**Supplementary Methods.**

**“A new, triglycyl peptide linker for antibody-drug conjugates (ADCs) with improved targeted killing of cancer cells”**

**Supplementary Methods (S1-S3).** Supplementary Method S1: Synthesis of di-, tri-, tetra-glycyl, and valine-citrulline-glycine peptide linkers and catabolites. Supplementary Method S2: Test of *in vitro* cytotoxicity, cell-cycle inhibition, and bystander cytotoxic activity of ADCs. Supplementary Method S3: Catabolism of [3H]-labeled and non-radiolabeled CX and SMCC ADCs by cancer cells.

**Supplementary Method S1**: Synthesis of di-, tri-, tetra-glycyl, and valine-citrulline-glycine peptide linkers and catabolites.

C:\Users\rsingh\Desktop\CX Article, 4-2014\Revised CX Linker, 2-19-2016\Figs, Revised manuscript\Revised Supplementary Figures S1-4, 3-9-2016\Scheme synthesis, Supplementary method S1.tif

Benzyloxycarbonyl (CBz)-protected di-, tri-, or tetra-glycyl peptides (**2a-2c**) were coupled to the *tert*-butyl ester of -alanine (**3**) followed by hydrogenolysis of the CBz protecting group to give **5a-5c**. The carboxy-protected peptides (**5a-5c**) were coupled with 4-maleimidobutyric acid (**6**) to give **7a-7c**, followed by cleavage of the *tert*-butyl ester with trifluoroacetic acid to give **8a-8c**. Each of these was then converted to the activated NHS ester (**1a-1c**) using *N*-(3-dimethylaminopropyl)-*N’*-ethylcarbodiimide hydrochloride (EDC) and NHS.

**General method for the preparation of Z-(Gly)n-β-Ala-OtBu (4a – 4c):** In a 100 mL flask, Z-Glyn-OH (**2a**-**2c**, 4.0 mmol), tert-butyl-3-aminopropionate (4.0 mmol), hydroxybenzotriazole (0.651 g, 4.25 mmol) and *N*-(3-dimethylaminopropyl)-*N’*-ethylcarbodiimide hydrochloride (EDC, 0.81 g, 4.23 mmol) were dissolved in dimethylformamide (20 mL) and reacted for 3 h at room temperature. The solvent volume was reduced under vacuum to approximately (10 mL) and crude product was precipitated by trituration with anhydrous diethyl ether (45 mL) and collected by vacuum filtration and washed with diethyl ether (35 mL).

**Z-Gly2-β-Ala-OtBu (4a)**: 60.2% yield as a white solid, 1H NMR (400 MHz, DMSO-*d*6) δ 1.40 (s, 9H), 2.36 (t, *J* = 7.0 Hz, 2H), 3.10 – 3.37 (m, 2H), 3.53 – 3.76 (m, 4H), 5.04 (s, 2H), 7.25 – 7.39 (m, 5H), 7.43 – 7.58 (m, 1H), 7.76 – 7.93 (m, 1H), 8.00 – 8.25 (m, 1H). HRMS calcd. (M+Na)+ 416.1792; found 416.1788.

### **Z-Gly3-β-Ala-OtBu (4b):** 75% yield as a white solid, 1H NMR (d6-DMSO) δ 1.39 (s, 9H), 2.35 (t, *J* = 6.8 Hz, 2H), 3.25 (q, *J* = 6.1 Hz, 2H), 3.67 (t, *J* = 6.4 Hz, 4H), 3.74 (d, *J* = 5.6 Hz, 2H), 5.04 (s, 2H), 7.25 – 7.4 (m, 5H), 7.82 (t, *J* = 5.2 Hz, 1H), 8.10 (t, *J* = 5.2 Hz, 1H), 8.16 (t, *J* = 5.2 Hz,1H); 13C NMR (d6-DMSO) 27.70, 34.78, 34.89, 41.90, 42.10, 43.56, 65.51, 79.89, 127.69, 127.76, 128.30, 136.94, 156.49, 168.63, 169.00, 169.61, 170.45. HRMS (M +Na)+ Calcd. 473.2012; found 473.1995.

**Z-Gly4-β-Ala-OtBu (4c):** 50% yield as a white solid,1H NMR (400 MHz, DMSO-d6) δ 1.39 (s, 9H), 2.34 (t, J = 7.0 Hz, 2H), 3.24 (q, J = 6.8 Hz, 2H), 3.66 (dd, J = 8.8, 6.0 Hz, 4H), 3.74 (dd, J = 11.1, 5.7 Hz, 4H), 5.03 (s, 2H), 7.26 – 7.40 (m, 5H), 7.48 (t, J = 6.1 Hz, 1H), 7.82 (t, J = 5.7 Hz, 1H), 8.04 – 8.11 (m, 1H), 8.10 – 8.20 (m, 2H). HRMS (M+Na)+ calcd: 530.2221; found: 530.2217.

