Impact of ligand size and conjugation chemistry on the performance of universal chimeric antigen receptor T-cells for tumor killing

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Supplementary information

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CAR-T production and design

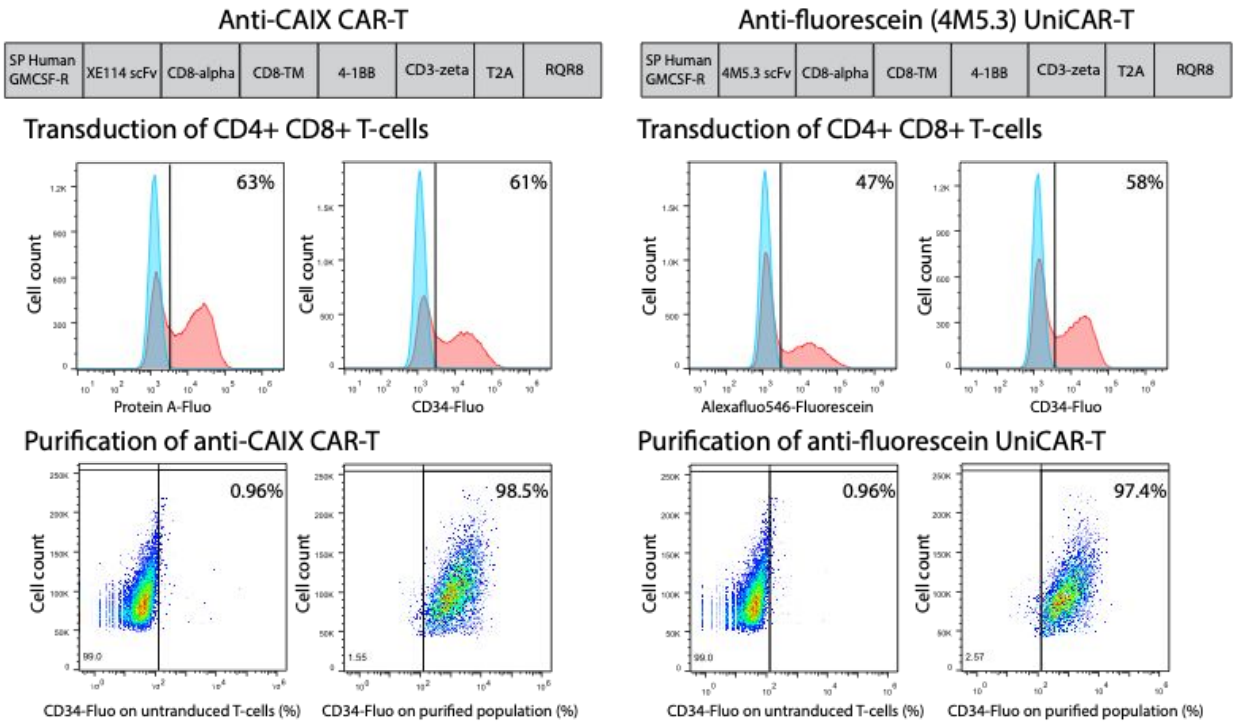


Fig S1. The CAR-T design was kept the same for both αCAIX and UniCAR-T constructs. The transduction percentage was 53% for the αCAIX CAR-T and 57% for the UniCAR-T. After one week of cell enrichment, the CAR T-cell population was purified via anti-FITC magnetic MACs beads (Miltenyi). The purity obtained was 98.5% for the αCAIX CAR-T and 97.4% for the UniCAR-T. After the purification process, the CAR-T were cultured for a week for further enrichment. The cell numbers reached roughly 2 x 10^8 and were cryopreserved until required.

Cell culture

HEK293T cells (ATCC® CRL-3216™) were kept in DMEM media (Gibco), supplemented with 10% FBS (Gibco, #10270106), 2mM Glutamine and 1% antibiotic-antimycoticum (Gibco, #15240062). Human PBMCs were kindly provided by Cerus (Intercept blood systems, California, USA). T-cells were expanded in Advanced RPMI (Gibco) supplemented with 10% FBS (Gibco, #10270106), 2mM Ultraglutamate (Lonza, #BE17-605E/U1), 1% antibiotic-antimycoticum (Gibco, #15240062) and 100 UI of human IL-2/ml (Proleukin, Roche Diagnostics). SK-RC-52 cells were kindly provided by Professor E. Oosterwijk (Radbound University Nijmegen Medical Centre, Nijmegen, the Netherlands) and expanded in RPMI-1640 media (Gibco, #21875034) supplemented with 10% FBS (Gibco, #10270106) and 1% Anti-Anti

(Gibco, #15240062). The cells were detached through each passage step via EDTA-Trypsin 0.05% (Invitrogen).

Cryopreservation of cells

Cryopreservation of cells was performed as recommended by ATCC®. For CAR T-cell cryopreservation, 10 x 10^6 cells were frozen in 1 mL of freezing media (composed of 90% FBS and 10% Dimethyl sulfoxide (Sigma-Aldrich)) and transferred to cryovials (Greiner Bio-one). Vials were put in Mr Frosty (Nalgene, Sigma-Aldrich) and transferred to -80°C. After 3 days, T-cells were then transferred to liquid nitrogen tanks and stored until further use.

Virus production

HEK293T-cells were seeded at a density of 5 x 10^6 in 15 mL of complete DMEM media (as previously described) in a T75 cell culture flask.

The sPAX2 packaging and pCAG-VSVG envelope plasmids were both kindly provided by Dr. Patrick Salmon (University of Geneva, Switzerland). The Lentiviral plasmid (pCDH-EF1α-MCS-T2A-GFP; System Biosciences, Palo Alto, CA, USA) was cloned into the EF1α-CAR-T2A-RQR8 backbone that was provided by Dr Renier Myburgh and Prof Dr Markus G Manz [1].

The EF1-UniCAR-T2A-RQR8 was cloned using restriction sites NheI (NEB) and XhoI (NEB) to insert the scFv 4M5.3[2]. The RQR8 sequence was kindly provided by Dr Brian Philip and Dr Martin Pule and was inserted as a marker for expression[3]. The transfection mixture was added to the HEK293T cells and incubated at 37°C and 5%CO_2. The media was exchanged the next morning with fresh medium and the virus was harvested the following day. To harvest the virus the supernatant was transferred to a 50ml Falcon tube and spun (400 g, 5 min, RT) and filtered using 0.45 µm filter (TPP, Switzerland) to remove cells and debris. To concentrate the virus, PEG-it (System Biosciences, CA, USA) was added according to the manufacturer's instructions and samples incubated overnight at 4°C. The next morning, samples were centrifuged (1500 g, 30 min, 4°C). After aspirating the supernatant, the pellet was centrifuged again (1500 g, 30 min, 4°C) to remove residual supernatant. The pellet was resuspended in 1/200 of the original volume and stored at -80°C until further use.

Viral transduction of T-cells

Human T-cells were activated in T-cell media using CD3/CD28 Dynabeads (ThermoFisher) the day prior to transduction. For the transduction, Polybrene (Santa Cruz Biotechnology, #134220) was added to a final concentration of 8 µg/ mL and 10 µL virus supernatant was added on the cells and mixed. After centrifugation (1000 g, 90 min, 37°C) the cells were incubated overnight (37°C, 5% CO_2). At the following day, the media was exchanged, and the T-cells were incubated overnight (37°C, 5% CO_2). The beads were removed using a magnet incubate for 2 min and centrifuged the supernatant (400 g, 5 min, 37°C). The pellets were then resuspended in fresh T-cell media and expand them at 0.5 x 10^6 cells/mL. Once the population of transduced T-cells reached roughly 2 x 10^7, the CAR T-cells were purified via magnetic cell sorting.

