Enhanced Therapeutic Activity of Non-Internalizing Small Molecule-Drug Conjugates Targeting Carbonic Anhydrase IX in Combination With Targeted Interleukin-2

SUPPLEMENTARY INFORMATION

Samuele Cazzamalli [a,§], Barbara Ziffels [a,§], Fontaine Widmayer [a], Patrizia Murer [a], Giovanni Pellegrini [b], Francesca Pretto [c], Sarah Wulhfard [c] & Dario Neri [a,*]

a) Department of Applied Biosciences, Swiss Federal Institute of Technology (ETH Zürich), Vladimir-Prelog-Weg 4, CH-8093 Zurich (Switzerland)

b) Laboratory for Animal Model Pathology, Institute of Veterinary Pathology, University of Zurich, Winterthurerstrasse 268, CH-8057 Zurich (Switzerland)

c) Philochem AG, Libernstrasse 3, CH-8112 Otelfingen (Switzerland)

§) These authors contributed equally to this work

*) Corresponding Author

Tel: +41-44-6337401

e-mail: neri@pharma.ethz.ch

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Abbreviations

AA	Antibiotic-Antimycotic
AAZ	Acetazolamide
AAZ^{+}	Affinity matured acetazolamide
Arg	Arginine
Boc	*tert*-Butoxycarbonyl
CAIX	Carbonic Anhydrase IX
Cit	Citrulline
Cys	Cysteine
CT	Chlorotrityl
CuAAC	Azide-Alkyne Huisgen Cycloaddition
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
eq	Equivalents
FA	Formic Acid
FCS	Fetal Calf Serum
Fmoc	9-Fluorenylmethoxycarbonyl
HATU	O-(7-azabenzotriazol-1-yl)-tetramethyl-uronium hexafluorophosphate
HBSS	Hank's Balanced Salt Solution
hCAIX	Human Carbonic Anhydrase IX
HOAt	1-Hydroxy-7-azabenzotriazole
HPLC	High Performance Liquid Chromatography
IC	Inhibitory Capacity

IVIS	In Vivo Imaging System
Lys	Lysine
MC	Maleimidocaproyl
MeCN	Acetonitrile
MeOH	Methanol
MMAE	Monomethyl Auristatin E
MS	Mass Spectroscopy
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyl phenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MW	Molecular Weight
PABC	*p*-aminobenzylalcoholcarbonate
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PBS	Phosphate-Buffered Saline
ppm	Part per million
RCC	Renal Cell Carcinoma
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
SEM	Standard Error of the Mean
SMDC	Small Molecule-Drug Conjugate
SPPS	Solid Phase Peptide Synthesis
*t*Bu	*tert*-Butyl
Tc-99m	Technetium-99m
TFA	Trifluoroacetic Acid
TIS	Triisopropylsilane

R_f	Retention factor
Trt	Trityl
UPLC	Ultra Performance Liquid Chromatography
Val	Valine

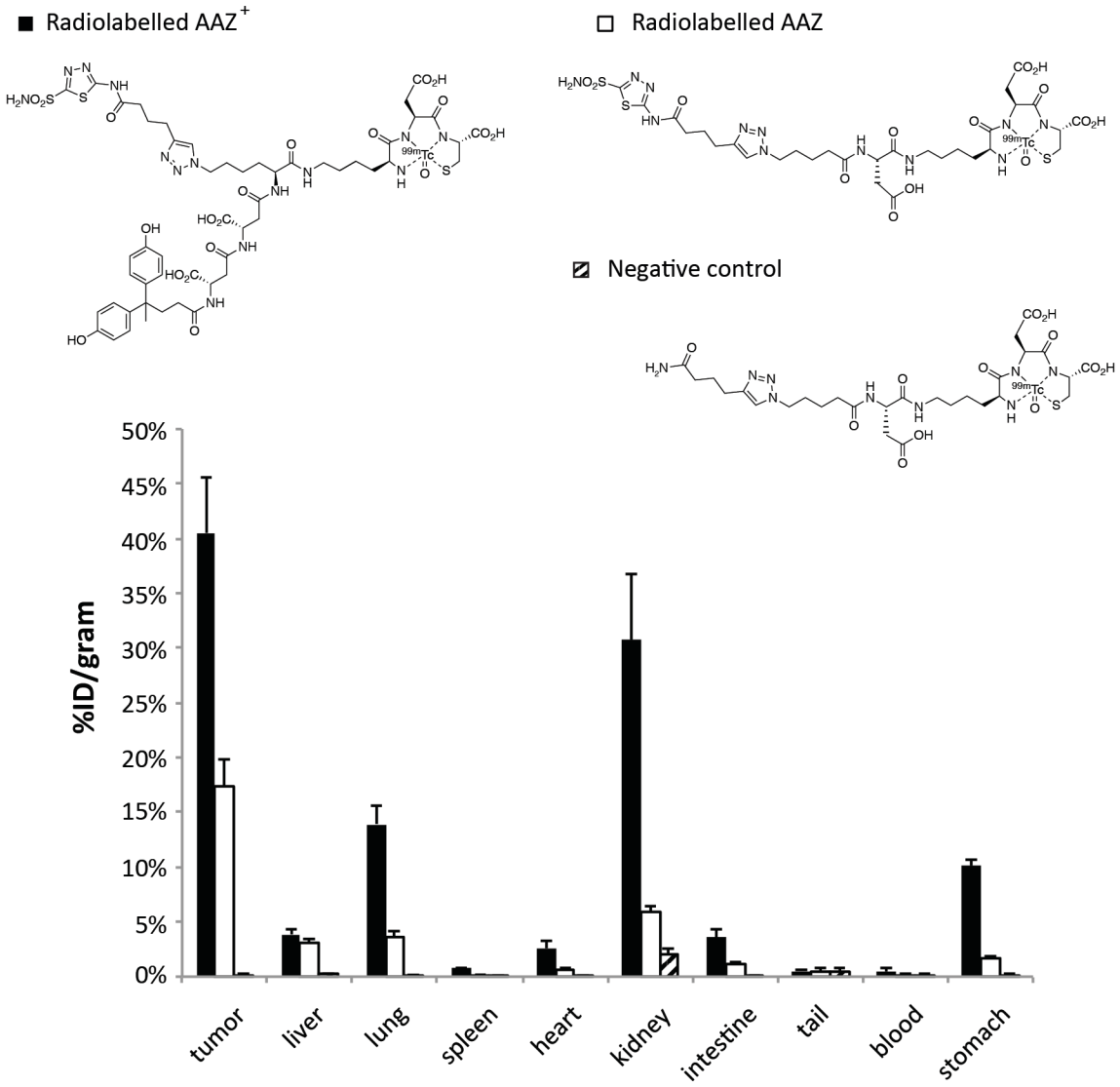
General Remarks and Procedures

Anhydrous solvents were purchased from Acros or Fluka. Peptide grade *N,N*-dimethylformamide (DMF) for solid phase synthesis was bought from ABCR. All other solvents were used as supplied by Fisher Chemicals, Merck or Sigma Aldrich in HPLC or analytical grade. H-Cys(Trt)-2-CT-polystyrene resin was purchased from RAPP Polymere. Ultra-Technekow DTE Mo-99/Tc-99m Generator was purchased from Mallinckrodt Pharmaceuticals (675 McDonnell Blvd. St. Louis, MO 63042 USA). Iodine-125 radionuclide was purchased from Hartmann Analytic GmbH (Steinriedendamm 15, Geb. 1G, 38108 Braunschweig, Germany). Maleimidocaproyl-ValCit-*p*-aminobenzylalcohol-MMAE and free MMAE were purchased from Levena Biopharma (No.9 Weidi Road, Qixia District, Nanjing, 210046, China). Maleimidoethyl-IRDye680RD was purchased from LI-COR Biosciences (4647 Superior Street Lincoln, Nebraska USA 68504-5000). L19-IL2 was produced by Philogen S.p.A. (Via Bellaria, 35, 53018 Sovicille SI, Italy) and diluted to the concentration used for therapy studies with the appropriate formulation buffer (Philogen). QIFIKIT® was purchased from Agilent (Santa Clara, CA 95051 United States). All other reagents were purchased from Sigma Aldrich, Acros, ABCR or TCI and used as supplied. All reactions using anhydrous conditions were performed using oven-dried glassware under an atmosphere of argon. Silica for flash column chromatography (high-purity grade, pore size 60 Å) was purchased from Sigma Aldrich. Concentration under reduced pressure was performed by rotary evaporation at 40 °C at the appropriate pressure. Yields refer to chromatographically purified and spectroscopically pure compounds, unless noted otherwise.

High-Resolution Mass Spectrometry (HRMS) spectra and analytical Reversed-Phase Ultra Performance Liquid Chromatography (UPLC) were recorded on a Waters Xevo G2-XS QTOF coupled to a Waters Acquity UPLC H-Class System with PDA UV detector, using a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml min^{-1} with linear gradients

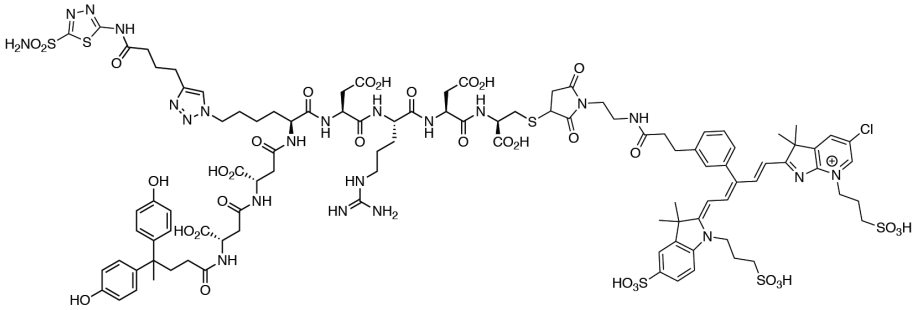
of solvents A and B (A = Millipore water with 0.1% FA, B = MeCN with 0.1% FA). Preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) were performed on a Waters Alliance HT RP-HPLC with PDA UV detector, using a Synergi 4μm, Polar-RP 80Å 10 × 150 mm C18 column at a flow rate of 4 ml min^{-1} with linear gradients of solvents A and B (A = Millipore water with 0.1% TFA, B = MeCN with 0.1% TFA).