**General method for the preparation of (Gly)n-β-Ala-OtBu (5a-5c):** To a 250 mL capacity Parr shaker flask was added a solution of Z-Glyn-β-Ala-OtBu (**4a** – **4c**, 2.89 mmol) in 95:5 methanol:deionized water (80 mL) and 10% palladium on carbon (0.12 g). The flask was shaken under a hydrogen atmosphere (42 PSI) for 3 h then vacuum filtered through celite filter aid. The filtrate was concentrated by rotary evaporation under vacuum.

**Gly2-β-Ala-OtBu** (**5a**): 100% yield as a colorless oil, 1H NMR (400 MHz, DMSO-*d*6) δ 1.39 (s, 9H), 2.35 (t, *J* = 7.0 Hz, 2H), 3.00 – 3.19 (m, 3H), 3.21 – 3.27 (m, 2H), 3.31 (s, 1H), 7.84 – 7.95 (m, 1H), 8.08 (s, 1H). HRMS calcd. (M+Na)+ 282.1424; found 282.1418.

**Gly3-β-Ala-OtBu (5b):** 96% yield as a colorless oil, 1H NMR (d6-DMSO) δ 1.39 (s, 9H), 2.35 (t, *J* = 6.8 Hz, 2H), 3.13 (s, 2H), 3.24 (q, *J* = 6.0 Hz, 2H), 3.28 (bs, 2H), 3.64 (d, *J* = 5.9 2H), 3.75 (s, 2H), 8.08 (t, *J* =1.6 Hz, 1H), 8.12 (t, *J* = 1.6 Hz, 2H); 13C NMR (d6-DMSO) 27.71, 34.78, 34.88, 41.95, 44.65, 79.89, 168.70, 169.18, 170.46, 173.38. HRMS (M + H)+ calcd. 317.1825; found 317.1801.

**Gly4-β-Ala-OtBu (5c):** 92% yield as a colorless oil,1H NMR (400 MHz, DMSO-d6) δ 1.24 (s, 2H), 1.40 (s, 9H), 1.61 (s, 2H), 2.30 – 2.40 (m, 2H), 3.18 – 3.26 (m, 2H), 3.60 – 3.81 (m, 6H), 8.52 (s, 2H). HRMS (M+H)+ calcd. 374.2034; found 374.2029.

**General method for the preparation of GMB-(Gly)n-β-Ala-OtBu (7a – 7c):** Maleoyl--aminobutyric acid (**6**, 513 mg, 2.8 mmol), Glyn-β-Ala-OtBu (**5a**-**5c**, 2.8 mmol) and *N*-(3-dimethylaminopropyl)-*N’*-ethylcarbodiimide hydrochloride (583 mg, 3.0 mmol) were dissolved in anhydrous dimethyl formamide (12 mL) and stirred for 3 h. The reaction mixture was purified in four equal portions by reverse phase HPLC using a 5.0 cm x 25 cm C18 column. The column was eluted at 100 mL/min with deionized water containing 0.3 % formic acid and 5% acetonitrile for 10 min followed by a 13 min linear gradient from 5% - 33 % acetonitrile. Fractions of desired product were combined, frozen and lyophilized.

**GMB-Gly2-β-Ala-OtBu** (**7a**): 29.1% yield as a white solid, 1H NMR (400 MHz, DMSO-*d*6) δ 1.39 (s, 9H), 1.67 – 1.78 (m, 2H), 2.13 (t, *J* = 8.4, 6.9 Hz, 2H), 2.34 (t, *J* = 7.0 Hz, 2H), 3.23 (dd, *J* = 7.1, 5.6 Hz, 2H), 3.41 (t, *J* = 6.9 Hz, 2H), 3.63 (d, *J* = 5.9 Hz, 2H), 3.66 (d, *J* = 5.7 Hz, 2H), 7.01 (s, 2H), 7.79 (t, *J* = 5.6 Hz, 1H), 8.06 (t, *J* = 5.9 Hz, 1H), 8.13 (t, *J* = 5.7 Hz, 1H). HRMS calcd. (M+Na)+ 447.1850; found 447.1844.

### **GMB-Gly3-β-Ala-OtBu (7b):** 62% yield as a white solid, 1H NMR (d6-DMSO) δ 1.39 (s, 9H), 1.67 – 1.76 (m, 2H), 2.132 (t, *J* = 7.2 Hz, 2H), 2.348 (t, *J* = 6.8, 2H), 3.19-3.26 (m, 2H), 3.1-3.33 (m, 1H), 3.41 (t, *J* = 6.8, 2H), 3.64 (d, *J* = 5.6 Hz, 2H), 3.747 (t, *J* = 6.0 Hz, 3H), 7.0 – 7.15 (m, 1H), 8.10-8.16 (m, 2H), 8.07 (t, *J* = 4.8 Hz, 1H). 13C NMR (d6-DMSO) 23.95, 27.83, 32.26, 34.71, 34.82, 37.38, 41.83, 42.05, 79.83, 134.37, 168.56, 168.96,169.48,170.39, 170.98, 171.80. HRMS (M + Na)+ calcd. 504.2070; found 504.2046.