Purification of CAR T-cells via magnetic cell sorting

The transduced T-cells were centrifuged (400 g, 5 min, 4°C) and resuspended in 10 mL MACS buffer (2 mM EDTA and 0.5% FBS in PBS). After counting the cells, they were centrifuged (400 g, 5 min, 4°C) again and resuspended in 10 µL MACS buffer per 1 x 10^6 cells. Anti-CD34 antibody coupled with FITC (QBEND-10, Sigma-Aldrich) was added in a 1:20 dilution to the cells and incubated for 30 min at 4°C in the dark. Then, 25 mL MACS buffer was added, and cells were spun down (400 g, 5 min, 4°C). The supernatant was aspirated, and cells were resuspended in 10 µL MACS buffer per 1 x 10^6 cells. Anti-FITC magnetic micro beads (Miltenyi Biotech) were added in a dilution of 1:10. Cells were incubated with the beads for 30 min at 4°C and washed using 25 µL MACS buffer. Cells were spun down (400 g, 5 min, 4°C), supernatant removed, and cells resuspended in 500 µL MACS buffer. In the meantime, magnetic columns (Miltenyi Biotech) were put in magnetic separators (Miltenyi Biotech) and equilibrated using 3 mL MACS buffer. The transduced T-cell mixture was added onto the column trough a cell strainer (Miltenyi Biotech). The column was washed three times using 3 mL of MACS buffer per washing step. After the washing, the column was taken out of the magnetic separator and T-cells eluted in 5 mL MACS buffer. A small aliquot was taken for cell counting as well as subsequent flow cytometry analysis of the purified T-cell population. Purified CAR T-cells were then resuspended in 1 mL T-cell media per 1 x 10^6 cells and expanded until reaching sufficient cell numbers.

Protein production

Proteins were produced by transient transfection of CHO-S cells and purified by protein-A affinity chromatography as described previously[4-6]. The XE114 (anti-CAIX IgG with mutated cysteines) was previously cloned in our laboratory and published[7]. The diabody was cloned to contain the sequences of the VH and VL of the XE114 antibody with a 5-amino acid linker (GGSGG). The sequence was cloned in pcDNA 3.1 and produced using the same protocol referenced above.

Protein sequence of the site-specific diabody construct

The full sequence for the C-terminus modified cysteine is shown below. The terminal serine residue on the heavy chain was modified to a cysteine.

Light chain:

SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTA
SLTITGAQAEDEADYYCQSSKWSWDPVVFGGGTKLTVLG

Heavy chain:

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAIDGSGGSTYYADSVKGRF
TISRDNSKNTLYLQMNSLRAEDTAVYYCVKGPPVFDYWGQGTLVTV**C**S

Non-site-specific fluorescein conjugation

The protein sample with a concentration of 2 mg/mL was dialyzed against 0.1 M $NaCO_3$ (pH 9) overnight at 4°C. The Fluorescein isothiocyanate (Sigma-Aldrich) was dissolved in DMSO (3 mg in 3 mL) to obtain a final concentration of 10% (v/v) when added to the protein. The mixture was incubated for 8 h at 4°C in a 360° rotator. After that, the protein was purified by a PD-10 size exclusion column (GE Healthcare). The collected fractions containing the product were pooled. Protein aliquots were snap frozen and stored at -80°C until further use.

Site-specific fluorescein conjugation

A 1.0 mg/mL antibody (IgG or diabody) solution was reduced with 20mM of DTT (1,4-Dithio-DL-threitol) in PBS (pH = 7.4) overnight. Complete reduction of the cysteines was monitored by mass Spectrometry. After reduction, the protein was added to a Vivaspin centrifugation concentrator (Sartorius) and 4 mL of PBS (pH=7.4) were added to dilute the DTT (centrifuged at 4000 g, 20 min, 4°C). After three washing steps, the protein solution was adjusted to a final

concentration of approximately 1.0 g/mL and moved to an Eppendorf reaction tube. Commercially available fluorescein-5-maleimide (30 eq.) was dissolved in DMSO (Sigma-Aldrich) (4.3 mg in 1 mL) to obtain a final concentration of 10% (v/v) when added to the reduced protein. The mixture was stirred for 1 hour at RT. The completion of conjugation was proofed by mass Spectrometry. The final product was purified by PD-10 size exclusion column (GE Healthcare). Fraction containing protein were pooled and the final protein was concentrated using Vivaspin centrifugation concentrator (Sartorius). Protein aliquots were snap frozen and stored at -80°C until further use.

To calculate the fluorescein:protein ratios the following formula was used:

$$\text{F:P} = (3.3 \times OD^{495}) + [OD^{280} - (0.35 \times OD^{495})]$$

Equation 1: The equation gives a value quantifying the average number of fluorescein molecules per protein molecule.

Determination of dissociation constants

The dissociation constant of the targeting ligands was investigated on SK-RC-52 cells via flow cytometry analysis. Media was removed from cell culture dishes and cells were washed with PBS (Gibco). Per 25 cm^2 surface area 1 mL Accutase (Sigma-Aldrich) was added to detach the cells from the flask. The cells were spun down (400 g, 5 min, RT) and resuspended in FACS buffer (PBS (Gibco), 1% Bovine Serum Albumin (Sigma-Aldrich), 2 mM EDTA (Janssen Pharmaceuticals)), strained (35 µm nylon mesh) and incubated for 30 min on ice. Triplicates were performed for every concentration of targeting ligand. Therefore, 150'000 cells in 100 µL per well were distributed in a round bottom 96 well plate (Cellstar, Greiner Bio One) and spun down (400 g, 5 min, 4°C). The supernatant was removed by flicking, and the pellets were resuspended in FACS buffer containing different concentrations of targeting ligands. The samples were incubated for 1 h in the dark on ice. Subsequently, the cells were centrifuged (400 g, 5 min, 4°C), and the supernatant removed. The washing step was repeated once for small molecules and three times for antibody derivatives. Finally, the pellet was resuspended in 200 µL of FACS buffer and analysed gating for FITC channel by Cytoflex flow cytometer (Beckman Coulter Life Sciences). The flow cytometry data was analysed using FlowJo software (Treestar).

The concentration of CAIX in FACs was calculated to be approximately in the subnanomolar range (0.21 nM). The equation used to determine the concentration is shown below:

$$n_{CAIX} = \frac{N_{cells} \times N_{CAIX/cell}}{N_A}$$

$$[CAIX] = \frac{n_{CAIX}}{V}$$

Where:

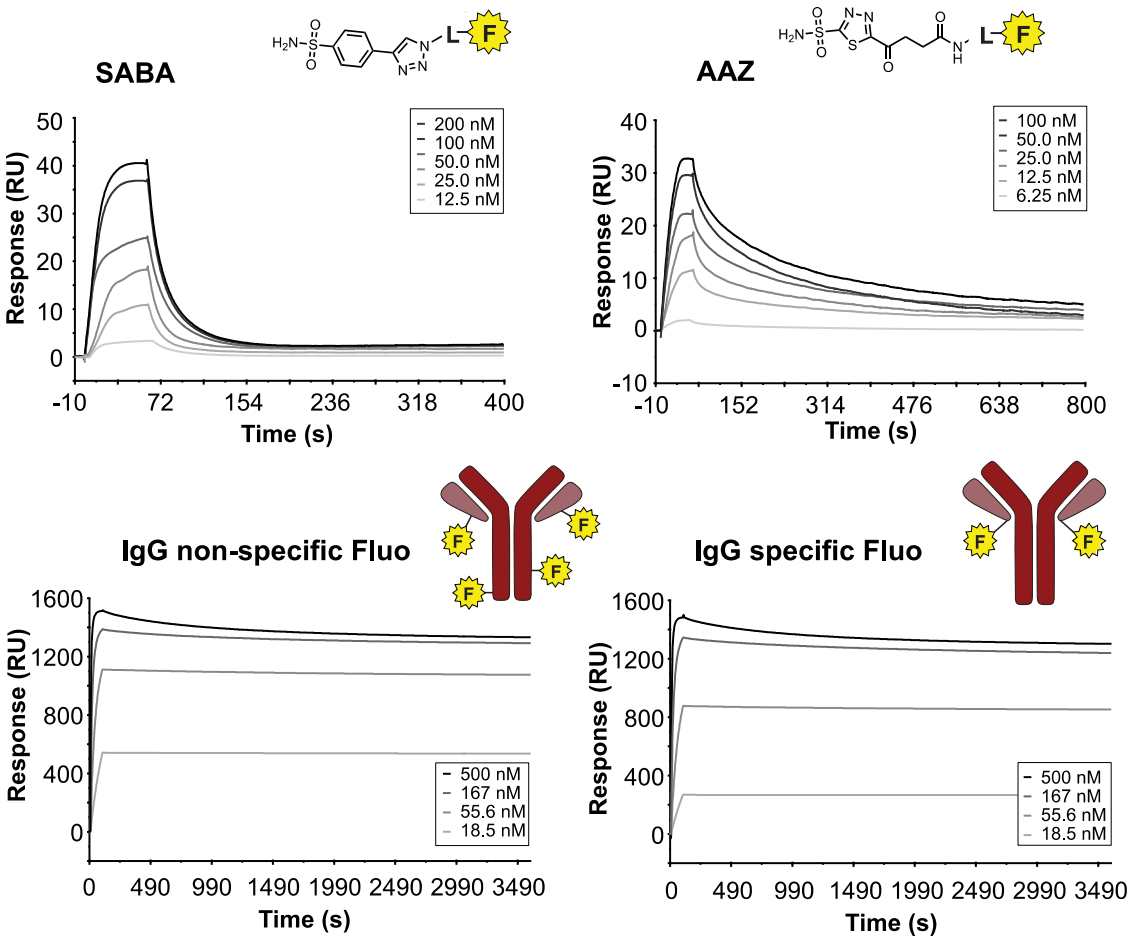
N_{cells} = total number of cells (150'000),