Quantitative and qualitative biodistribution of AAZ^+-99mTc and of AAZ-99mTc in the SKRC-52 model

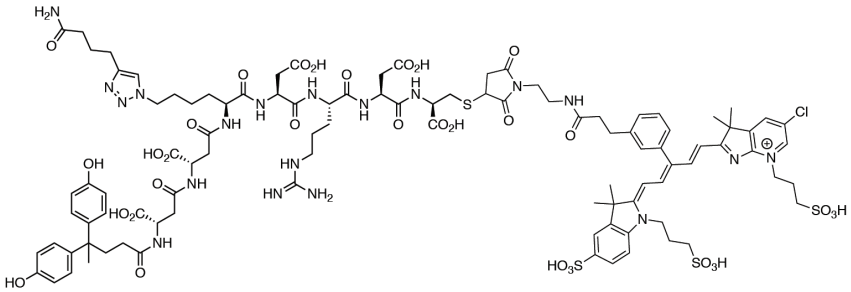


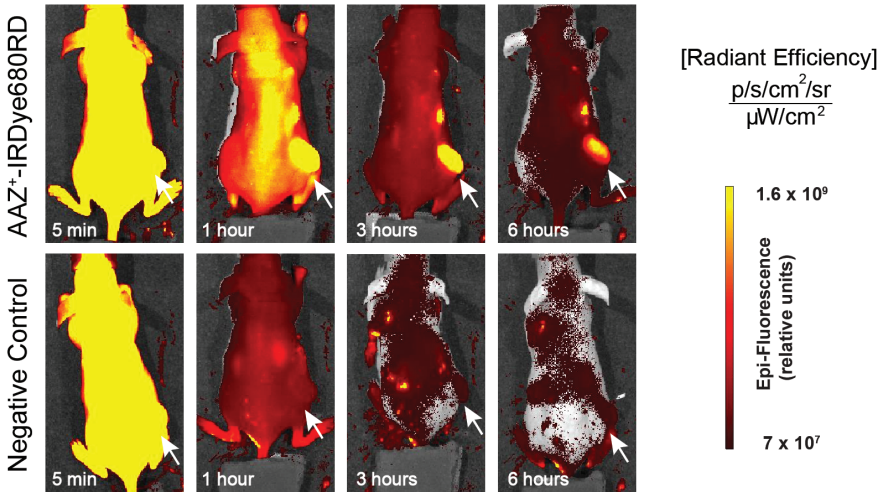
Supplementary Figure 1 Chemical structures and organ distribution of ^{99m}Tc-radiolabeled AAZ and AAZ^+ (compounds **7** and **8**, respectively) in BALB/c nu/nu mice bearing SKRC-52 xenografts (n = 3 per group). A compound devoid of the anti-CAIX targeting moiety (compound **9**) served as negative control for the experiment. The data, expressed as mean % Injected Dose/gram of tissue ± SD (%ID/gram), correspond to the 6 hours time point after the intravenous administration of the radiolabeled compound.

AAZ^{+}-IRDye680RD :



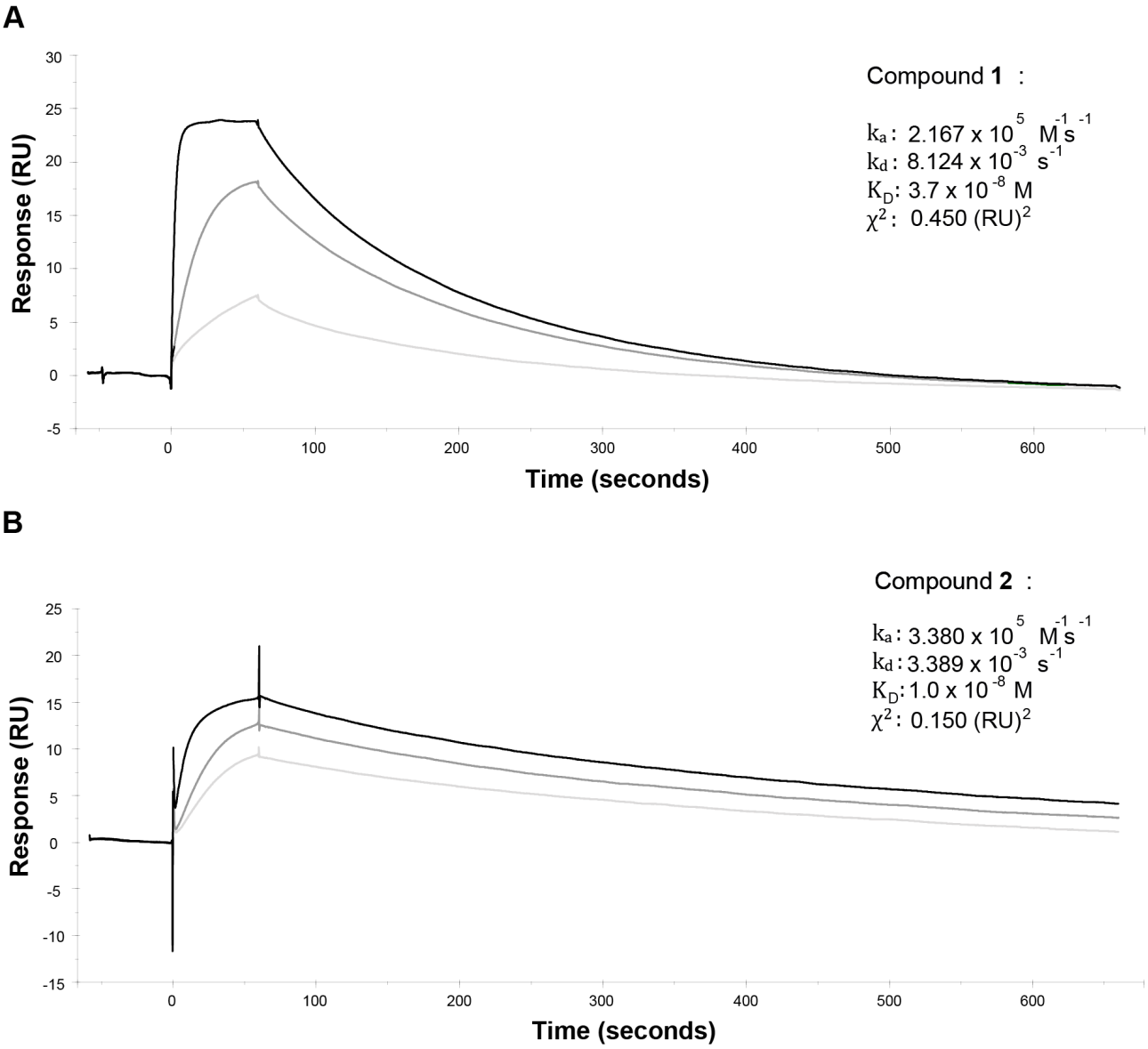
Negative Control :





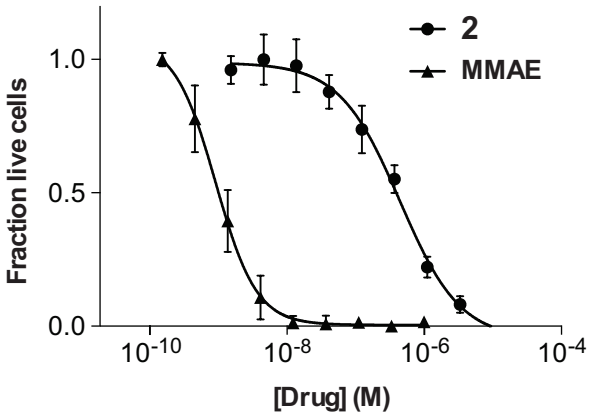
Supplementary Figure 2 Structures and qualitative biodistribution of AAZ^{+}-IRDye680RD (**13**) and of the corresponding negative control (**14**), devoid of the acetazolamide-targeting moiety. A selective tumor uptake of AAZ^{+}-IRDye680RD can be observed at early time points (1, 3 and 6 hours) in immunodeficient BALB/c nu/nu mice bearing SKRC-52 tumors (white arrows) by near-infrared fluorescence imaging.

Surface Plasmon Resonance (SPR) - SMDCs on human CAIX



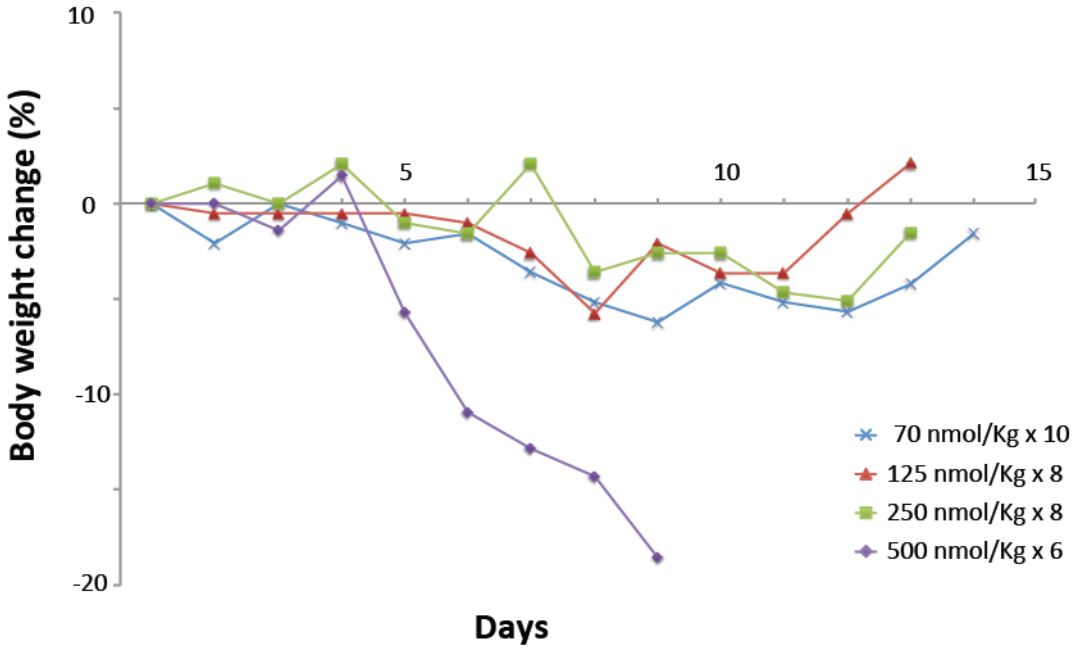
Supplementary Figure 3 SPR sensorgrams and fitting data of a dilution series of (A) AAZ-ValCit-MMAE (**1**; 1 μM to 250 nM in steps of 1:2) and (B) AAZ^+-ValCit-MMAE (**2**; 500 nM to 125 nM in steps of 1:2) binding to immobilized human carbonic anhydrase IX (hCAIX). Binding kinetics were analyzed with the BIAcore™ S200 evaluation software version using a 1:1 Langmuir binding model. BIAcore™ methodology may overestimate the K_D of small organic ligands and it can measure only apparent k_{on} values (lower than k_{on} values measured in solution).

In vitro cytotoxicity of AAZ^+-ValCit-MMAE on SKRC-52 cells



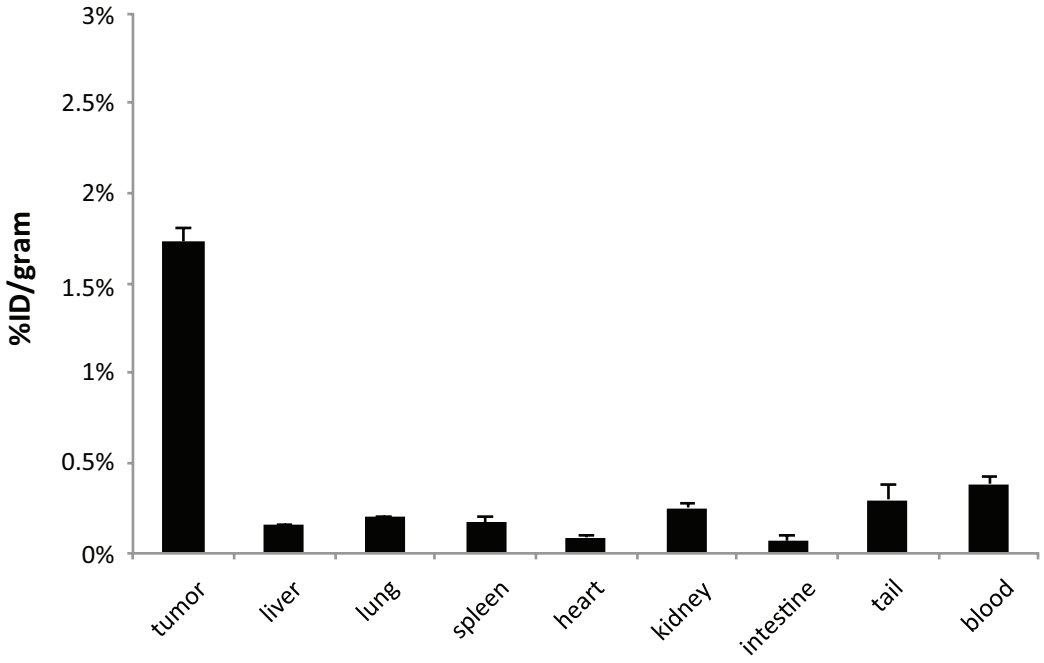
Supplementary Figure 4 Cells were incubated for 72 hours in the presence of various concentrations of the test compound at 37°C. Data points are averages of three experiments. Error bars indicate standard deviations. Compound **2** behaves as a prodrug, being found to be less potent *in vitro* than the corresponding free drug (IC50 value: 440 nM for **2**, 0.9 nM for **MMAE**). This experimental data confirms the absence of internalization of the affinity matured AAZ^+ ligand for CAIX.