**GMB-Gly4-β-Ala-OtBu (7c)**: 37% yield as a white solid, 1H NMR (400 MHz, DMSO-d6) δ 1.39 (s, 9H), 1.69 – 1.74 (m, 2H), 2.13 (t, J = 7.6 Hz, 2H), 2.35 (t, J = 7.1 Hz, 2H), 3.21 – 3.26 (m, 2H), 3.38 – 3.42 (m, 2H), 3.63 – 3.73 (m, 8H), 7.00 (s, 2H), 8.03 – 8.21 (m, 5H). HRMS (M+Na)+ calcd: 561.2279; found: 561.2270.

**General method for the preparation of GMB-(Gly)n-β-Ala-OH (8a-8c):** GMB-Glyn-β-Ala-OtBu (**7a** – **7c**, 820 mg, 1.7 mmol) was dissolved in 95:5 trifluoroacetic acid: deionized water (9.0 mL) and magnetically stirred for 3 h. Solvent was removed by rotary evaporation under vacuum to give 730 mg (100%) of the desired compound.

**GMB-Gly2-β-Ala-OH** (**8a**): 91% yield as a colorless oil, 1H NMR (400 MHz, DMSO-*d*6) δ 1.67 – 1.77 (m, 2H), 2.13 (t, *J* = 8.5, 6.8 Hz, 2H), 2.37 (t, *J* = 7.0 Hz, 2H), 3.21 – 3.27 (m, 2H), 3.40 (t, *J* = 7.0 Hz, 2H), 3.64 (d, *J* = 5.8 Hz, 2H), 3.67 (d, *J* = 5.7 Hz, 2H), 7.00 (s, 2H), 7.82 (t, *J* = 5.6 Hz, 1H), 8.05 (t, *J* = 5.9 Hz, 1H), 8.12 (t, *J* = 5.6 Hz, 1H), 12.22 (s, 1H). HRMS calcd. (M+H)+ 369.1405; found 369.1400.

### **GMB-Gly3-β-Ala-OH (8b):** 100% yield as a colorless oil,1H NMR (d6-DMSO) δ 1.67-1.77 (m, 2H), 2.14 (q, *J* = 8.0 Hz, 2H), 2.38 (t, *J* = 7.2 Hz, 2H,), 3.26 (q, *J* = 5.6 Hz, 2H), 3.41 (t, *J* = 7.2 Hz, 2H), 3.65 (d, *J* = 6.0 Hz, 2H), 3.71 (t, *J* = 6.0 Hz, 4H), 7.00 (s, 2H), 7.82 (t, *J* = 6.0 Hz, 1H), 8.05-8.20 (m, 3H), 12.1 (bs, 1H); 13C NMR (d6-DMSO) 23.97, 32.28, 33.69, 34.70, 36.75, 41.84, 42.07, 134.38, 168.51, 168.99, 169.50, 171.01,171.83, 172.70. HRMS (M + Na)+ calcd. 448.1444; found 448.1465.

**GMB-Gly4-β-Ala-OH** (**8c**)**:** 1H NMR (400 MHz, DMSO-d6) δ 1.72 (t, J = 7.3 Hz, 2H), 2.13 (dd, J = 8.8, 6.5 Hz, 2H), 2.83 (m, 2H), 3.39 (dt, J = 8.4, 6.5 Hz, 4H), 3.63 – 3.81 (m, 8H), 7.01 (s, 2H), 7.93 – 8.16 (m, 5H), 12.1 (s, 1H). HRMS calcd. (M+Na)+ 505.4338, found: 505.4334.

**General method for the preparation of GMB-(Gly)n-β-Ala-NHS (1a-1c):** GMB-Glyn-β-Ala-OH (0.18 mmol), *N*-(3-dimethylaminopropyl)-*N’*-ethylcarbodiimide hydrochloride (72 mg, 0.376 mmol) and *N*-hydroxysuccinimide (66 mg, 0.575 mmol) were dissolved in 1.0 mL of dimethyl sulfoxide. After 2 h the reaction mixture was purified in two equal portions by reverse phase HPLC using a 1.9 cm x 10 cm C8 column. The column was eluted at 18 mL/min with deionized water containing 0.2 % formic acid and 5% acetonitrile for 3 min followed by a 15 min linear gradient from 5% to 30% acetonitrile. Fractions containing desired product were collected in a flask and immediately frozen in a dry ice acetone bath then lyophilized.