$N_{CAIX/cell}$ = average number of molecules of CAIX for each SKRC52 cell (84'700)[8],

N_A = Avogadro's number (6.022×10^{23}),

n_{CAIX} = mol of CAIX, V = volume of FACS buffer (100 µL).

The dissociation constants for acetazolamide (AAZ) and the IgG (XE114) were analysed by BIAcore 3000 (Biacore) to visualize the K_{off} and K_{on} interactions in binding to CAIX. A total of 2'100 response units (RU) of recombinant His-tagged CAIX were immobilized onto a CM5 chip (Biacore). For this purpose, 15 µl of each supernatant was allowed to flow over the coated chip at a flow rate of 10 µl/min.



	k_{on} (1/Ms)	k_{off} (1/s)	K_d (M)
SABA	$5.470 \cdot 10^{+5}$	0.05844	$1.1 \cdot 10^{-7}$
AAZ	$1.688 \cdot 10^{+6}$	0.005613	$3.3 \cdot 10^{-9}$
Non-specific Fluo IgG	$2.333 \cdot 10^{+5}$	$1.915 \cdot 10^{-5}$	$8.2 \cdot 10^{-11}$
Spcific Fluo IgG	$2.141 \cdot 10^{+5}$	$1.265 \cdot 10^{-5}$	$5.9 \cdot 10^{-11}$

Fig S2. The BIAcore profiles for SABA and AAZ demonstrate their differences in Koff and Kon. In the lower part of the figure is depicted the BIAcore profile of the IgG non-specific fluo and IgG specific fluo conjugation.

In vitro Killing assay

UniCAR T-cells, αCAIX CAR T-cells and non-transduced T-cells were thawed and grown in culture of 1×10^6 cells per ml Advanced RPMI (Gibco) without IL-2 (Proleukin, Roche Diagnostics). On day 0, SK-RC-52 cells were harvested and membrane stained using PKH26 Red Fluorescent Cell Linker Kit for General Membrane (Sigma-Aldrich) following manufacturer instructions. After the staining procedure was completed, the stained SK-RC-52 cells were

seeded at a density of 30’000 cells per well, in a 96 well plate and incubated overnight (37°C, 5% CO_2). The next day, SK-RC-52 cells in extra wells were detached and counted. T-cells or UniCAR-Ts were resuspended in 100 µL Advanced RPMI (Gibco) containing different concentrations of bispecific adapters and added on the SK-RC-52 cells in a 1:1 target to effector cell ratio. Anti CAIX CAR-Ts and non-transduced T-cells served as a positive and negative control respectively. After addition, the plate was spun down (400 g, 1 min, RT) and incubated for 24 h (37°C, 5% CO_2). The killing rate was assessed for the different condition via FACS. Therefore, the supernatant was transferred to a round bottom 96 well plate. After washing the wells with 100 µL of PBS, the PBS was also collected. Then, 50 µL Accutase (Millipore) was added to each well and incubated for 5 minutes at 37°C to detach the target cells. The detached cells were added to the corresponding well of the round bottom 96 well plate. The plate was spun down (400 g, 5 min, RT), flicked to remove the supernatant and the pellets were resuspended in 150 µL FACS buffer. After 30 min of incubation with the FACS buffer at 4°C in the dark, the cells were spun down (400 g, 5 min, 4°C). Immediately before measurement, the pellets were resuspended in a 1:10’000 dilution of the live/dead staining TOTO -3 Iodide (ThermoFisher) in FACs buffer, strained (30 µm nylon mesh) and analyzed via flow cytometry (Cytoflex, Beckman Coulter). The flow cytometry data was analyzed using FlowJo software (Treestar). The figure below illustrates the gating strategy for the cell lysis calculation.

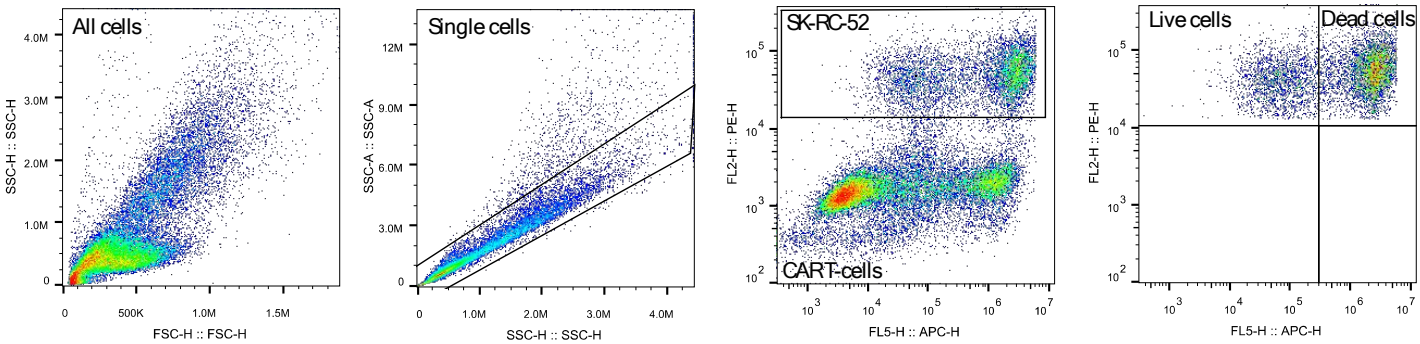


Fig S3. The gating strategies used to determine the UniCAR T-cell induced tumor cell lysis.