Dose escalation for AAZ^{+}-ValCit-MMAE in nude mice bearing SKRC-52 xenografts



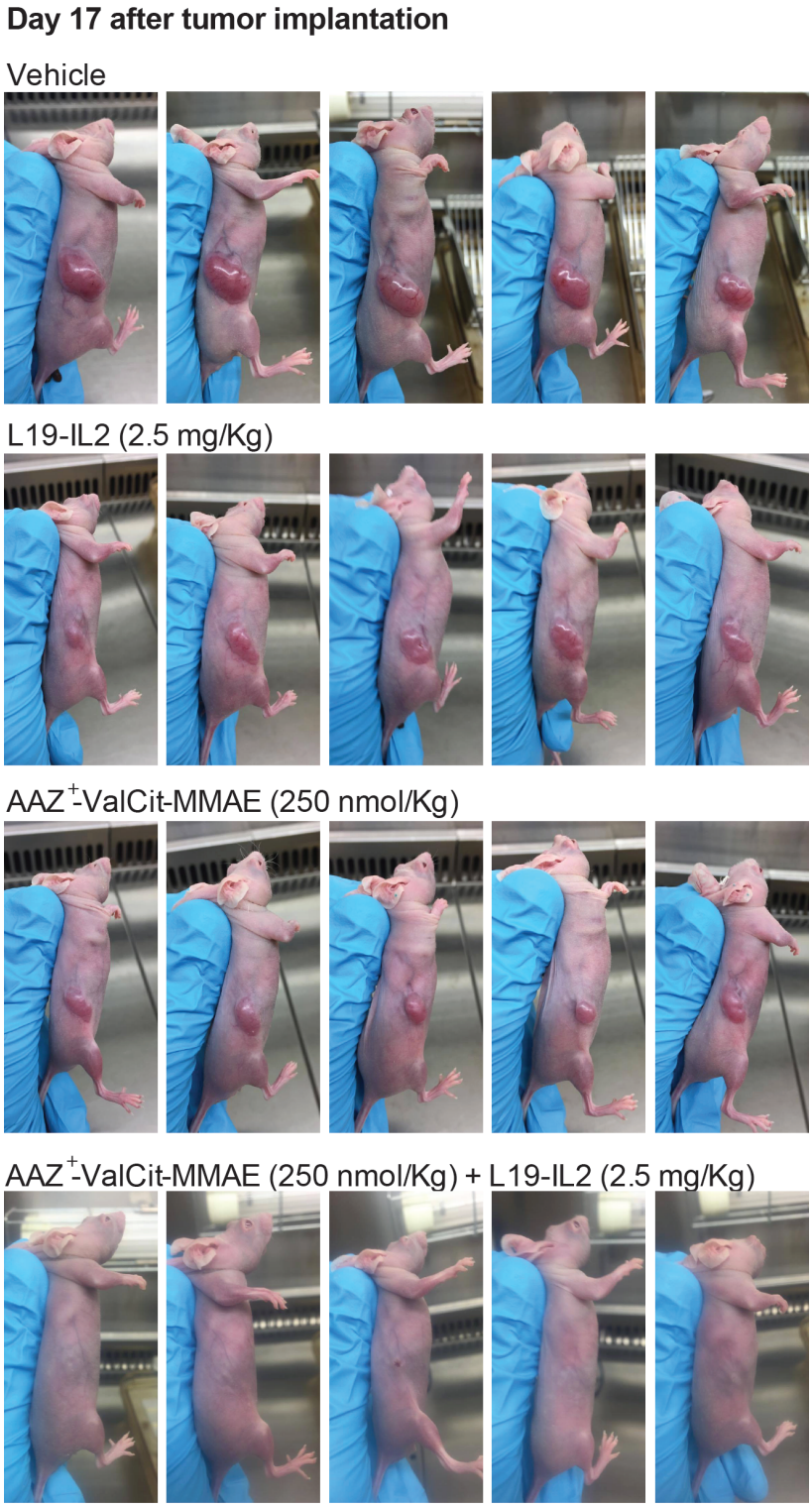
Supplementary Figure 5 Animals (n =1 per group) that were treated daily with AAZ^{+}-ValCit-MMAE (**2**; IV injections) did not loose more than 5% of their initial body weight over 14 days after the initial injection of 70, 125 or 250 nmol/Kg. The dose of 250 nmol/Kg was considered as well tolerated. Mice treated with 500 nmol/Kg suffered of a major body weight loss (20% at day 9 after six IV injections).

Quantitative biodistribution of L19-IL2 in the SKRC-52 model



Supplementary Figure 6 Organ distribution of radioiodinated L19-IL2 in BALB/c nu/nu mice bearing SKRC-52 xenografts (n = 3). The data, expressed as mean % Injected Dose/gram of tissue ± SD, correspond to the 24 hours time point after the intravenous administration of the radiolabeled protein.

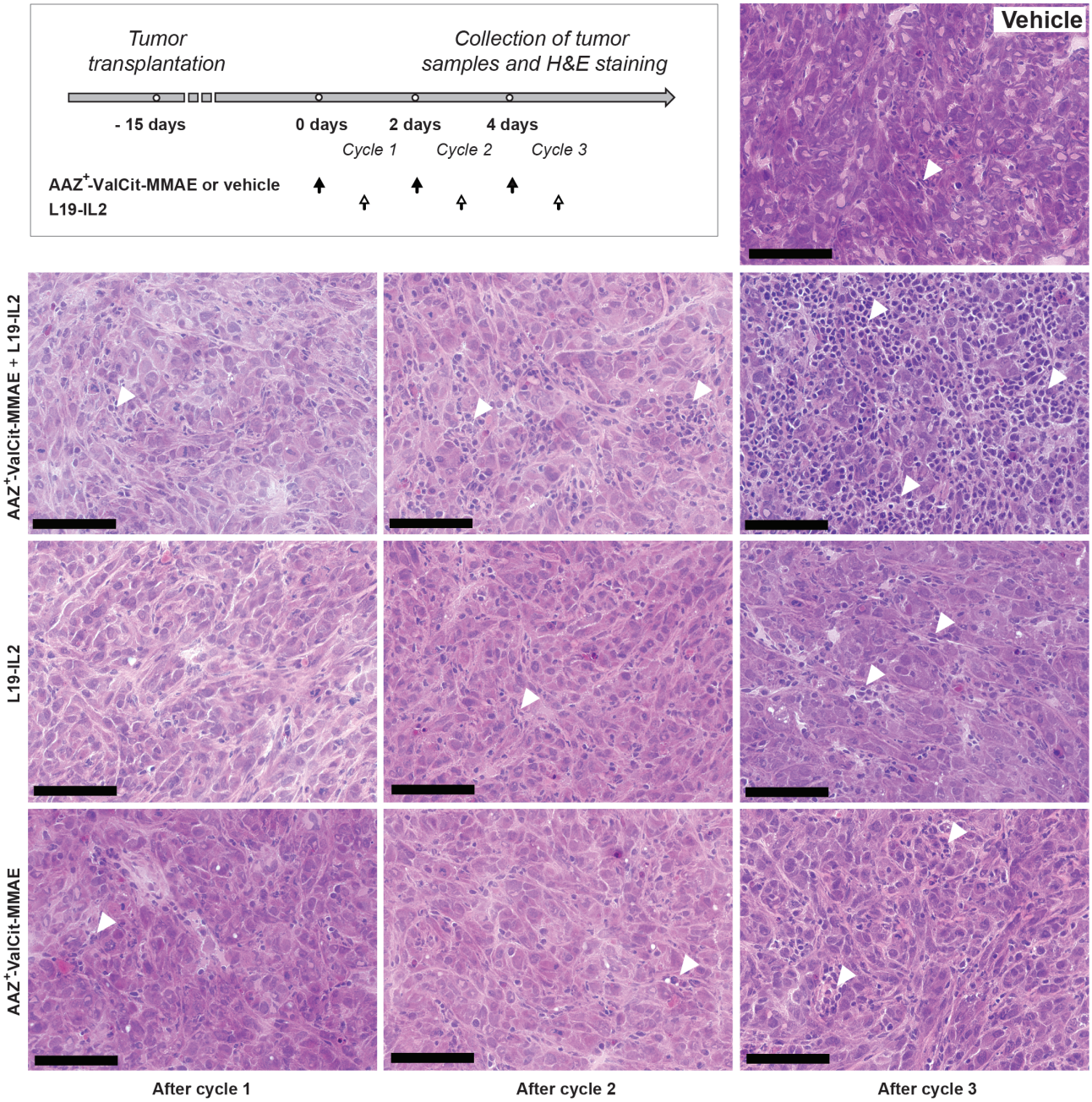
Therapeutic effect of AAZ^{+}-ValCit-MMAE/L19-IL2 on SKRC-52 tumor bearing mice



Supplementary Figure 7 Photos of BALB/c nude mice bearing subcutaneous SKRC-52 tumors, on day 17 after tumor implantation, after having received different treatments. Potent antitumor activi-

ty was observed for L19-IL2 and AAZ^{+}-ValCit-MMAE (**2**), but only the combination of the two agents led to the complete regression of the tumor in the model.