**GMB-Gly-Gly-Gly-β-Ala-NHS (1b, CX):** 42% yield as a white solid, 1H NMR (d6-DMSO) δ 1.69-1.72 (m, 2H), 1.83-1.93 (m, 2H), 2.13 (t, *J* = 7.6 Hz), 2.84 ( s, 4H), 3.0-3.2 (m, 4H), 3.6-3.75 (m, 6H), 7.00 (s, 2H), 7.99 (t, *J* = 6.4 Hz, 1H), 8.08-8.11 (m, 3H). HRMS (M + Na)+ calcd. 545.1608; found 545.1638.

**GMB-Gly-Gly-β-Ala-NHS (1a)**: 27% yield, 1H NMR (400 MHz, DMSO-*d*6) δ 1.67 – 1.78 (m, 2H), 2.13 (t, *J* = 8.2, 7.0 Hz, 2H), 2.37 (t, *J* = 7.1 Hz, 2H), 2.59 (s, 4H), 3.22 – 3.26 (m, 2H), 3.41 (t, *J* = 7.0 Hz, 2H), 3.64 (d, *J* = 5.9 Hz, 2H), 3.67 (d, *J* = 5.8 Hz, 2H), 7.01 (s, 2H), 7.82 (t, *J* = 5.6 Hz, 1H), 8.05 (t, *J* = 5.9 Hz, 1H), 8.12 (t, *J* = 5.7 Hz, 1H). HRMS calcd. (M+H)+ 466.1569; found 466.1564.

**GMB-Gly4-β-Ala-NHS (1c):** 1H NMR (400 MHz, DMSO-d6) δ 1.72 (t, J = 7.3 Hz, 2H), 2.13 (dd, J = 8.8, 6.5 Hz, 2H), 2.83 (d, J = 13.1 Hz, 6H), 3.39 (dt, J = 8.4, 6.5 Hz, 4H), 3.63 – 3.81 (m, 8H), 7.01 (s, 2H), 7.93 – 8.16 (m, 5H). HRMS (M+Na)+ found: 602.1805, calculated: 602.1817.

The GMB-L-Val-L-Cit-Gly-β-Ala-NHS linker was prepared by solid and liquid phase reactions as follows:



**GMB-*L*-Val-*L*-Cit-Gly-β-Ala-OH**: Fmoc-β-alanine Wang resin (9-fluorenylmethyloxycarbonyl)-β-alanine-4-alkoxybenzyl alcohol resin; 0.735 meq/g, 5 g) was swelled in a fritted reaction vessel with dimethyl formamide (100 mL) overnight. Solvent was removed by filtration, and the resin was treated with piperidine (20% v/v in dimethyl formamide, 100 mL) by agitating with nitrogen bubbling for 40 min.The solution was drained, and the resin was washed with dimethyl formamide with agitation (4 x 100 mL). The resin was then treated with a solution of Fmoc-glycine (1.1 eq) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexaﬂuorophosphate (2.5 eq) in dimethyl formamide (100 mL) for 5 min. Then diisopropyl ethyl amine (3 eq) was added, and the reaction proceeded with nitrogen agitation for 2 h. Solvent was removed by filtration and the resin was washed with dimethyl formamide (4 x 100 mL). The cycle of piperidine deprotection and Fmoc-amino-acid reaction was repeated with Fmoc-*L*-citrulline, then Fmoc-*L*-valine. The resin was treated with piperidine and washed, then treated with diisopropyl ethyl amine (10 eq) in dimethyl formamide (100 mL) followed by a solution of γ-maleimidobutyric acid NHS ester (3 eq) in a minimal volume of dimethyl formamide, then agitated with nitrogen bubbling for 2 h. The solution was removed by filtration, and the resin was washed with dimethyl formamide (4 x 100 mL) then washed with methanol (100 mL) and dried under vacuum overnight.

To release the peptide, the resin was suspended in a solution of 95:5 trifluoroacetic acid:water (100 mL), agitating with nitrogen for 4 h. The filtrate was collected, and the resin was washed with 95:5 trifluoroacetic acid:deionized water (2x100 mL). The combined filtrate and washes were concentrated *en vacuo*, then redissolved in 1:1 acetonitrile:deionized water (50 mL), frozen, and lyophilized to give an off-white solid which was used without further purification.