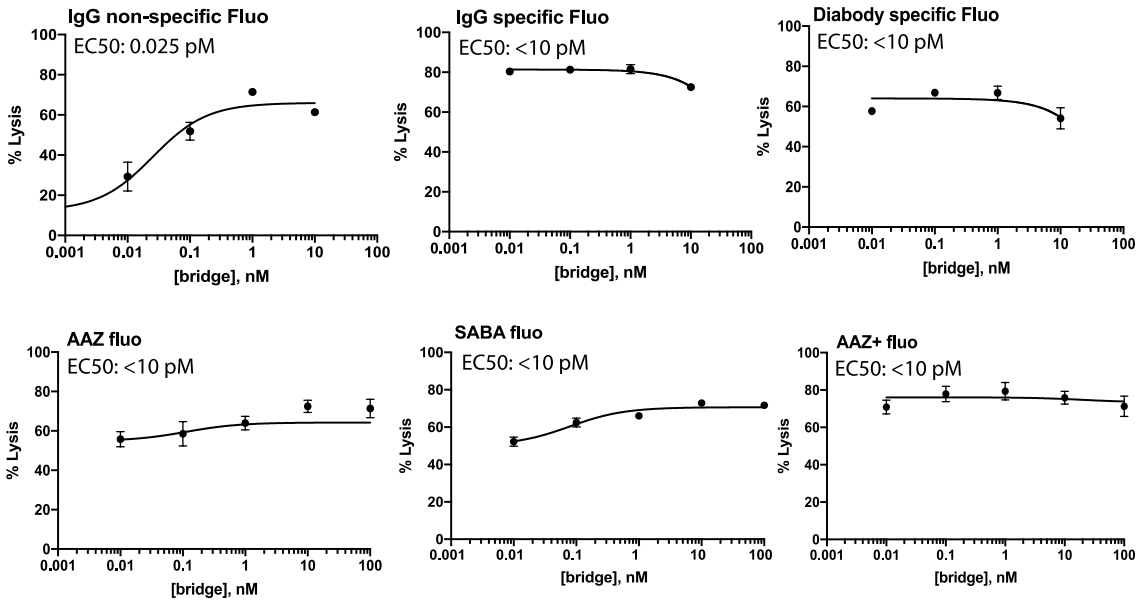


Fig S4. The killing assays were plotted to estimate the EC50 for each bridge construct. The EC50 values reported are an estimation derived from the data achieved in the killing assays reported in ***Figure 4****.*

Solid phase synthesis of small molecules "bridges"

The synthesis of small molecules bridges was performed on solid phase using pre-loaded Fmoc-Lys(Boc) on Wang resin (0.5 mmol/g, REF, Bachem).

Fmoc Deprotection procedure. The Fmoc protecting group was removed by treating the resin three times with piperidine:DMF=1:4 solution (20 mL/g, 1x30 minutes, 2x10 minutes). After deprotection, the resin was washed several times with fresh DMF.

Amino-acid and carboxylic acid coupling procedure

The deprotected resin was allowed to react for 4 hours with an activated solution of Fmoc-protected amino-acid or carboxylic acid (4 equivalents of acid, 4 equivalents of HATU, 8 equivalents of DIPEA) in dry DMF (5 mL / mmol). After the coupling reaction, the resin was washed several times with DMF. The coupling performance was confirmed by TNBS test. The TNBS (2,4,6-trinitrobenzenesulfonic acid) test can only be used for detecting primary amino groups. The beads turn orange-red in presence of free primary amino group. Few beads was poured into a solution of 2,4,6-trinitrobenzenesulfonic acid in DMF:DIPEA=9:1 and incubated for 5 minutes.

Resin cleavage. The resin was treated with cleavage solution (trifluoroacetic acid : H_2O : triisopropylsilane = 95: 2.5 : 2.5, 20 ml / g) for 1 hour at room temperature. In order to increase the cleavage yield, the procedure was repeated for additional 30 minutes. The TFA solution of cleaved product was poured into cold (-20 °C) diethyl ether and centrifuged at 5000 rpm (-10 °C) for 30 minutes. The supernatant was discarded and the pellet was dried and re dissolved in 1 mL of H_2O: Acetonitrile = 1:1. The products were purified by RP-Chromatography on C18 40 µM irregular column (12 g) with H_2O : Acetonitrile (0.1% FA) in concentration gradient as mobile phase. Purified products were lyophilized and characterized by UPLC-MS.

Synthesis of tripeptide linker (L) and of compound 1 (NH_2-L-NHFmoc)

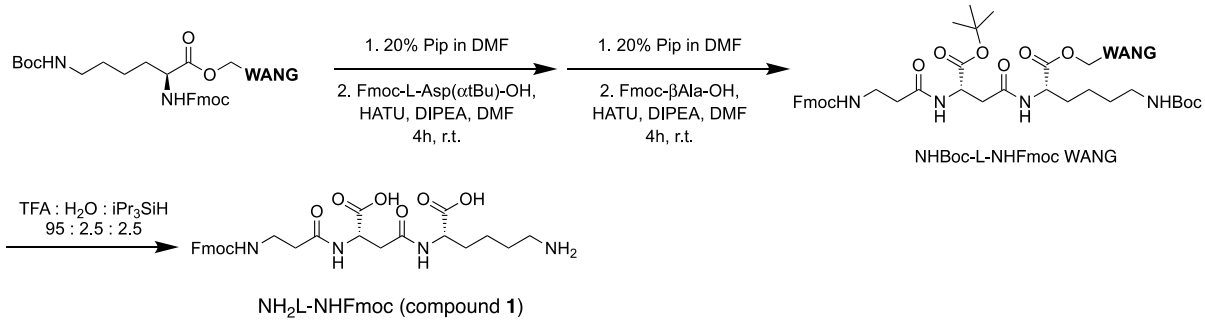


Figure S5. The synthesis of the linker is illustrated above. For each small molecule based bispecific adapter, the same linker was conjugated.

1.0 g of commercially pre-loaded Fmoc-Lys(Boc) Wang resin (0.5 mmol) was swollen in DMF (30 mins). The resin was deprotected as previously describe an assembled using the Fmoc-Amino-acid coupling procedure with the following sequence: Aspartic acid (αtBu ester), β-Alanine.

The tripeptide NH_2L-NHFmoc (compound **1**) was obtained by cleaving a portion of Fmoc-protected resin (100 mg, 0.05 mmol) which was subsequentely purified. **Yield** = 22%. **^{1}H NMR** (600 MHz, DMSO-d6) δ 8.02 (s, 1H), 7.91 – 7.87 (m, 2H), 7.69 (d, *J* = 7.6 Hz, 2H), 7.45 – 7.30 (m, 6H), 4.33 – 4.18 (m, 5H), 4.12 (td, *J* = 8.2, 4.2 Hz, 2H), 3.21 (dt, *J* = 9.6, 7.1 Hz, 3H), 2.75 (d, *J* = 7.5 Hz, 2H), 2.60 (d, *J* = 10.5 Hz, 1H), 2.47 – 2.40 (m, 1H), 2.29 (t, *J* = 7.2 Hz, 2H), 1.77 – 1.62

(m, 1H), 1.50 (dt, *J* = 31.6, 9.6 Hz, 3H), 1.43 – 1.21 (m, 3H). **^{13}C NMR** (151 MHz, DMSO) δ 173.95, 173.41, 169.83, 169.25, 163.89, 155.91, 143.78, 142.43, 140.56, 139.28, 137.29, 128.81, 127.48, 127.18, 126.98, 125.12, 121.27, 119.91, 109.66, 65.33, 51.06, 46.56, 39.80, 38.07, 37.16, 35.83, 30.30, 26.06, 21.58. **m/Z** calculated for $C_{28}H_{34}N_4O_8$: 554.24, detected (TOF MS ES+): 555.2159.

