_2$ (4.0 mL) was added, and the mixture was stirred at 0 °C for 1 h. The mixture was
subsequently filtered, and the filtrate was washed with sat. NH₄Cl (25 mL) and concentrated to give the crude WW product as a gray solid. LCMS: RT = 0.76 min, m/z = 564.0 [M+1]⁺.

**Final Assembly**

TFA (4.00 mL, 1.65 mmol) was added to a mixture of compound QQ (600 mg, 0.82 mmol) in CH₂Cl₂ (10.0 mL) and the mixture was stirred at 25 °C for 2 h. The reaction mixture was subsequently concentrated and the orange solid thus obtained was washed with EtOAc (25 mL x 3) to give compound XX (500 mg, 99%). LCMS: RT = 0.66 min, m/z = 616.2 [M+H]⁺.

TsOH (25 mg, 0.13 mmol) and compound WW (440 mg, 0.78 mmol) were added sequentially to a solution of compound XX (160 mg, 0.26 mmol) and EDCI (399 mg, 2.08 mmol) in dimethylacetamide (10 mL). The mixture was stirred at 25 °C for 12 h, then was concentrated. The residue was purified by flash column chromatography (eluting 0-10% CH₃OH in CH₂Cl₂) to give compound YY (420 mg, 93%) as a yellow solid. LCMS: RT = 0.90 min, m/z = 854.6 [M/2+1]⁺.
LiOH (51.6 mg, 1.23 mmol) was added to a solution of compound YY (420 mg, 0.25 mmol) in a mixture of CH$_3$OH (5.0 mL), THF (5.0 mL), and water (3.0 mL). The mixture was stirred at 25 °C for 1 h then was concentrated. Water (10 mL) and HCl (2.0 M) were added to the residue to adjust the pH to 6, and the resulting mixture was freeze-dried to give crude compound ZZ (330 mg, 100%) as a red solid. LCMS: RT = 1.32 min, m/z = 1340.0 [M-1]-.

DIEA (158 mg, 1.23 mmol) was added to a solution of compound ZZ (330 mg, 0.25 mmol) and HATU (112 mg, 0.29 mmol) in DMF (8.0 mL). The mixture was stirred at 25 °C for 10 min whereupon compound AAA (107 mg, 0.49 mmol) was added. The resulting mixture was stirred at 25 °C for 30 min then was purified by prep-HPLC (acetonitrile 50-80/0.225% FA in water) to afford compound 12 (65 mg, 17%) as a yellow solid. HRMS: m/z = 1506.5228 [M+1]+, 1508.5233[M+1+2]+.
Synthesis of TDCs

Anti-CD22 10F4v3 LC-K149C, anti-CD22 10F4v3 LC-V205C, anti-CD22 10F4v3 HC-A140C, anti-NaPi2b 10H1.11.4B LC-K149C, anti-Ly6E 9B12.v12 LC-K149C, and anti-gD 5B6 LC-K149C were conjugated to compounds 10, 11, and 12 via the engineered LC-K149C, LC-V205C, or HC-A140C cysteine residues. Solutions of a given antibody at 10 mg/mL in 50 mM Tris pH 8.5 were reduced with 50 molar excess of DTT at ambient temperature for 16-18 hours. The reduced antibody was purified using SP HP cationic exchange chromatography. The purified antibody in 50 mM Tris pH 8 was re-oxidized using 15 molar excess of DHAA dissolved in DMA at ambient temperature for 2-3 hours. The antibody was again purified using SP HP cationic exchange column to remove DHAA and aggregates. Three to five-fold molar equivalents of compounds 10, 11, or 12 in DMF were added to a 5 to 10 mg/mL solution of the purified antibody in 100 mM Tris pH 7.5 followed by additional DMF for a final concentration of 10% DMF. The coupling reaction was then incubated at ambient temperature for 3 to 4 hours. The conjugated antibody was purified using cationic exchange chromatography using HiTrap SP HP resin. The resulting purified conjugate was formulated using dialysis with 10 kDa MWCO Slide-a-Lyzer dialysis cassette into 20 mM histidine acetate pH 5.5, 240 mM sucrose, 0.02% polysorbate-20.

TDCs produced typically afforded protein yields of 50 to 90%. All of the TDCs were characterized in regards to aggregation (SEC HPLC), drug to antibody ratio (LC/MS), amount of free drug (LC-MS) present in the final TDCs and endotoxin (LAL assay). Detailed characterization data for each conjugate are provided in Table S4 below.
Table S4. TDCs prepared from compounds 10, 11, and 12.

<table>
<thead>
<tr>
<th>Conjugate Name (antigen-site-linker-drug)</th>
<th>Site&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DAR&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Aggregation&lt;sup&gt;c&lt;/sup&gt; (%)</th>
<th>Free LD&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCD22-LC-K149C-10</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>1.2 / 0</td>
<td>&lt;5 / 1</td>
<td>54 / 78</td>
</tr>
<tr>
<td>aNaPi2b-LC-K149C-10</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>1.4</td>
<td>&lt;5</td>
<td>67</td>
</tr>
<tr>
<td>aLy6E-LC-K149C-10</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>0.9 / 1.0</td>
<td>&lt;5 / 2</td>
<td>83 / 84</td>
</tr>
<tr>
<td>agD-LC-K149C-10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>2.8 / 3.4</td>
<td>&lt;5 / 5</td>
<td>86 / 80</td>
</tr>
<tr>
<td>aCD22-LC-K149C-11</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>1.0 / 3.2</td>
<td>&lt;1 / &lt;1</td>
<td>64 / 58</td>
</tr>
<tr>
<td>aNaPi2b-LC-K149C-11</td>
<td>LC-K149C</td>
<td>1.8</td>
<td>1.5</td>
<td>&lt;1</td>
<td>73</td>
</tr>
<tr>
<td>aLy6E-LC-K149C-11</td>
<td>LC-K149C</td>
<td>2.0</td>
<td>1.2</td>
<td>&lt;5</td>
<td>74</td>
</tr>
<tr>
<td>aCD22-LC-V205C-10</td>
<td>LC-V205C</td>
<td>2.0</td>
<td>1.1</td>
<td>&lt;2</td>
<td>81</td>
</tr>
<tr>
<td>aCD22-HC-A140C-10</td>
<td>HC-A140C</td>
<td>2.0</td>
<td>0.6</td>
<td>&lt;2</td>
<td>70</td>
</tr>
<tr>
<td>aCD22-HC-A140C-11</td>
<td>HC-A140C</td>
<td>1.9</td>
<td>1.2</td>
<td>&lt;1</td>
<td>67</td>
</tr>
<tr>
<td>aCD22-LC-K149C-12&lt;sup&gt;e&lt;/sup&gt;</td>
<td>LC-K149C</td>
<td>1.9</td>
<td>2.0</td>
<td>&lt;5</td>
<td>66</td>
</tr>
</tbody>
</table>

<sup>a</sup>Site of Cys mutation used for linker attachment. The nomenclature used to depict the mAb attachment sites follows that described in: E. A. Kabat, T. T. Wu, C. Foeller, H. M. Perry, K. S. Gottesman “Sequences of Proteins of Immunological Interest” Diane Publishing, 1992 ISBN 094137565X.  
<sup>b</sup>Drug-antibody ratio.  
<sup>c</sup>Percent aggregated material observed during conjugation process. Such aggregates were separated from the TDCs during subsequent purification process.  
<sup>d</sup>Amount of unconjugated linker-drug present in purified TDCs.  
<sup>e</sup>Viral antigen (non-targeting control). Multiple entries reflect separate (repeat) preparations of the same conjugate.