**GMB-*L*-Val-*L*-Cit-Gly-β-Ala-NHS**: GMB-L-Val-L-Cit-Gly-β-Ala-OH (30.0 mg, 0.053 mmol) was dissolved in dimethyl formamide (1 mL) and treated with diisopropyl ethyl amine (0.023 mL, 0.132 mmol), N-hydroxysuccinimide (9.12 mg, 0.079 mmol), and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (0.028 mL, 0.159 mmol). The reaction was stirred at room temperature under argon for 1 h. The crude reaction was purified by reverse-phase HPLC (Kromasil C8, 21.2 x 250 mm), using deionized water containing 0.1% formic acid using an acetonitrile gradient as follows: (minutes, acetonitrile%) (0, 5) (5, 5) (30, 33) (31, 5). The product fractions were quickly combined, frozen in a dry ice/acetone bath, and lyophilized to give 19 mg of desired product as a white solid (54 % yield). 1H NMR (400 MHz, DMSO-*d*6) δ 8.24 – 8.14 (m, 1H), 8.11 – 7.97 (m, 1H), 7.96 – 7.71 (m, 2H), 6.93 (d, *J* = 3.0 Hz, 2H), 5.85 (d, *J* = 5.9 Hz, 1H), 5.29 (s, 1H), 4.15–4.04 (m, 2H), 3.69–3.47 (m, 1H), 3.37–3.21 (m, 4H), 2.90– 2.83 (m, 2H), 2.81–2.70 (m, 4H), 2.52 (s, 1H), 2.19 (s, 1H), 2.15–1.99 (m, 2H), 1.95–1.80 (m, 1H), 1.70–1.56 (m, 3H), 1.50–1.38 (m, 1H), 1.37–1.17 (m, 3H), 0.82–0.71 (m, 7H). HRMS (M+H)+ found: 665.2867, calcd: 665.2895.

**Cleavage of peptide linker conjugates by Cathepsin B:** The peptide cleavage of *L*-Val-*L*-Cit-Gly and CX (Gly-Gly-Gly) linker conjugates by Cathepsin B was tested *in vitro*. ADCs (2.3 M) were incubated with bovine Cathepsin B (1.8 M) in pH 6 buffer (containing 2 mM dithiothreitol and 0.6 mM CaCl2/MgCl2) at 37 °C, and the time course of cleavage of peptide linker over 24 h was monitored using Hisep HPLC. The ADC bearing *L*-Val-*L*-Cit-Gly linker was cleaved by Cathepsin B (~50% in 8 h), in contrast to the CX linker conjugate which was not cleaved by Cathepsin B.

**Preparation of catabolites:**

**DM-CX1 (9):** To a 160 mM solution of DM1 (59 mg, 0.080 mmol) in dimethylformamide was added γ–maleimidobutyric acid (22.0 mg, 0.12 mmol). Equal volumes of water and saturated sodium bicarbonate were added to raise the pH to 8.5, as measured with a pH meter. The reaction mixture was stirred at room temperature for 40 min, then neutralized with 1N hydrochloric acid. The crude mixture was purified by reverse-phase HPLC using a 19 x 150 mm C8 column with deionized water containing 0.1% formic acid and a linear gradient of 10-95% acetonitrile over 20 min. Fractions containing the two isomers of desired product were frozen then lyophilized to give 62 mg (84%) of desired product as a white solid. 1H NMR (400 MHz, Chloroform-d) δ 0.80 (d, J = 4.8 Hz, 3H), 1.30 (dd, J = 6.4, 1.7 Hz, 3H), 1.34 – 1.39 (m, 2H), 1.65 (s, 3H), 1.90 – 1.96 (m, 1H), 2.16 – 2.23 (m, 2H), 2.37 – 2.43 (m, 2H), 2.58 – 2.66 (m, 2H), 2.74 – 2.82 (m, 3H), 2.93 (d, 3H), 2.95 – 3.00 (m, 2H), 3.06 – 3.14 (m, 3H), 3.20 (d, J = 6.9 Hz, 3H), 3.36 (s, 3H), 3.45 – 3.50 (m, 1H), 3.56 (t, J = 6.2 Hz, 2H), 3.59 – 3.64 (m, 2H), 3.69 – 3.74 (m, 1H), 3.77 – 3.84 (m, 1H), 3.99 (d, 3H), 4.28 – 4.37 (m, 1H), 4.76 – 4.87 (m, 1H), 4.99 (s, 1H), 5.14 (s, 1H), 5.58 – 5.65 (m, 1H), 6.37 – 6.47 (m, 2H), 6.49 – 6.57 (m, 2H), 6.66 (dd, J = 8.9, 1.8 Hz, 1H), 6.84 (dd, J = 4.8, 1.8 Hz, 1H). HRMS (M+Na)+ calcd. 943.3173; found: 943.3156.