Synthesis of compound 2 (SABA-L-NH_2)

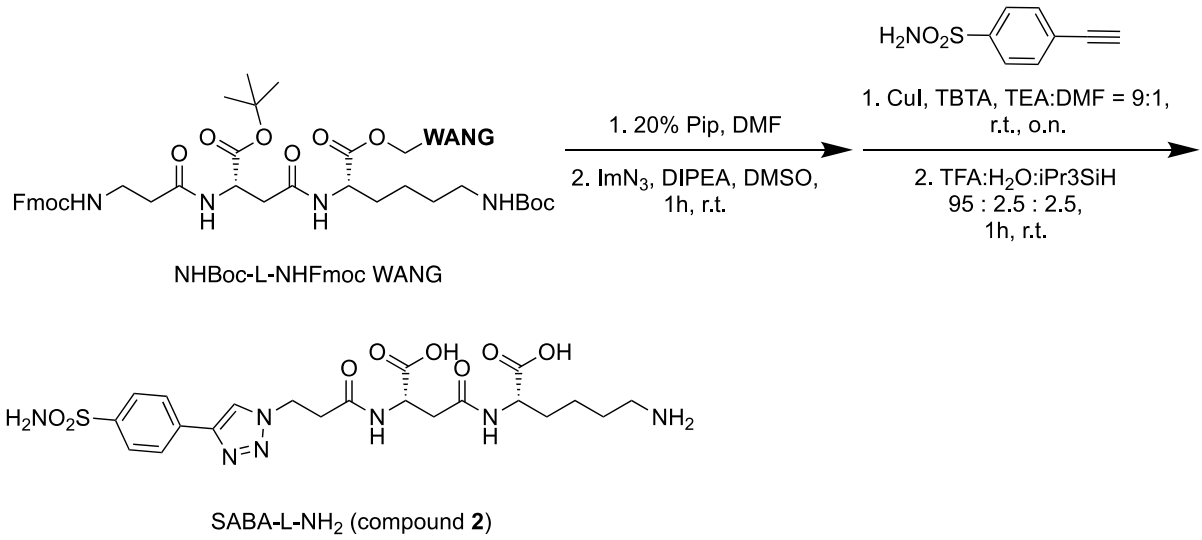


Figure S6. The synthesis steps of SABA-L-NH_2 is reported in this figure.

Pre-synthesized **NHBoc-L-NHFmoc** WANG resin (200 mg, 0.1 mmol) was deprotected as reported in the general procedure. The free amino derivative was allowed to react with Imidazole-1-sulfonyl azide hydrochloride (51 mg, 0.3 mmol, CAS 952234-37-6) and DIPEA (180 µL) for 1 hour at room temperature. The product was washed several times with fresh DMF. Commercially available 4-ethynylbenzenesulfonamide (72 mg, 0.4 mmol, CAS 1788-08-5), copper Iodide (CuI, 4 mg, 0.02 mmol), Tris(benzyltriazolylmethyl)amine (TBTA, 0.04 mmol., 21 mg) were dissolved in 2 mL of degassed DMF and added to the tripeptide azido-derivative. The reaction was kept overnight at room temperature, then washed several times with EDA (0.5M in water, pH=8) and fresh DMF. The final product **2** (**SABA-L-NH_2**) was cleaved from the resin and purified as previously described. **Yield** = 32%. **^{1}H NMR** (600 MHz, DMSO-d6) δ 8.71 (s, 1H), 8.03 (dq, *J* = 8.6, 2.2 Hz, 2H), 7.91 – 7.84 (m, 2H), 7.39 (s, 2H), 4.63 (t, *J* = 6.8 Hz, 2H), 4.30 (dt, *J* = 7.7, 5.6 Hz, 1H), 4.14 (ddd, *J* = 9.4, 7.9, 4.3 Hz, 1H), 2.82 (t, *J* = 6.7 Hz, 2H), 2.75 (t, *J* = 6.8 Hz, 2H), 2.61 – 2.56 (m, 1H), 2.44 (dd, *J* = 14.2, 5.9 Hz, 1H), 1.81 – 1.26 (m, 8H). **^{13}C NMR** (151 MHz, DMSO) δ 173.67, 172.96, 169.72, 168.11, 144.84, 142.87, 133.89, 126.26, 125.21, 122.57, 51.50, 50.84, 46.09, 38.07, 37.79, 35.28, 30.05, 25.96, 21.70. **m/Z** calculated for $C_{21}H_{29}N_7O_8S$: 539.18, detected (TOF MS ES+): 540.1931.

Synthesis of compound 3 (AAZ-L-NH_2)

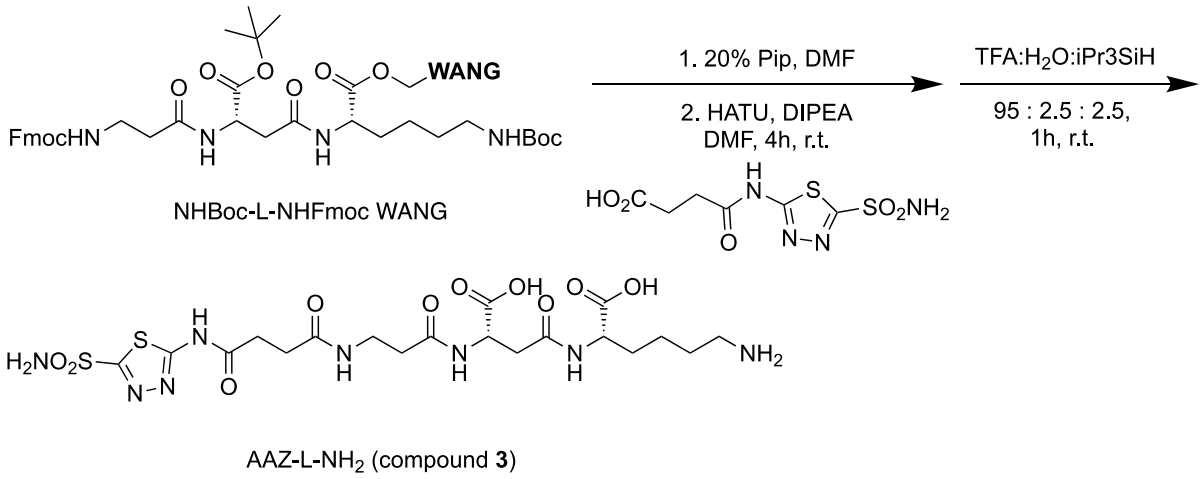


Figure S7. A schematic representation of the synthesis steps of AAZ-L-NH_2 are shown.

Pre-synthesized **NHBoc-L-NHFmoc** WANG resin (200 mg, 0.1 mmol) was deprotected as reported in the general procedure. A solution of 4-oxo-4-((5-sulfamoyl-1,3,4-thiadiazol-2-yl)amino)butanoic acid (**AAZ-CO_2H**, 95 mg, 0.4 mmol, CAS: 113411-20-4) was added to the resin and coupled as reported in the general procedure. The final product **3** was cleaved and purified as previously described. **Yield** = 34%. **m/z** calculated for $C_{19}H_{30}N_8O_{10}S_2$ = 594.15 g/mol; Mass detected (MS TOF ES+) = 595.2102.

Synthesis of compound 4 (AAZ^+-L-NH_2)