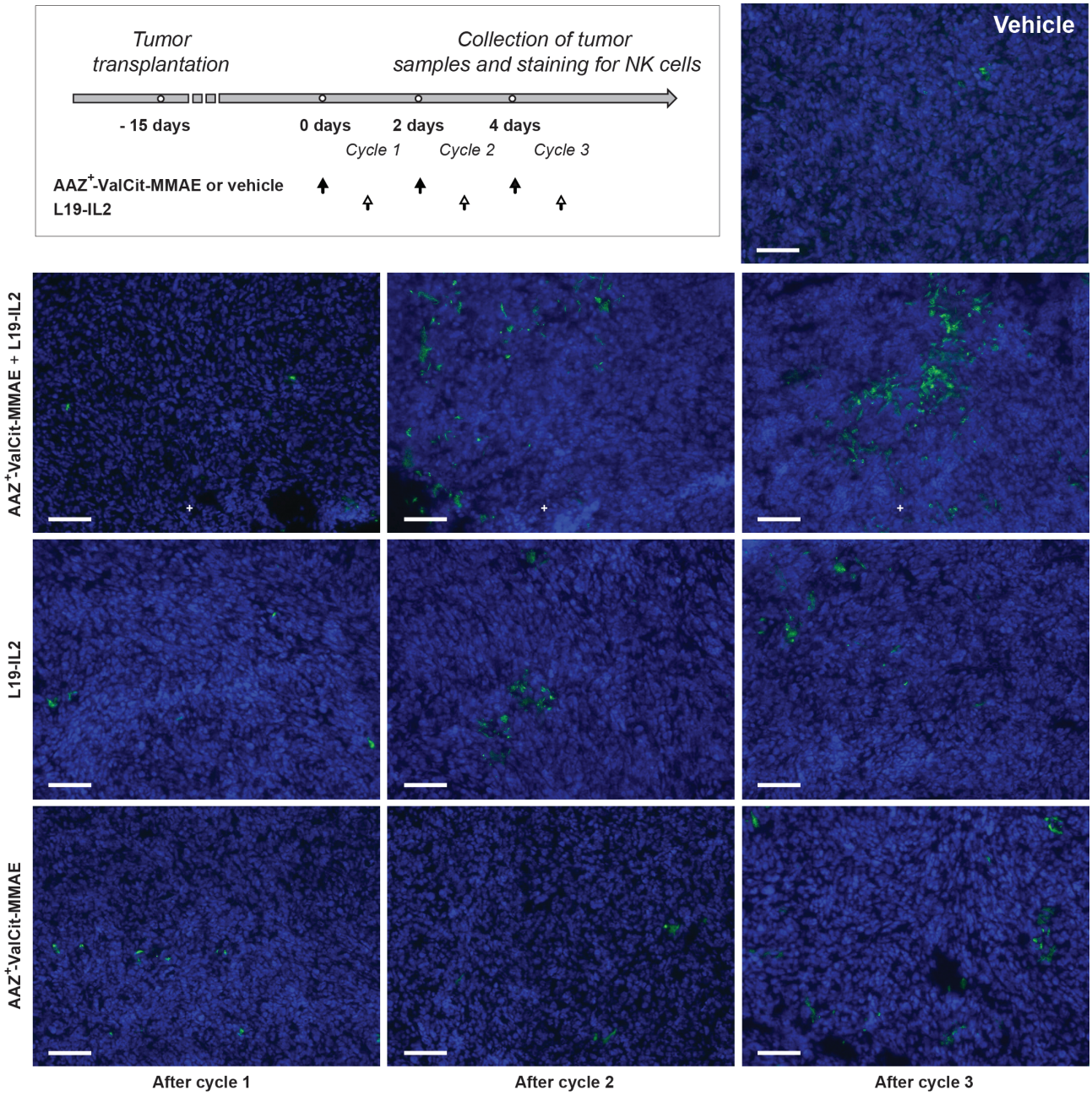
Structural analysis by H&E staining of SKRC-52 tumors after therapy



Supplementary Figure 8 Nude mice bearing subcutaneous SKRC-52 xenografts were treated with the indicated therapeutics. The figure shows representative H&E stainings of tumor sections obtained after 1, 2 or 3 cycles of therapy (as indicated in the scheme above). Inflammatory cells are indicated in the figure with a white arrowhead. Increased numbers of inflammatory cells in the xen-

ografts exposed to combination therapy are already observed following the second cycle. Scale bars: 100 μm.

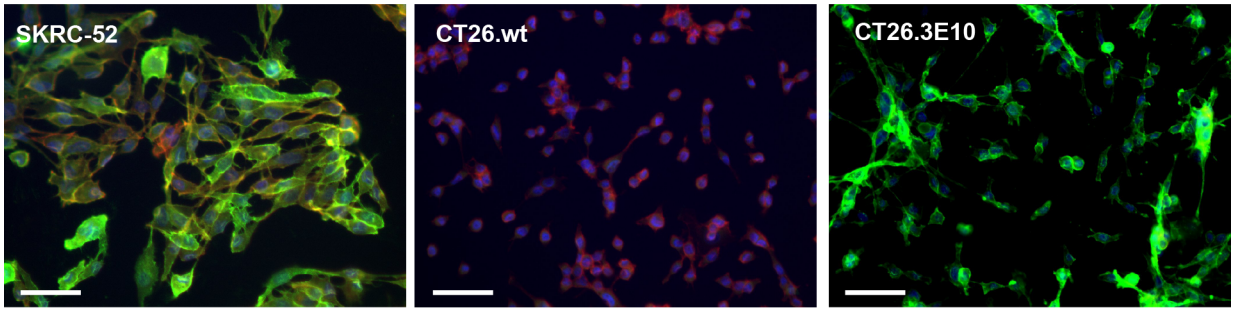
Immunofluorescence analysis of SKRC-52 tumor-infiltrating NK cells after therapy



Supplementary Figure 9 Nude mice bearing subcutaneous SKRC-52 xenografts were treated with the indicated therapeutics. The figure shows representative fluorescent images of tumor sections obtained after 1, 2 or 3 cycles of therapy (as indicated in the scheme above) and stained for NK cells (green, NKp46). Scale bars: 100 µm. Treatment with the SMDC/L19-IL2 combination, but not

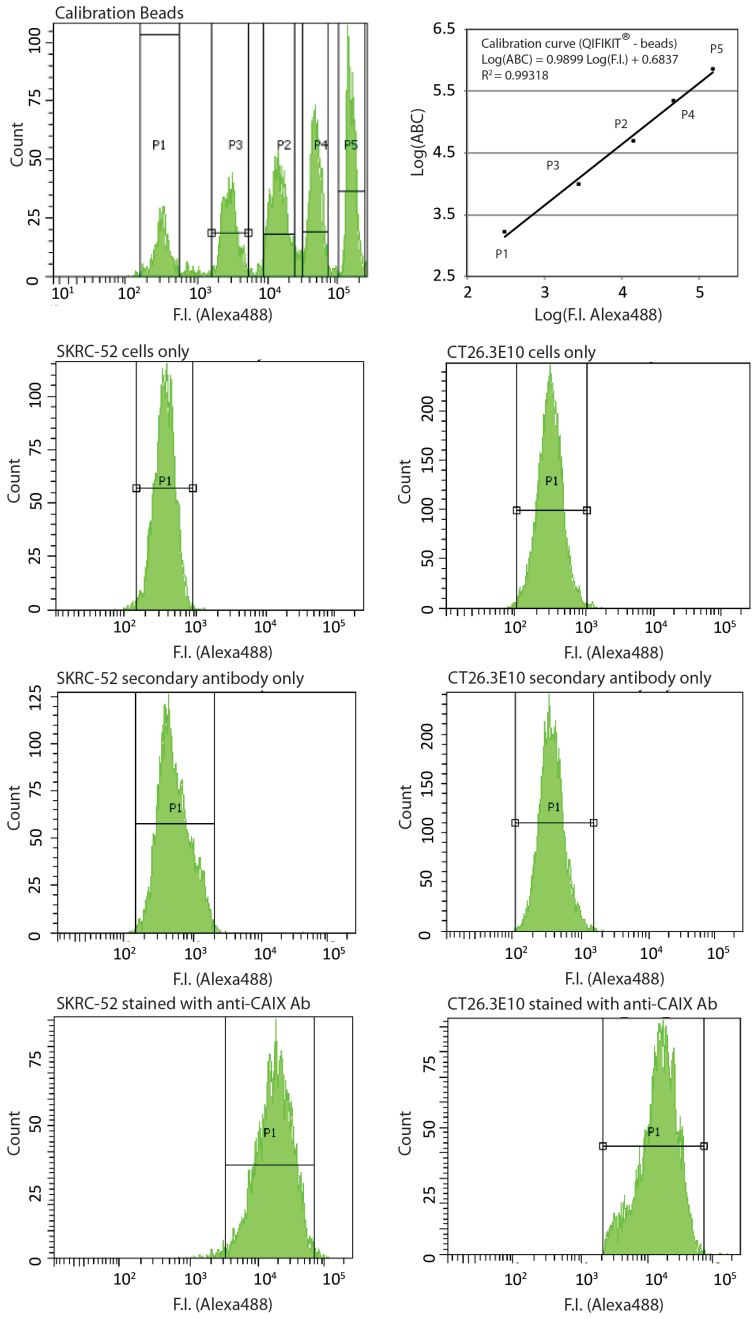
with single agents injected in a monotherapy setting, leads to the recruitment of spots of NK cells at the tumor site, which is evident in mice treated with three cycles of the combination.

Immunofluorescence analysis of the expression of hCAIX on CT26.3E10



Supplementary Figure 10 Antigen expression by CT26.3E10 transfected monoclonal cell line was checked by immunofluorescence, FACS and confocal microscopy analysis and the cells were then used for further *in vivo* experiments. Green = human CAIX staining by a human anti-CAIX specific antibody; Red = cytoskeleton staining by phalloidin; Blue = nuclei staining (DAPI); Scale bars: 50 μm.