**DM-CX2 (10):** To a 198 mM solution of DM1 (73 mg, 0.099 mmol) in dimethylformamide and deionized water (0.030 mL) was added a 792 mM solution of L-Glycine (30 mg, 0.396 mmol) in 250 mM sodium bicarbonate, and a 714 mM solution of γ–maleimidobutyric acid NHS ester (33 mg, 0.119 mmol) in dimethylformamide. A minimal amount of 1:1 deionized water:dimethylformamide was added to dissolve the precipitate, and 250 mM sodium bicarbonate solution was added to make the pH approximately 8. The reaction, which was slightly pink, was stirred at room temperature for 2 h, then neutralized with 1N hydrochloric acid. The crude reaction mixture was purified by reverse-phase HPLC on a (19 x 150 mm) C8 column, using deionized water containing 0.1% formic acid and a 10-60% gradient of acetonitrile over 15 min. Fractions containing the two isomers of desired product were frozen then lyophilized to give 25 mg (26% yield) of desired product as a white solid. 1H NMR (400 MHz, Chloroform-d) δ 0.79 (d, J = 5.5 Hz, 3H), 1.23 – 1.30 (m, 4H), 1.34 – 1.38 (m, 2H), 1.42 – 1.50 (m, 2H), 1.65 (s, 3H), 2.20 – 2.31 (m, 4H), 2.33 – 2.45 (m, 2H), 2.53 – 2.70 (m, 3H), 2.81 – 2.98 (m, 6H), 3.07 – 3.15 (m, 3H), 3.20 (d, J = 3.1 Hz, 2H), 3.35 (s, 3H), 3.44 – 3.49 (m, 1H), 3.54 – 3.64 (m, 3H), 3.73 (dd, 1H), 3.80 (dd, J = 9.1, 3.9 Hz, 1H), 3.99 (s, 3H), 4.00 – 4.12 (m, 2H), 4.28 – 4.39 (m, 2H), 4.77 – 4.85 (m, 1H), 5.06 – 5.24 (m, 2H), 5.58 – 5.67 (m, 1H), 6.36 – 6.46 (m, 2H), 6.48 – 6.56 (m, 2H), 6.68 (d, J = 1.8 Hz, 1H), 6.82 – 6.85 (m, 1H). HRMS (M+Na)+ calcd: 1000.3387; found: 1000.3371.

**DM-CX-Lysine (11):** To a 19.0 mM solution of DM1 (7.0 mg, 0.0095 mmol) in 4:1 dimethylacetamide:water was added a 27.5 mM solution of L-Lysine (5.9 mg, 0.011 mmol) in 150 mM EPPS buffer pH 8.5. GMB-Gly3--Ala-NHS **1b** (2.0 mg, 0.014 mmol) was added to the reaction mixture, which was stirred under argon at room temperature for 30 min. The crude reaction mixture was purified by HPLC using a 20 x 150 mm C18 column. The column was eluted with deionized water containing 0.1% formic acid and linear gradient of 10% to 22% acetonitrile for 1 min then 22% to 50% acetonitrile. Fractions containing the two isomers of desired product were frozen then lyophilized to give 1.2 mg (10% yield) of product as a white solid. 1H NMR (400 MHz, DMSO-d6) δ 0.77 (d, J = 2.1 Hz, 3H), 1.14 (dd, J = 22.5, 6.5 Hz, 6H), 1.20 – 1.28 (m, 2H), 1.31 – 1.50 (m, 6H), 1.59 (s, 3H), 1.60 – 1.69 (m, 4H), 1.99 – 2.07 (m, 2H), 2.08 – 2.17 (m, 3H), 2.18 – 2.23 (m, 2H), 2.28 – 2.34 (m, 1H), 2.35 (d, J = 4.0 Hz, 1H), 2.65 – 2.68 (m, 1H), 2.71 (s, 3H), 2.76 – 2.80 (m, 2H), 2.81 – 2.93 (m, 3H), 2.94 (s, 1H), 2.31 – 2.34 (m, 1H), 2.99 – 3.06 (m, 3H), 3.10 (d, 3H), 3.15 – 3.22 (m, 4H), 3.25 (s, 3H), 3.46 – 3.51 (m, 2H), 3.61 – 3.66 (m, 2H), 3.69 – 3.74 (m, 4H), 3.83 – 3.89 (m, 1H), 3.90 – 3.95 (m, 4H), 4.02 – 4.11 (m, 1H), 4.52 (dd, J = 12.0, 2.8 Hz, 1H), 5.27 – 5.35 (m, 1H), 5.52 – 5.59 (m, 1H), 6.55 (td, J = 5.9, 3.4 Hz, 2H), 6.89 (s, 1H), 7.17 (dd, J = 7.9, 1.8 Hz, 1H), 7.73 – 7.83 (m, 2H), 8.25 – 8.32 (m, 2H), 8.62 – 8.74 (m, 1H). HRMS (M+H)+ calcd. 1290.5251; found: 1290.525.

**Supplementary Method S2**: Test of *in vitro* cytotoxicity, cell-cycle inhibition, and bystander cytotoxic activity of ADCs.

The *in vitro* cytotoxicity of anti-EGFR SMCC and CX ADCs with typical, 3-4 DAR and anti-EpCAM CX ADCs with high, 8-9 DAR were measured in antigen-expressing cells using WST-8 assay (Supplementary Figs. S1, S2). The high DAR CX ADCs were extremely potent toward a relatively low antigen-expressing cell line, RPMI 8226, with an *IC*50 ~ 0.1 nM, in comparison with the *IC*50 of 0.3 nM for an ADC with a typical DAR of ~3-4 (based on Ab concentration; Supplementary Fig. S2).