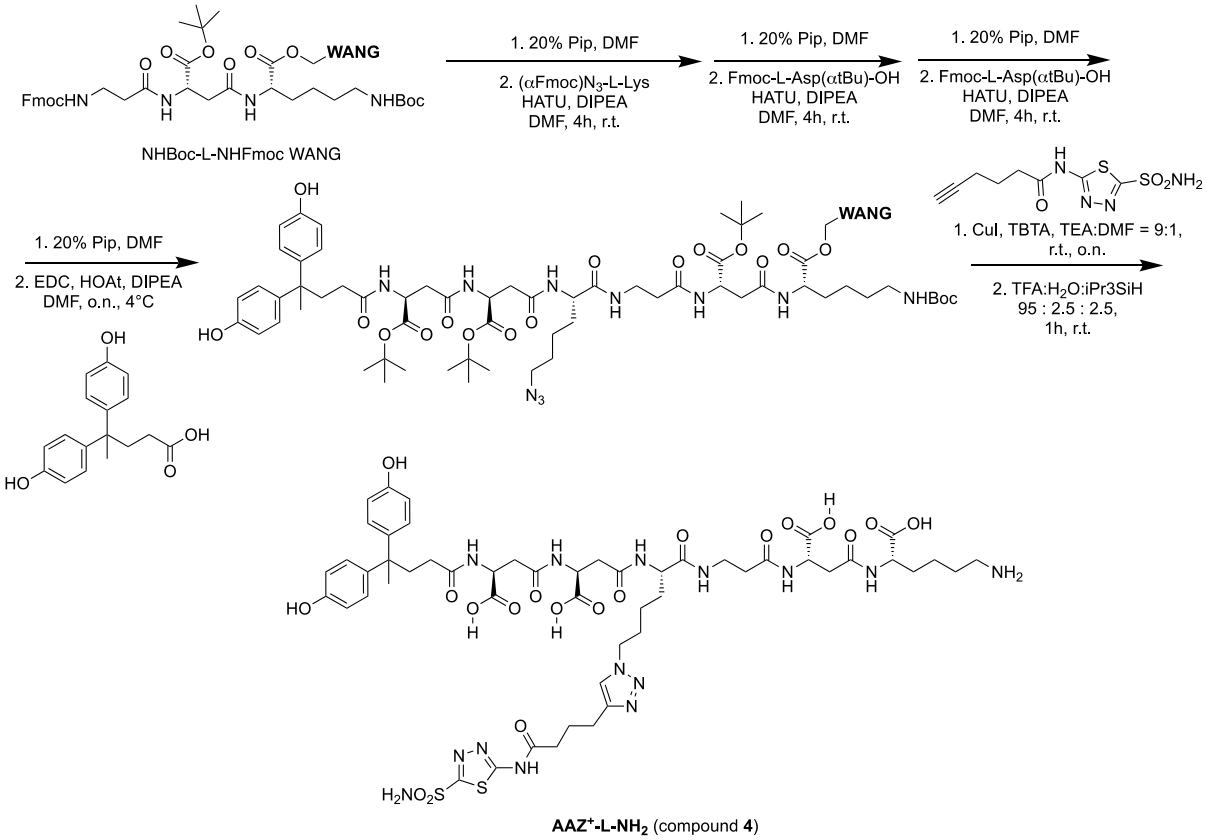


Figure S8. The synthesis steps for AAZ^+-L-NH_2 are delineated.

Pre-synthesized NHBoc-L-NHFmoc WANG resin (350 mg, 0.175 mmol) was deprotected as reported in the general procedure. The peptide was assembled by adding in the order the following protected aminoacids: N^2(Fmoc)-N^6-diazo-L-lysine (276 mg, 0.7 mmol); Fmoc-L-Asp(αtBu)OH (288 mg, 0.7 mmol); Fmoc-L-Asp(αtBu)OH (288 mg, 0.7 mmol). 4,4-Bis(4-hydroxyphenyl)valeric acid (CAS: 126-00-1, 75 mg, 0.26 mmol) was dissolved in 2 mL of dry DMF and activated at 4 °C by adding 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 0.3 mmol, 53 µL), 1-Hydroxy-7-azabenzotriazole (HOAt, 0.3 mmol, 40 mg) and DIPEA (0.78 mmol, 156 µL) and added to the deprotected hexapeptide. The reaction was kept overnight at 4°C, then the resin was washed several times with DMF.

Commercially available N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)hex-5-ynamide (192 mg, 0.7 mmol, CAS 1623789-42-3), copper Iodide (CuI, 7 mg, 0.035 mmol), Tris(benzyltriazolylmethyl)amine (TBTA, 37 mg, 0.07 mmol) were dissolved in 2 mL of degassed DMF and added to the azido-peptide. The reaction was kept overnight at room temperature, then washed several times with EDA (0.5M in water, pH=8) and fresh DMF. The

final product **4** (AAZ^+-L-NH_2) was cleaved from the resin and purified as previously described. MS calculated for $C_{52}H_{70}N_{14}O_{19}S_2$ = 1258.4383, Mass detected (MS TOF ES+) = 1259.4143.

Synthesis of 5-FITC labelled compounds

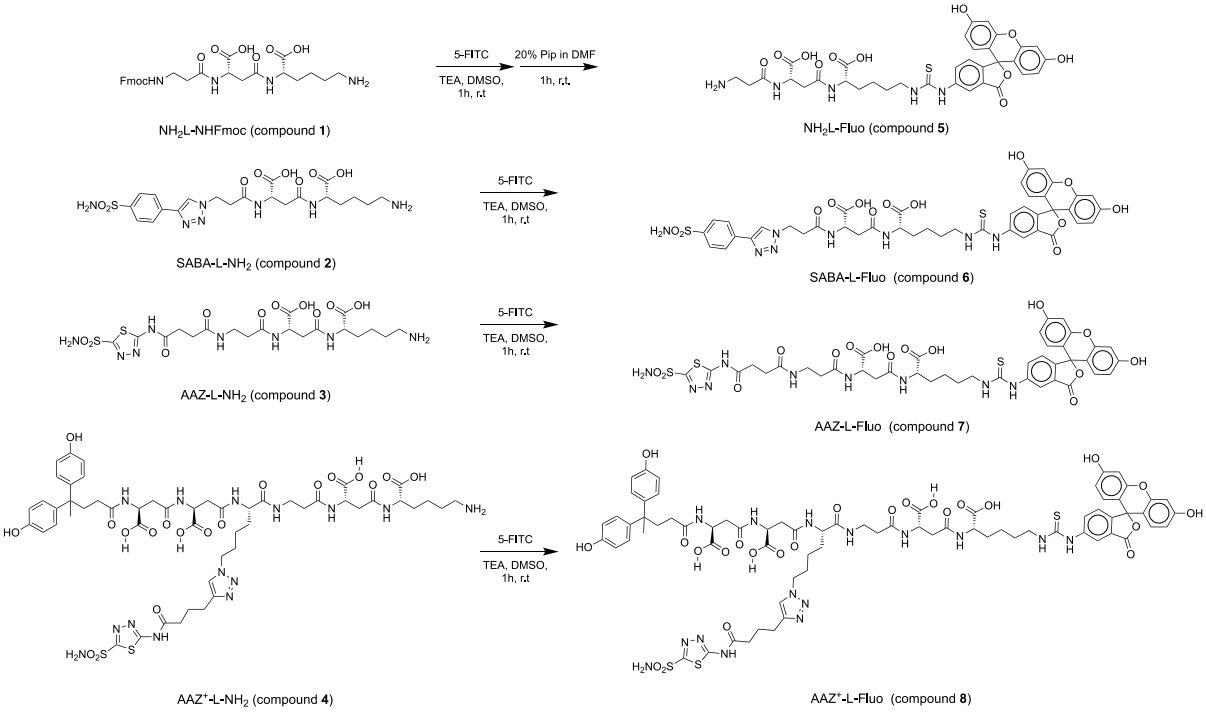


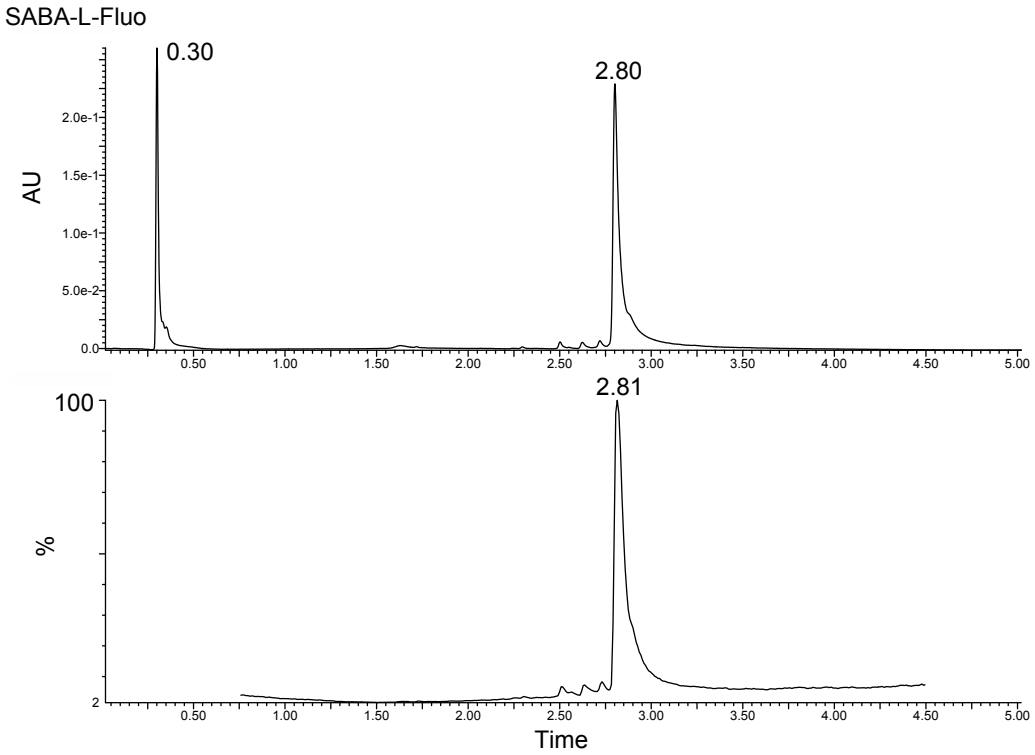
Figure S9. A scheme describing the conjugation of fluorescein to each small molecule based bispecific adapter bridges.