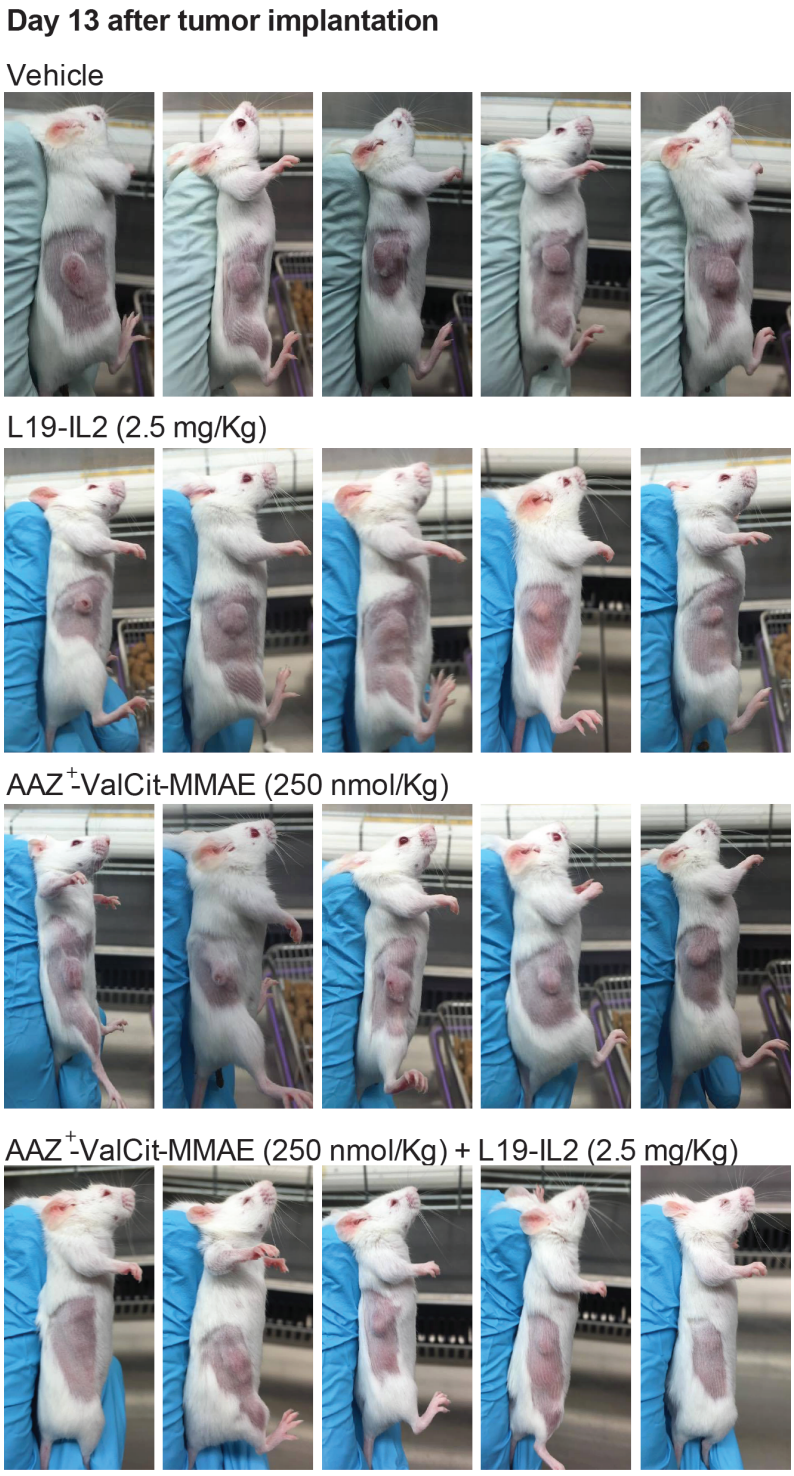
CAIX quantification on tumor cells



Supplementary Figure 11 Quantification of human CAIX antigen on the surface of SKRC-52 and CT26.3E10 tumor cell lines by FACS methodology. Calibration beads were stained with an Alexa 488 anti-murine Fc secondary antibody. The histogram of QIFIKIT® Calibration Bead populations and the relative calibration curve are shown. SKRC-52 and CT26.3E10 were analyzed as such (cells

only), after incubation with secondary antibody only or after CAIX staining. Antigen density calculated for SKRC-52 (84’600 copies of the protein per cell) was higher compared to the value obtained for CT26.3E10 cells (71’900 copies of the protein per cell).

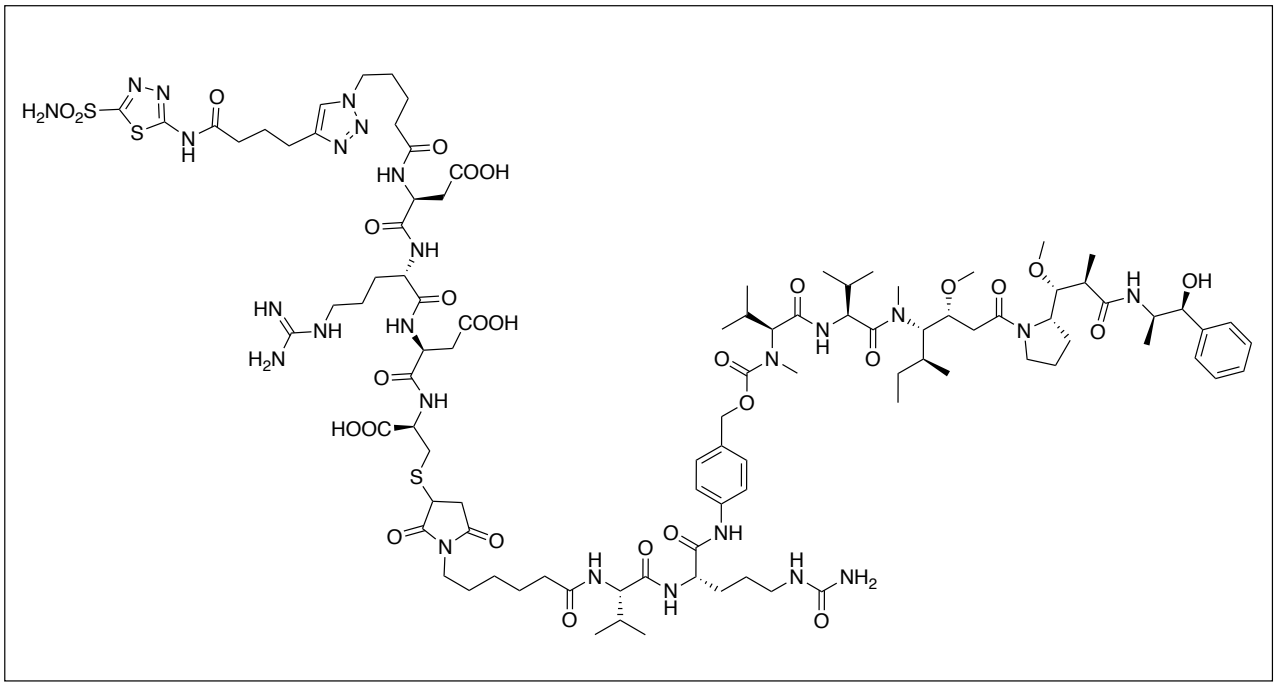
Therapeutic effect of AAZ^{+}-ValCit-MMAE/L19-IL2 on CT26.3E10 tumor bearing mice



Supplementary Figure 12 Photos of BALB/c immunocompetent mice bearing subcutaneous CT26.3E10 tumors, on day 13 after tumor implantation, after having received different treatments.

Experimental procedures and characterization data for present compounds

AAZ-ValCit-MMAE – Compound 1

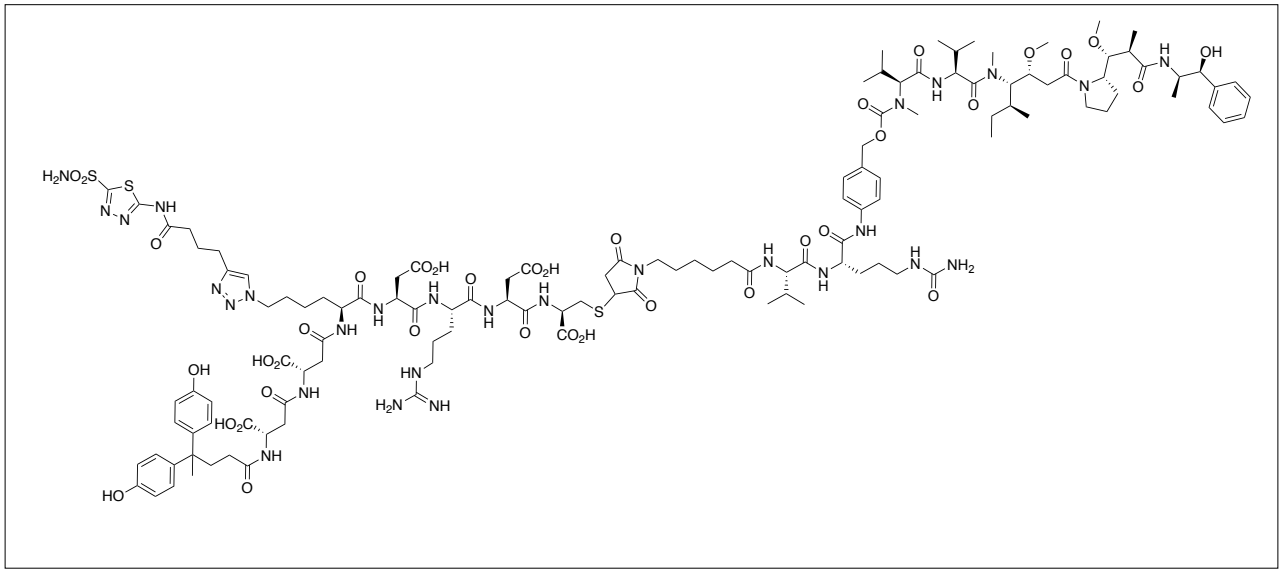


Chemical Formula: $C_{98}H_{151}N_{25}O_{28}S_3$

Molecular Weight: 2223.61

Compound **1** was prepared according to previously described procedures [1,2].

AAZ^{+}-ValCit-MMAE – Compound 2

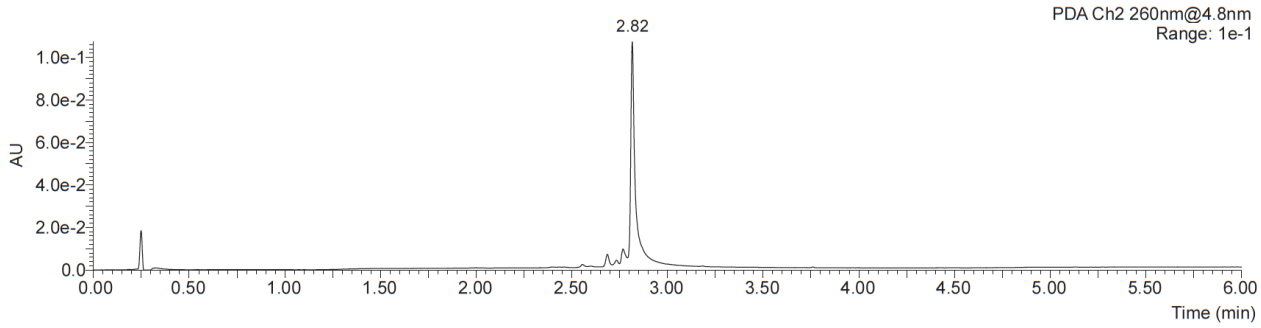


Chemical Formula: $C_{124}H_{180}N_{28}O_{37}S_3$

Molecular Weight: 2751.14

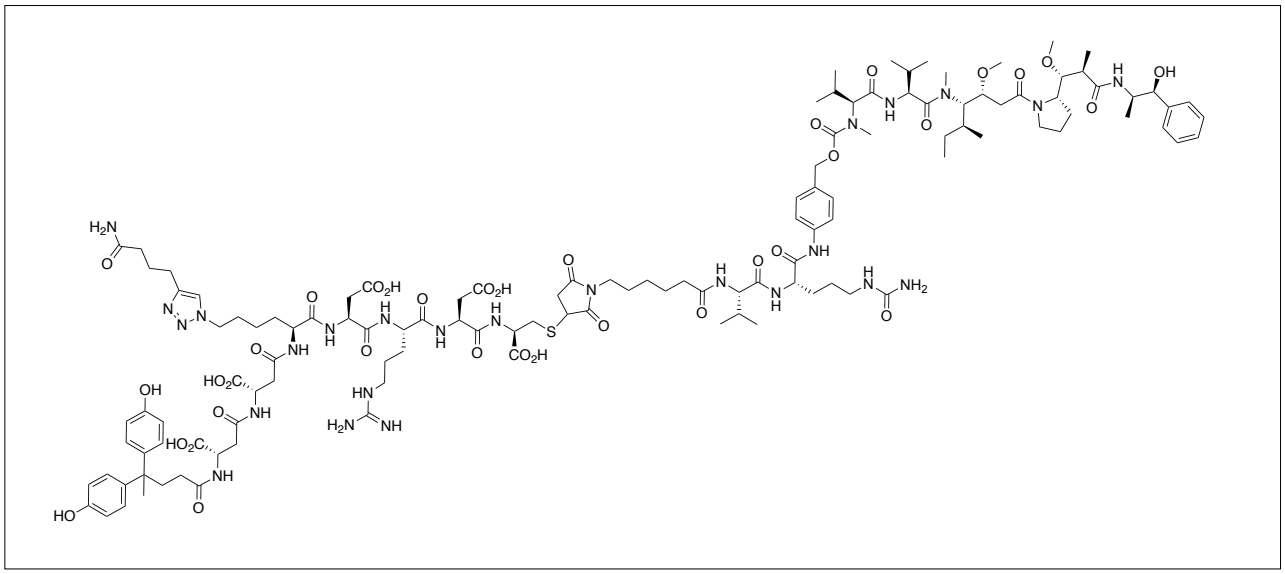
Compound **11** (3.5 mg, 2.44 µmol, 1.1 eq) was dissolved in degassed PBS (pH 7.4; 600 µl). Commercially available Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE (3.0 mg, 2.23 µmol, 1.0 eq) was added as a DMF solution (500 µl) and the mixture was stirred at room temperature. After 3 hours, UPLC-MS indicated completion. The solvents were removed under vacuum and the crude was diluted in a 1:1 H_2O/MeCN mixture (1 ml). The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve compound **2** as a white solid (3.1 mg; 1.13 µmol; 50.8% yield).