The effect of Bafilomycin A1, which blocks lysosomal processing via inhibition of the vacuolar ATPase, was tested on cell cycle arrest by the CX and SMCC ADCs. In the absence of Bafilomycin A1, treatment of asynchronously growing COLO 205 cells with anti-EpCAM CX and SMCC ADCs resulted in the growth arrest of about 80-83% cells in G2/M phase as compared to about 22% cells in G2/M phase upon treatment with control, non-binding CX and SMCC ADCs. In the presence of Bafilomycin A1, treatment of COLO 205 cells with anti-EpCAM CX and SMCC ADCs resulted in only 19-22% cells in G2/M phase, similar to that with non-binding CX and SMCC ADCs alone. Similarly, Bafilomycin A1 also suppressed the G2/M arrest of EGFR-expressing HSC-2 cells by anti-EGFR CX ADC from 53% to about 9%. This suppression by Bafilomycin A1 supports a lysosomal mechanism of activation for both CX and SMCC ADCs.

The bystander cytotoxic activity of anti-EGFR CX-DM1 conjugate was tested in mixed cultures of EGFR-positive (Ca9-22) and EGFR-negative (Ramos) cells. Cell mixtures (4000 Ca9-22 and 3000 Ramos cells) or Ramos or Ca9-22 cells alone were incubated in the absence or presence of conjugate (2 nM) in 96-well flat-bottom plate in 100 L of RPMI-1640 medium containing 10% FBS. After five days of growth, cell survival was measured using WST-8 reagent. The disulfide-linked SPDB-DM4 conjugate was used as a positive control for bystander cytotoxic activity (Y. V. Kovtun et al, Cancer Research, 2006, 66, 3214-3221). The SPDB-DM4 conjugate showed bystander killing of antigen-negative Ramos cells in mixed cell culture, in contrast to CX-DM1 conjugate which did not show bystander killing. Both conjugates killed antigen-positive cells but not antigen-negative cells when the cells were tested separately (Supplementary Fig. S4).

The *IC*50 of unconjugated, non-polar *S*-methyl DM1 in multidrug-resistant (MDR) LoVoDOX cells was about 3 nM, which was significantly greater than the *IC*50 of *S*-methyl DM1 in non-MDR cell lines (~0.1-0.3 nM) (data not shown).

**Supplementary Method S3**: Catabolism of [3H]-labeled and non-radiolabeled CX and SMCC ADCs by cancer cells.

Catabolism of [3H]-labeled CX and SMCC ADCs by cancer cells: Calu-3 and COLO 205 cells (~10-15 million cells in a T-150 cell culture flask) were treated at 25 °C for 20 min with 20 nM anti-EpCAM-SMCC-[3H]DM1 or CX-[3H]DM1 conjugate, bearing 4.3 maytansinoid molecules per antibody. The cells were washed to remove unbound conjugate, replaced with fresh culture media and incubated at 37 °C for an additional 24 hours, after which the cells were detached with trypsin-EDTA and separated from media by centrifugation (1500 x g, 5 min). No cytotoxicity was observed after the one day incubation. The media supernates (40 mL) were loaded onto a pre-equilibrated Sep-Pak (Waters) column, eluted with acetonitrile and evaporated to dryness. The cell suspension was mixed with 2-fold volume of ice-cold acetone, kept at -80 °C for at least one hour, thawed and centrifuged (2500 x g, 5 min) to remove the precipitated protein, and the supernates evaporated to dryness. Organic extracts of cells and media were separated by HPLC on an analytical C18 column (0.46x25 cm; Vydac) using a gradient of acetonitrile and water containing 0.025% trifluoroacetic acid. The effluent was directed to a dynamic flow LC-ARC system (AIM Research) for in-line radioactivity detection. Synthetic catabolite standards were used in HPLC to confirm the identity of the catabolites generated by cells. The total levels of [3H] catabolites generated in cells and media from CX and SMCC ADCs were similar: 0.84 and 0.86 pmol per million cells in the case of COLO 205 cells, and 0.30 and 0.26 pmol per million cells in the case of Calu-3 cells, respectively. The EpCAM expression on these cell lines was estimated from the initial cell-bound [3H]ADC levels to be 6.4x105 (COLO 205) and 1.3x105 (Calu-3) antibody binding sites per cell. [3H]-labeled catabolites were detected in the medium of COLO 205 cells, but not in Calu-3 cells. The relative proportions of the HPLC peaks for the DM-CX2 and DM-CX-lysine catabolites in the medium of COLO 205 cells (data not shown) were similar to the HPLC peaks for the intracellular catabolites (Fig. 3A).

Catabolism of non-radiolabeled ADCs by cancer cells: The catabolism of a non-radiolabeled anti-CanAg CX ADC by a high CanAg antigen-expressing cell line, COLO 205, was analyzed by HPLC using UV detection (C8 Kromasil column, 0.1% formic acid; acetonitrile gradient). The identity of the anti-CanAg CX ADC catabolite was confirmed by comparison with synthesized standards for HPLC retention time and mass spectrometry (Bruker esquire 3000 ion-trap MS, positive/negative ion mode).