The purified compounds were subsequently coupled to fluorescein isothiocyanate isomer I (5-FITC). Therefore, a solution of the respective compound (1.0 eq) in dry DMSO (200 µL/ 1 mg) was allowed to react in the dark with 5-FITC (1.5 eq) and TEA (10.0 eq) for 1 hour at room temperature. After that, the reaction was stopped with the addition of formic acid (20.0 eq) and diluted to 1 ml with a mixture of MeCN and H_2O (1:1) and purified over HPLC (95% H2O / 5% MeCN to 20% H2O / 80% MeCN over 20 min). After lyophilization the fluorescein derivatives were analysed by LC-MS TOF (ES).

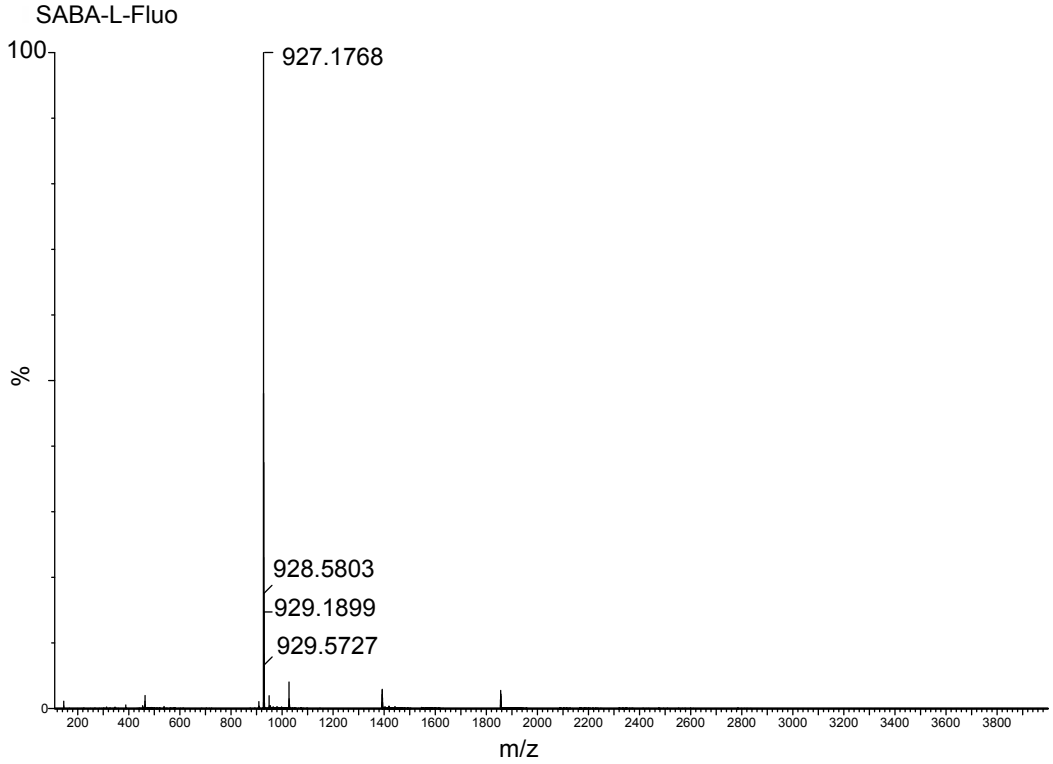
Compound	Formula	MS calculated	ionization	MS detected
NH_2-L-Fluo (**5**)	$C_{34}H_{35}N_5O_{11}S$	721.2054	ES -	720.2874
SABA-L-Fluo (**6**)	$C_{42}H_{40}N_8O_{13}S_2$	928.2156	ES +	927.1768
AAZ-L-Fluo (**7**)	$C_{40}H_{41}N_9O_{15}S_3$	983.1884	ES +	982.1185
AAZ^+-L-Fluo (**8**)	$C_{73}H_{81}N_{15}O_{24}S_3$	1647.4741	ES +	1647.3187

Table S1. The theoretical and detected mass (MS) are illustrated in this table.