MS (ESI) m/z calcd. for $[C_{124}H_{182}N_{28}O_{37}S_3]^{2+}$: 1375.6113 $[M+2H]^{2+}$, found: 1375.6986; m/z calcd. for $[C_{124}H_{183}N_{28}O_{37}S_3]^{3+}$: 917.4075 $[M+3H]^{3+}$, found: 917.4496.



Supplementary Figure 13 Analytical UPLC trace of compound **2** on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml min^{-1}, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.

NH_2^+-ValCit-MMAE negative control – Compound 3

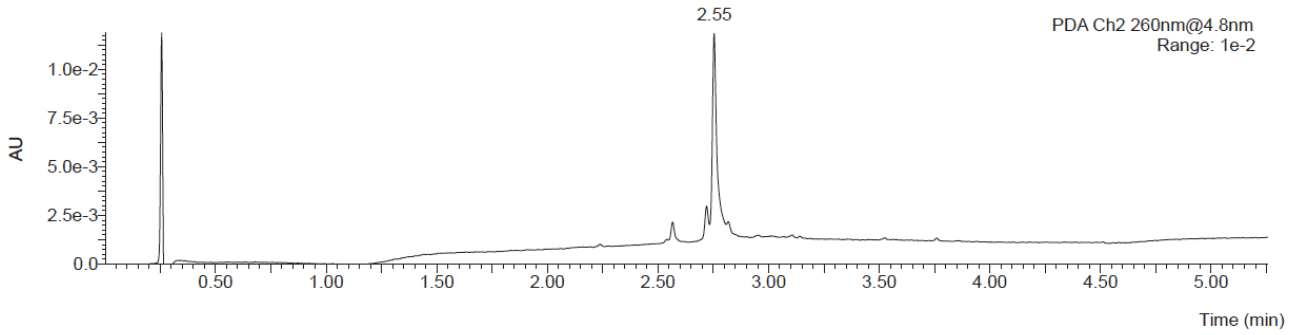


Chemical Formula: $C_{122}H_{179}N_{25}O_{35}S_1$

Molecular Weight: 2587.97

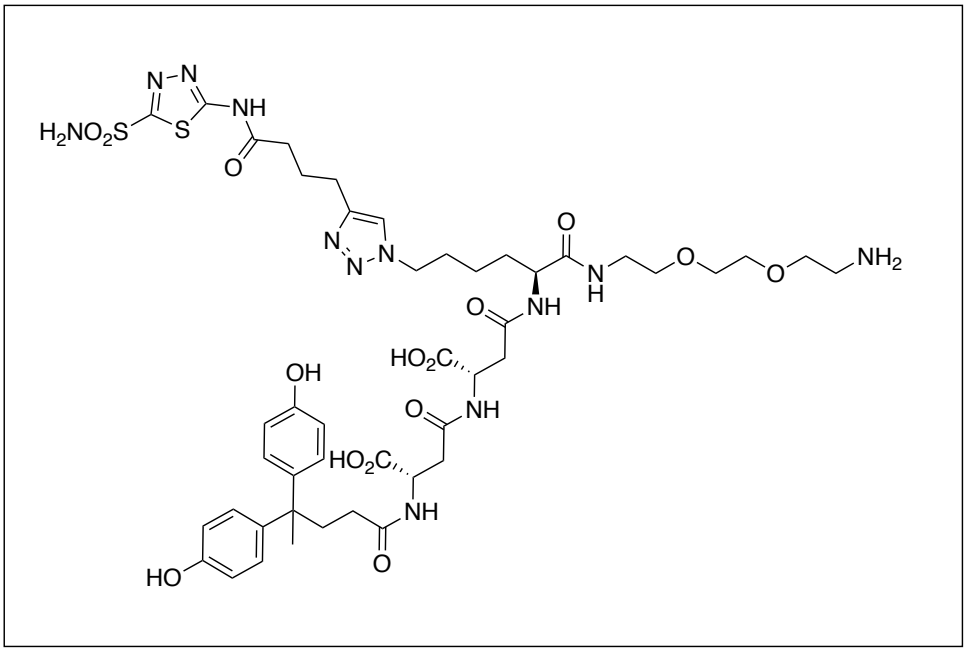
Compound **12** (2.5 mg, 1.97 µmol, 1.3 eq) was dissolved in degassed PBS (pH 7.4; 600 µl). Commercially available Maleimidocaproyl-ValCit-p-aminobenzylalcohol-MMAE (2.0 mg, 1.52 µmol, 1.0 eq) was added as a DMF solution (500 µl) and the mixture was stirred at room temperature. After 3 hours, UPLC-MS indicated completion. The solvents were removed under vacuum and the crude was diluted in a 1:1 H_2O/MeCN mixture (1 ml). The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve **3** as a white solid (2.2 mg; 0.85 µmol; 56.4% yield).

MS (ESI) m/z calcd. for $[C_{122}H_{181}N_{25}O_{35}S]^{2+}$: 1294.1358 $[M+2H]^{2+}$, found: 1294.2177; m/z calcd. for $[C_{122}H_{182}N_{25}O_{35}S]^{3+}$: 863.0905 $[M+3H]^{3+}$, found: 863.1285.



Supplementary Figure 14 Analytical UPLC trace of compound **3** on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml min^{-1}, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.

AAZ^{+}-NH$_2$ ligand – Compound 4

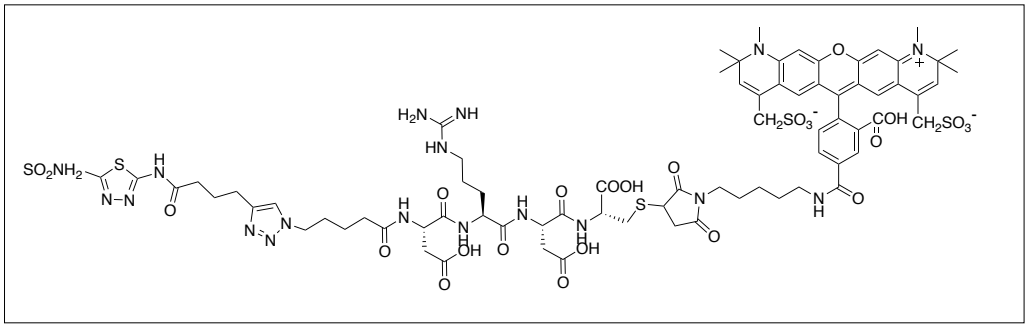


Chemical Formula: $C_{45}H_{62}N_{12}O_{15}S_2$

Molecular Weight: 1075.18

Compound **4** was prepared according to previously described procedures [3].

AAZ-Alexa594 – Compound 5

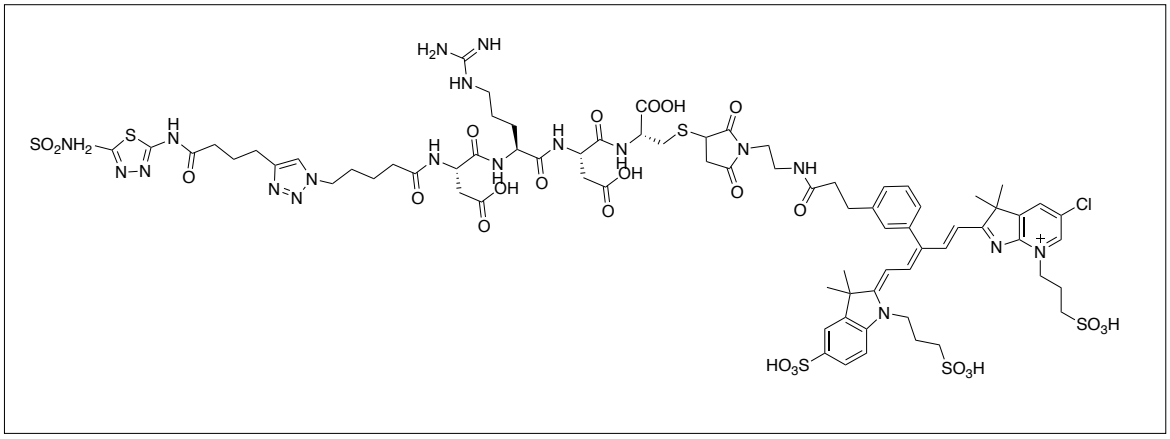


Chemical Formula: $C_{79}H_{91}N_{18}O_{25}S_5$

Molecular Weight: 1792.94

Compound **5** was prepared according to previously described procedures [1].

AAZ-IRDye680RD – Compound 6

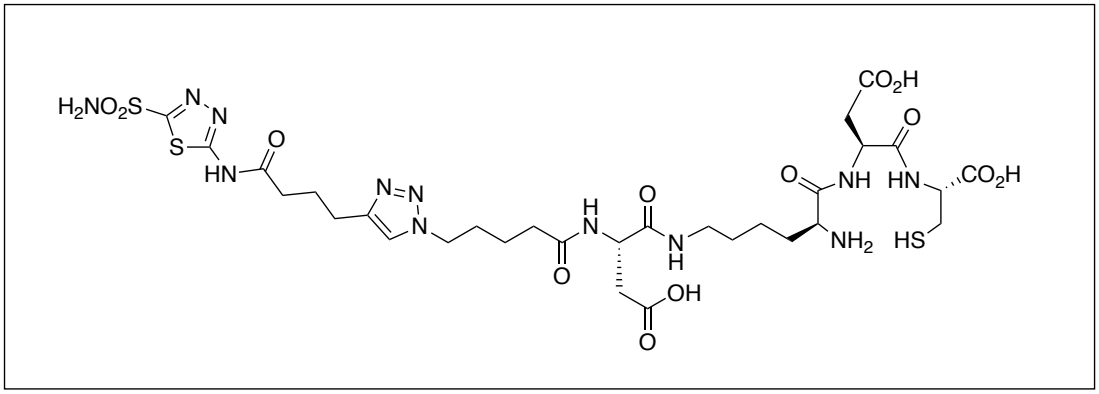


Chemical Formula: $C_{75}H_{97}ClN_{19}O_{25}S_6$

Molecular Weight: 1892.52

Compound **6** was prepared according to previously described procedures [1].