Quantitation of maytansinoid catabolites generated from non-radiolabeled ADCs using ELISA: Calu-3 cells were grown for 1 day in a T-75 cell culture flask to about 40% confluency, trypsinized, and washed with medium to remove trypsin. The cells were treated with a saturating concentration of non-radioactive anti-EpCAM-SMCC-DM1 or CX-DM1 conjugate (2 g/mL; 12 nM) and incubated in a petri dish without tissue culture coating for ~2 h (about 1.2 million cells per dish; 5 mL). The cells were washed with medium three times to remove unbound conjugate, suspended in fresh medium (5 mL), and incubated in two wells (2.5 mL each) of a six-well tissue culture plate at 37 °C with 5% CO2. After 1 day, supernates from the two wells were combined. The cells were lysed using 0.3 mL ice-cold methanol per well, scraped, and the cell suspensions from the two wells were combined. The cell suspensions were placed at -20 °C overnight and centrifuged (16,000 x g, 5 min) to prepare methanolic cell extracts. For adherent EGFR-positive cell lines (treated with anti-EGFR ADC) and for COLO 205 cells (treated with anti-EpCAM ADC), direct treatment with ADC was carried out without trypsinization, and other steps were as described above.

The catabolite levels in methanolic cell extracts and media were evaluated using maytansinoid-binding competition ELISA (P. L. Salomon and R. Singh, Molecular Pharmaceutics, 2015, 12, 1752-1761). The ELISA method is based on the competition by maytansinoid catabolite toward the binding of biotinylated anti-maytansine antibody to immobilized BSA−maytansinoid conjugate. BSA−DM4 conjugate (2 g/mL in sodium carbonate buffer, pH 9.6; 100 L per well) was added to wells of a 96-well Immulon-2HB plate and coated overnight at 4 °C. The wells were then blocked with 300 L blocking buffer containing BSA for 1 h, followed by washes. Separately, mixtures of biotinylated anti-maytansine antibody (16 ng/mL) with maytansinoid catabolites or maytansinoid standards (typically lysine-SMCC-DM1, ranging in concentrations from 0.031-2 nM; in 1 mL blocking buffer) were pre-incubated for >2 h, and then added in triplicate (0.25 mL each) to the wells containing coated BSA−DM4. For test samples containing 6% methanol, the maytansinoid standards also contained 6% methanol. After an incubation for ~2 h, the wells were washed and then incubated with streptavidin−HRP (1 g/mL; 100 L each well) for about 30 min at ambient temperature, followed by washes and incubation with ABTS/H2O2 substrate, containing 0.5 mg/mL ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), 0.03% w/w H2O2, in 0.1 M sodium citrate buffer, pH 4.2. The absorbance was measured at 405 nm using a multi-well plate reader, 5-10 min after substrate addition. Non-linear 4-PL fit (Graph Pad Prism) was used to estimate the maytansinoid concentrations in samples (tested at several dilutions) using the curve fit derived from maytansinoid standards (Supplementary Fig. S3). The estimated catabolite values tested at two different dilutions in ELISA were within 5%, with CV of less than 6% for the triplicates at each dilution. In separate experiments, media samples (generated from cells after 1 day ADC-processing) were treated with 3-fold volume of ice-cold methanol and incubated overnight at -20 °C to precipitate any dissociated conjugate, and the supernates were assayed by competition ELISA to confirm that inhibition was caused by small molecule catabolites in media and not by dissociated conjugate. All ELISA incubations, except for coating of plates, were at ambient temperature. Wash buffer contained 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20 (TBS-T). Blocking buffer contained 10 mg/mL bovine serum albumin (BSA) in TBS-T.

EpCAM expression was reported to be highest in cells seeded at low densities and at day 1 compared to 2 or 3 days after plating (S. Denzel et al, BMC Cancer, 2009, 9, 402). To increase the accessibility of EpCAM antigen, adherent Calu-3 cells were treated with trypsin/EDTA briefly to disaggregate and lift the cells off the plate, treated with conjugate for 2 h, washed and then allowed to adhere on a tissue culture plate for 1 day for the processing of the cell-bound conjugate. The EpCAM (EGP40) antigenic determinant was reported to be unaffected by trypsin/EDTA treatment (W. E. Corver et al., Cytometry, 1995, 19, 267-272). In separate control experiments, the antigen-specific processing of anti-EpCAM or anti-EGFR ADC was confirmed by incubation of cells with conjugate in the presence of excess, unconjugated anti-EpCAM or anti-EGFR antibody, respectively, which showed only about 5-10% of the amounts of catabolites (generated in both cells and medium) compared to the amounts of catabolites generated without competing excess, unconjugated antibody.

In a time course experiment in COLO 205 cells, the total catabolites increased in a time-dependent manner from 11 h to 24 h: 2.11 to 3.17 pmol (anti-EpCAM CX ADC), and 1.75 to 2.63 pmol (anti-EpCAM SMCC ADC).