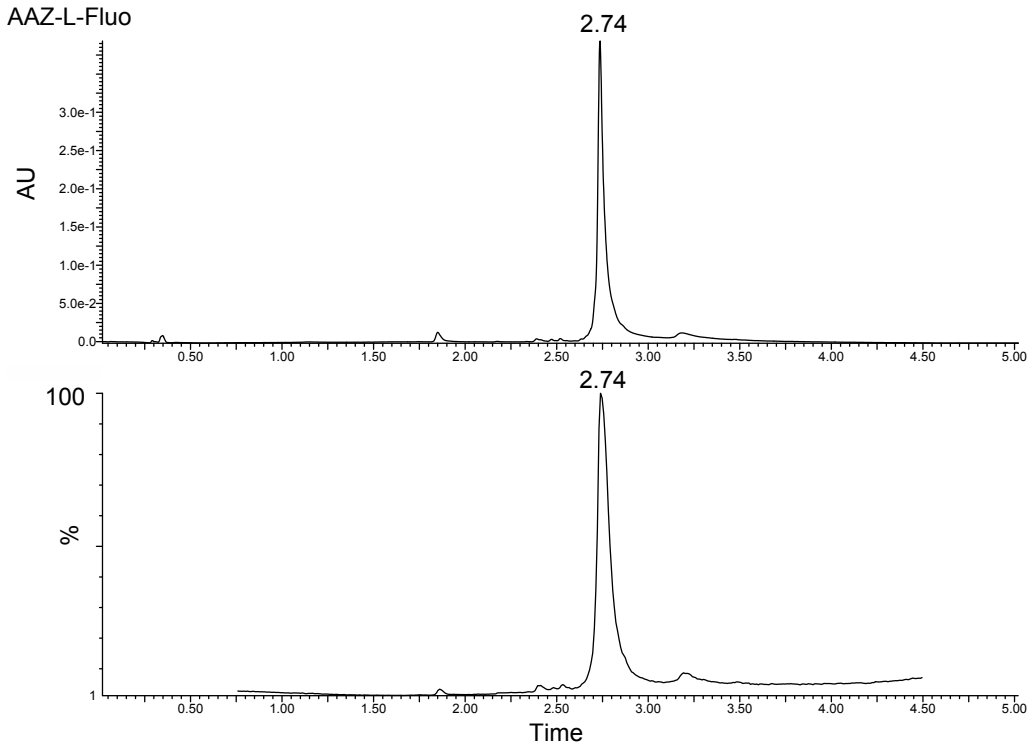
Appendix



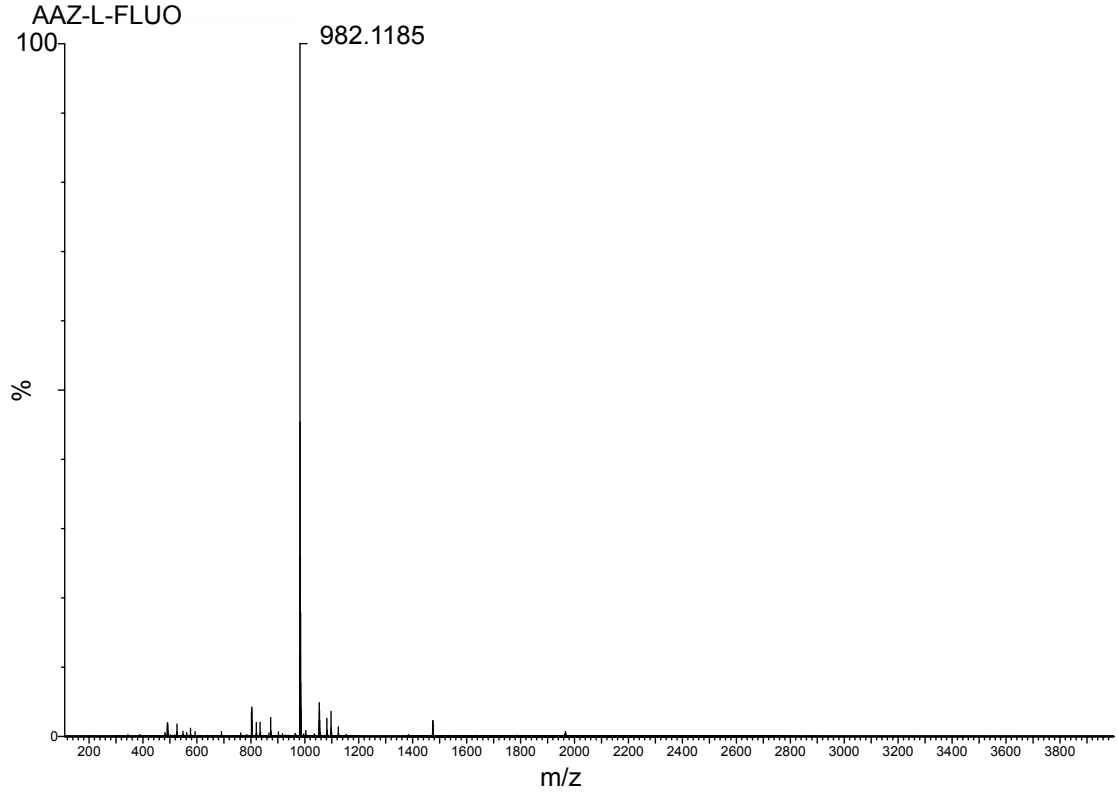
App. Figure1: LS-MS profile of SABA-L-Fluo at UV 260nm wavelength.



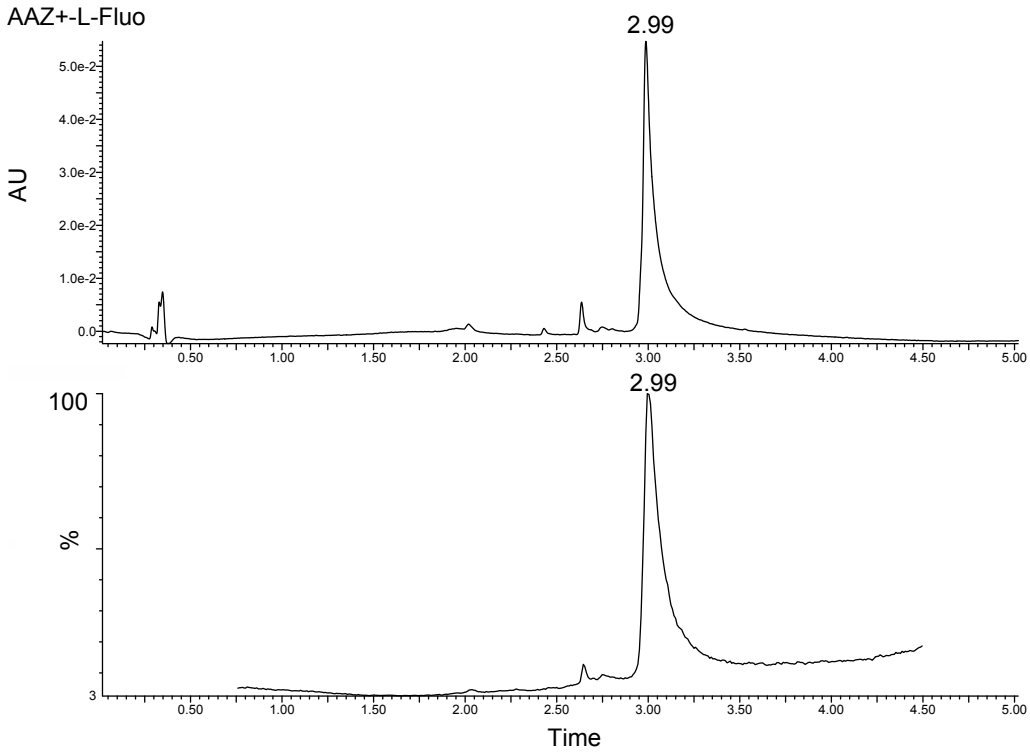
App. Figure 2: LS-MS profile of SABA-L-Fluo.



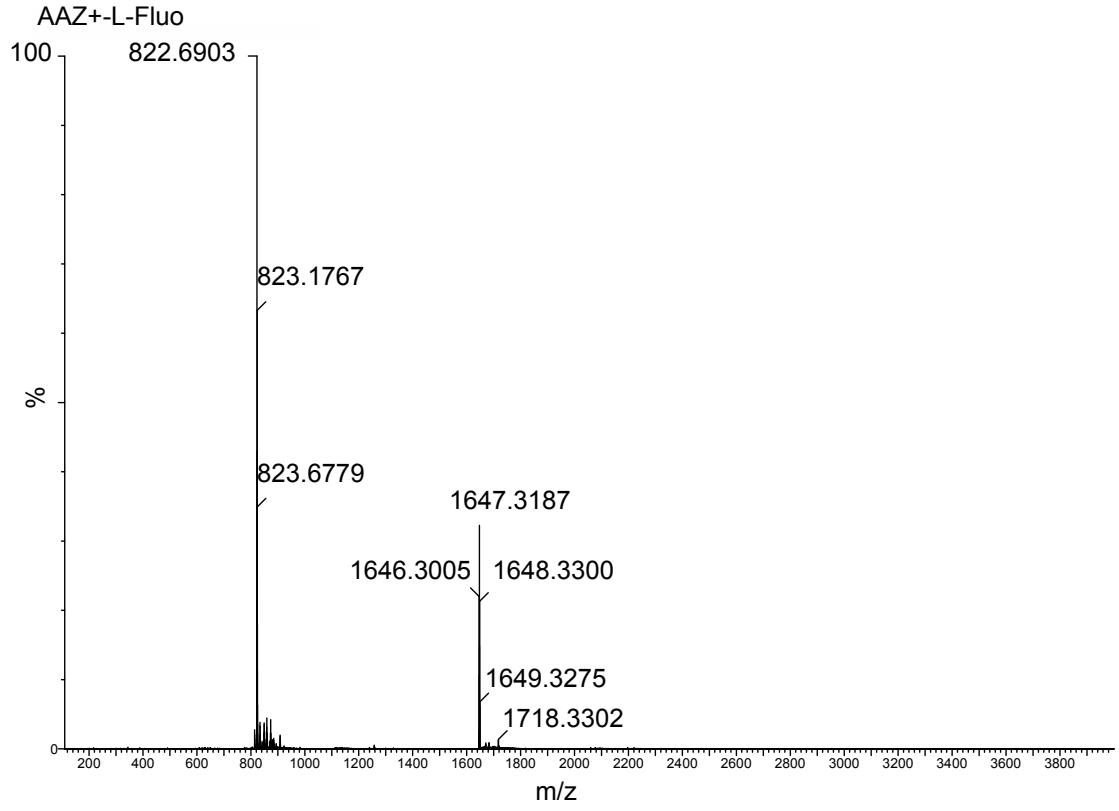
App. Figure 3: LS-MS profile of AAZ-L-Fluo at UV 260nm wavelength.



App. Figure 4: LS-MS profile of AAZ-L-Fluo.



App. Figure 5: LS-MS profile of AAZ⁺-L-Fluo at UV 260nm wavelength.



App. Figure 6: LS-MS profile of AAZ⁺-L-Fluo.

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