AAZ-99mTc chelator – Compound 7

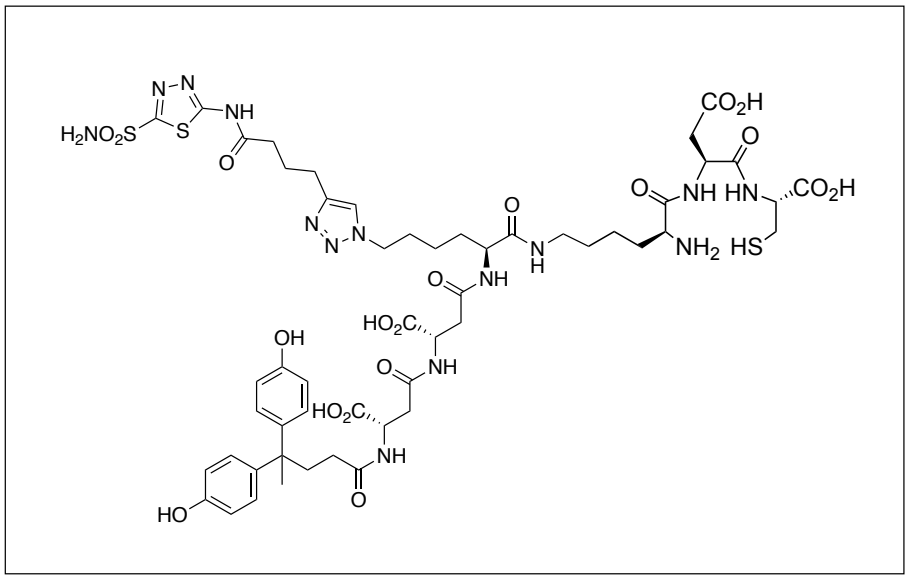


Chemical Formula: $C_{30}H_{46}N_{12}O_{13}S_3$

Molecular Weight: 878.95

Compound **7** was prepared according to previously described procedures [1,4].

AAZ^{+}-99mTc chelator – Compound 8



Chemical Formula: $C_{52}H_{70}N_{14}O_{19}S_3$

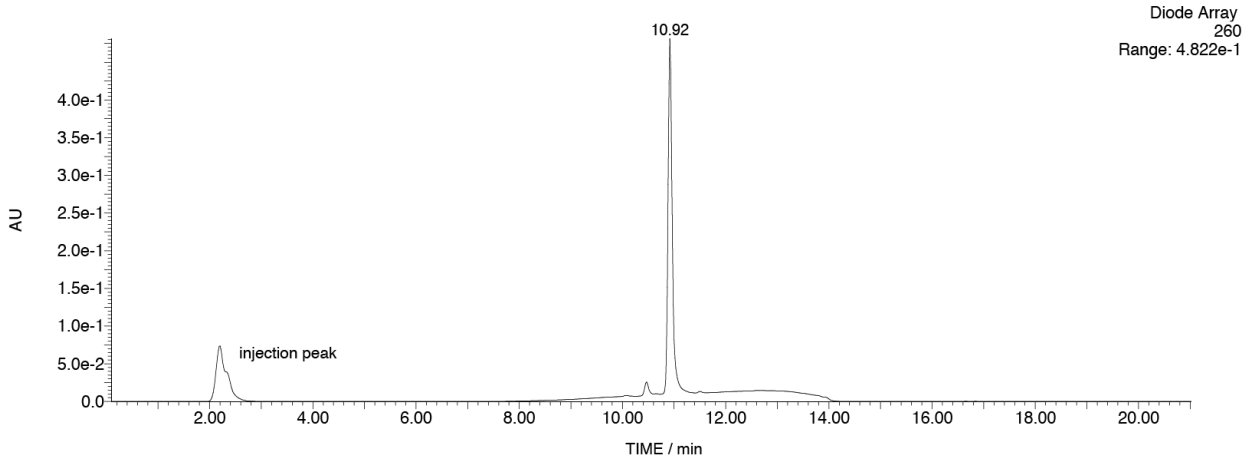
Molecular Weight: 1291.39

Commercially available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (500 mg, 0.32 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 ml) and the resin washed with DMF (4 × 10 min × 10 ml). Fmoc-Asp(OtBu)-OH (388 mg, 0.95 mmol, 3 eq) was activated with HATU (358 mg, 0.95 mmol, 3 eq), and DIPEA (328 µl, 1.88 mmol, 6 eq) in DMF (5 ml) at 0 °C for 5 min and then reacted with the resin for 1 h under gentle agitation. After washing the resin with DMF (4 × 10 min × 10 ml) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 ml) and the resin washed with DMF (4 × 10 min × 10 ml) before the peptide was extended with Boc-Lys(Fmoc)-OH (445 mg, 0.95 mmol, 3 eq), Fmoc-Lys(N_3)-OH (374 mg, 0.95 mmol, 3 eq), Fmoc-Asp(OH)-OtBu (388 mg, 0.95 mmol, 3 eq), Fmoc-Asp(OH)-OtBu (388 mg, 0.95 mmol, 3 eq) and 4,4-bis(4-hydroxyphenyl)valeric acid (162

mg, 0.95 eq, 3 eq) in the indicated order using the same coupling conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before.

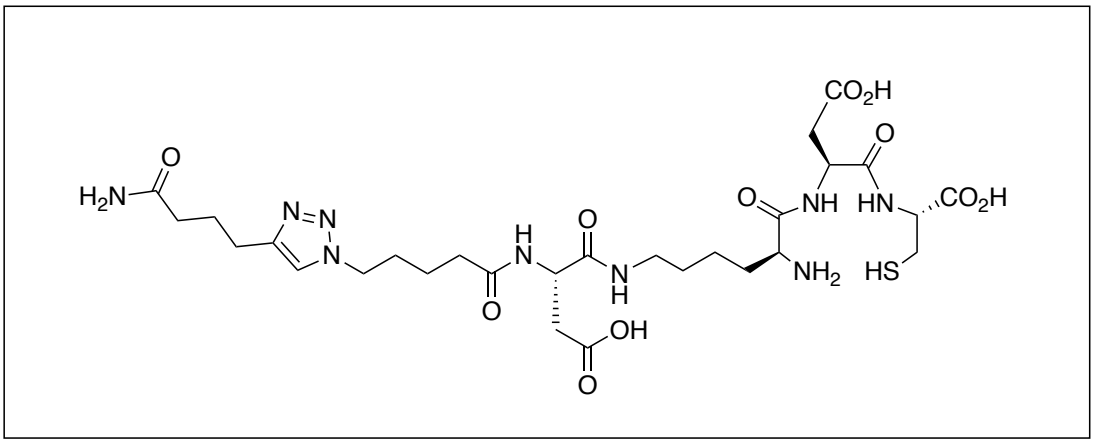
After the last peptide-coupling step, a solution of CuI (18 mg, 0.095 mmol, 0.3 eq), TBTA (16 mg, 0.031 mmol, 0.1 eq) and N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)hex-5-ynamide (261 mg, 0.95 mmol, 3 eq) in a mixture of DMF (2.5 ml) and THF (2.5 ml) was prepared and reacted with the resin at room temperature for 48 h. After washing with DMF (4 × 10 min × 10 ml), EDTA 50 mM (4 × 10 min × 10 ml) and DCM (4 × 10 min × 10 ml), the compound was cleaved from the resin by agitating with a mixture of TFA (8.6 ml), TIS (1.6 ml), H_2O (400 µl), m-Cresol (400 µl) and Thioanisol (400 µl) at room temperature for 1 h. Cleavage solution was added drop-wise to ice cold diethyl ether (50 ml) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride (30 eq). The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization compound **8** as collected as a white powder (91 mg, 70.5 µmol, 22% yield).

MS (ESI) m/z calcd. for $[C_{52}H_{71}N_{14}O_{19}S_3]^{1+}$: 1291.4104 $[M+H]^{1+}$, found: 1291.4219.



Supplementary Figure 15 Analytical HPLC trace of compound **8** on a Synergi RP Polar column at a flow rate of 4 ml min^{-1}, 5% MeCN in 0.1% aq. TFA to 80% MeCN in 20 min. Please disregard as an artifact the injection peak at around 2 minutes.

NH_2-99mTc chelator – Compound 9

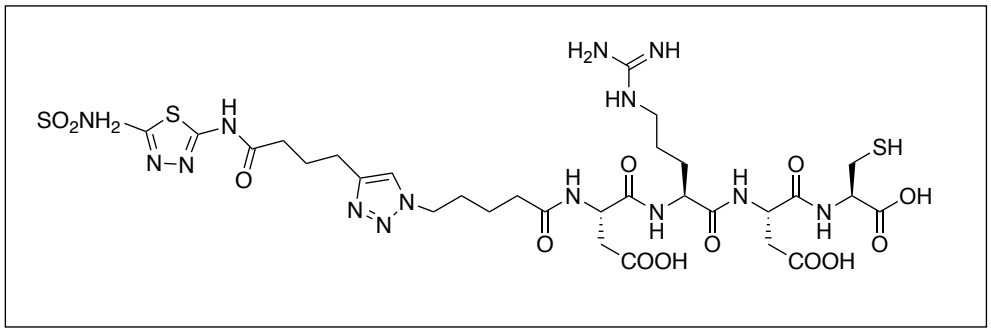


Chemical Formula: $C_{28}H_{45}N_9O_{11}S$

Molecular Weight: 715.78

Compound **9** was prepared according to previously described procedures [4].

Compound 10

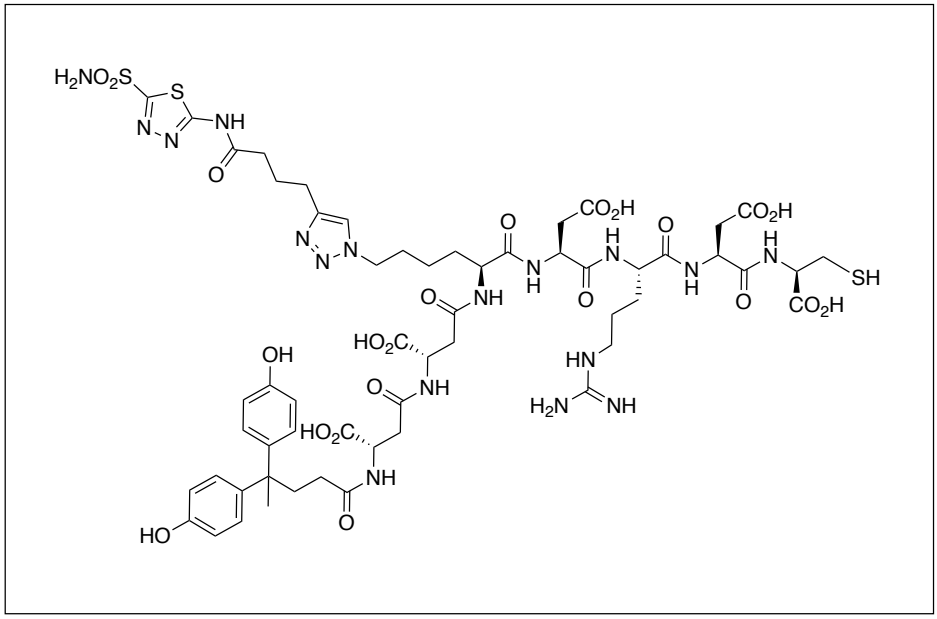


Chemical Formula: $C_{30}H_{46}N_{14}O_{13}S_3$

Molecular Weight: 906.96

Compound **10** was prepared according to previously described procedures [5].

Compound 11



Chemical Formula: $C_{56}H_{75}N_{17}O_{22}S_3$

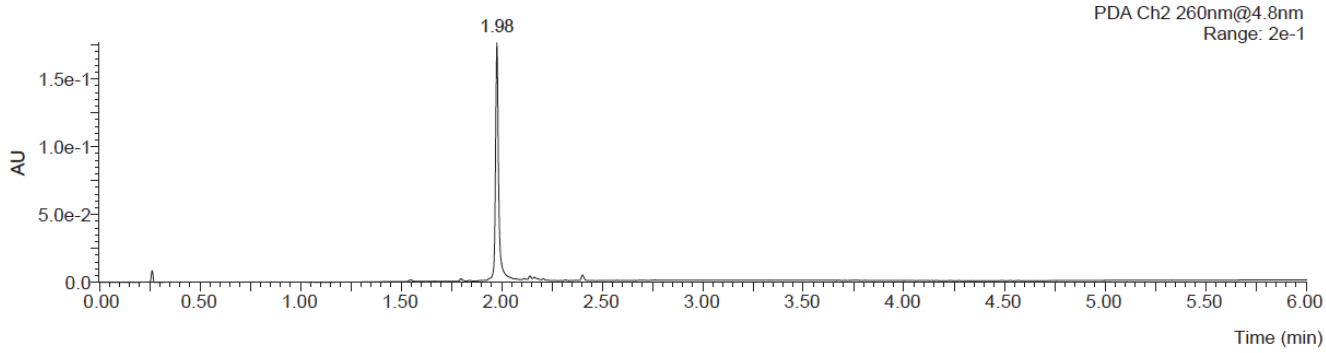
Molecular Weight: 1434.49

Commercially available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (300 mg, 0.19 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 ml) and the resin washed with DMF (4 × 10 min × 10 ml). Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq) was activated with HATU (215 mg, 0.57 mmol, 3 eq), and DIPEA (197 µl, 1.13 mmol, 6 eq) in DMF (3 ml) at 0 °C for 15 min and then reacted with the resin for 1 h under gentle agitation. After washing the resin with DMF (4 × 10 min × 10 ml) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 ml) and the resin washed with DMF (4 × 10 min × 10 ml) before the peptide was extended with Fmoc-Arg(Pbf)-OH (368 mg, 0.57 mmol, 3 eq), Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq), Fmoc-Lys(N_3)-OH (224 mg, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq) and 4,4-bis(4-hydroxyphenyl)valeric acid (162 mg, 0.57 eq, 3 eq) in the indicated order using the same coupling

conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before.

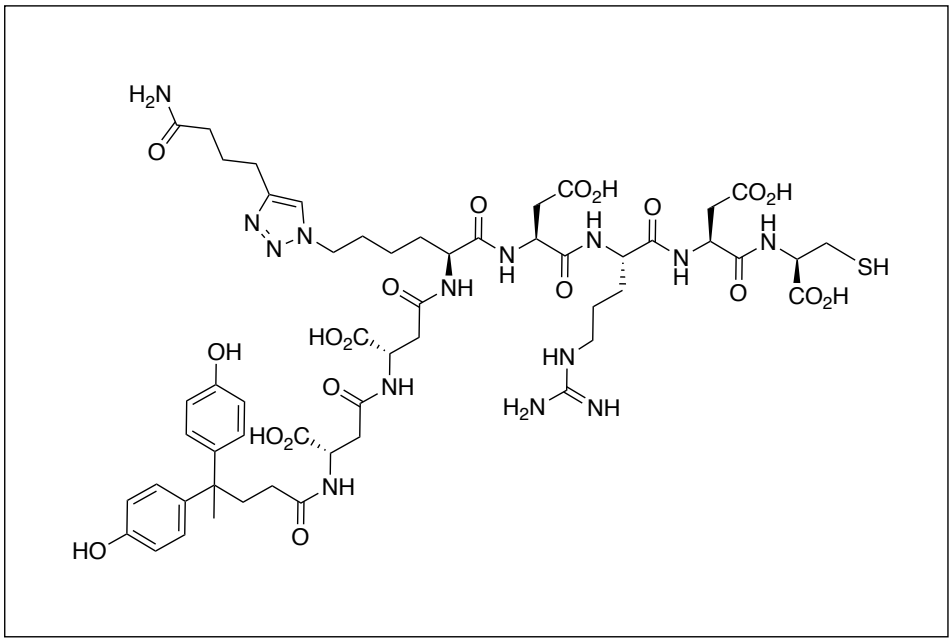
After the last peptide-coupling step, a solution of CuI (11 mg, 0.06 mmol, 0.3 eq), TBTA (10 mg, 0.02 mmol, 0.1 eq) and N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)hex-5-ynamide (155 mg, 0.57 mmol, 3 eq) in a mixture of DMF (1.5 ml) and THF (1.5 ml) was prepared and reacted with the resin at room temperature for 48 h. After washing with DMF (4 × 10 min × 10 ml), EDTA 50 mM (4 × 10 min × 10 ml) and DCM (4 × 10 min × 10 ml), the compound was cleaved from the resin by agitating with a mixture of TFA (6 ml), TIS (1.1 ml), H_2O (300 µl), m-Cresol (300 µl) and Thioanisol (300 µl) at room temperature for 1 h. Cleavage solution was added drop-wise to ice cold diethyl ether (50 ml) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride (30 eq). The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization compound **11** was collected as a white powder (25 mg, 17.4 µmol, 9% yield).

MS (ESI) m/z calcd. for $[C_{56}H_{76}N_{17}O_{22}S_3]^{1+}$: 1434.4435 $[M+H]^{1+}$, found: 1434.4501; m/z calcd. for $[C_{56}H_{77}N_{17}O_{22}S_3]^{2+}$: 717.7218 $[M+2H]^{2+}$, found: 717.7317.



Supplementary Figure 16 Analytical UPLC trace of compound **11** on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml min^{-1}, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.

Compound 12



Chemical Formula: $C_{54}H_{74}N_{14}O_{20}S$

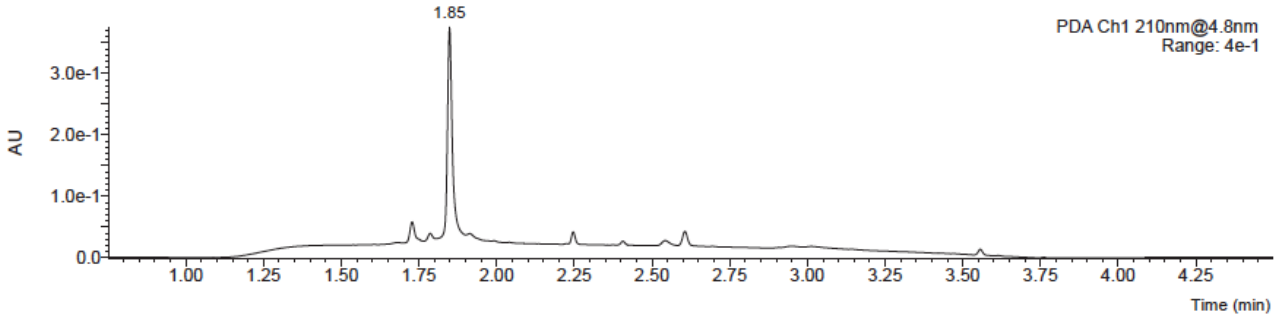
Molecular Weight: 1271.32

Commercially available pre-loaded Fmoc-Cys(Trt)-OH on polystyrene resin (300 mg, 0.19 mmol) was swollen in DMF for 15 min. The Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 ml) and the resin washed with DMF (4 × 10 min × 10 ml). Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq) was activated with HATU (215 mg, 0.57 mmol, 3 eq), and DIPEA (197 µl, 1.13 mmol, 6 eq) in DMF (3 ml) at 0 °C for 15 min and then reacted with the resin for 1 h under gentle agitation. After washing the resin with DMF (4 × 10 min × 10 ml) the Fmoc group was removed with 20% piperidine in DMF (3 × 10 min × 10 ml) and the resin washed with DMF (4 × 10 min × 10 ml) before the peptide was extended with Fmoc-Arg(Pbf)-OH (368 mg, 0.57 mmol, 3 eq), Fmoc-Asp(OtBu)-OH (233 mg, 0.57 mmol, 3 eq), Fmoc-Lys(N_3)-OH (224 mg, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq), Fmoc-Asp-OtBu (233, 0.57 mmol, 3 eq) and 4,4-bis(4-

hydroxyphenyl)valeric acid (162 mg, 0.57 eq, 3 eq) in the indicated order using the same coupling conditions (HATU/DIPEA), Fmoc-deprotection (20% piperidine in DMF) and washing step with DMF mentioned before.

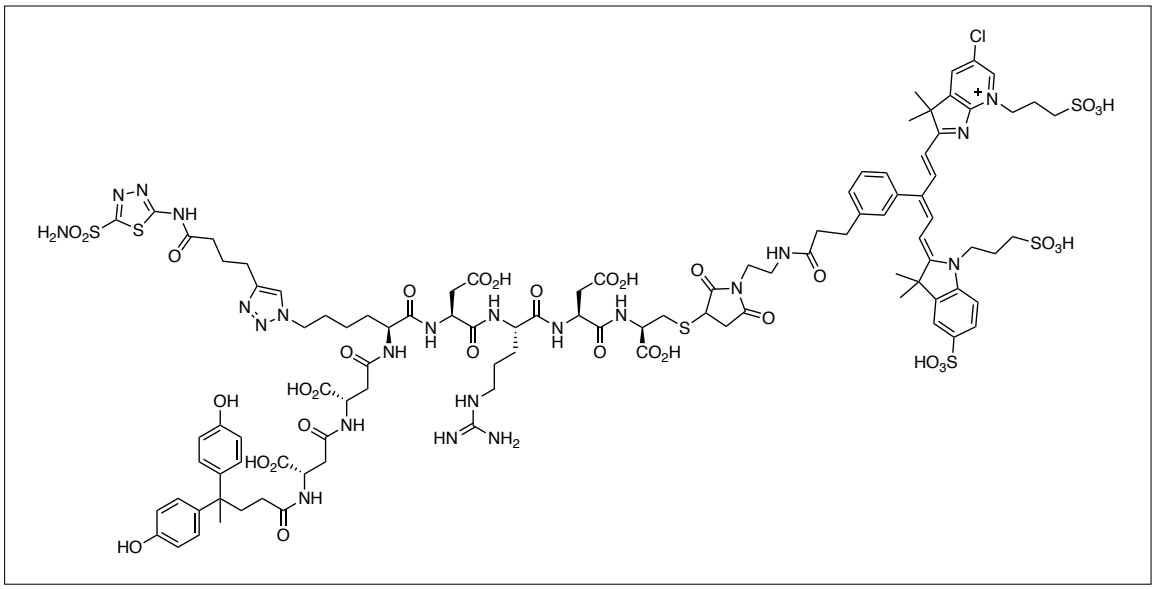
After the last peptide-coupling step, a solution of CuI (11 mg, 0.06 mmol, 0.3 eq), TBTA (10 mg, 0.02 mmol, 0.1 eq) and Hex-5-ynamide (63 mg, 0.57 mmol, 3 eq) in a mixture of DMF (1.5 ml) and THF (1.5 ml) was prepared and reacted with the resin at room temperature for 48 h. After washing with DMF (4 × 10 min × 10 ml), EDTA 50 mM (4 × 10 min × 10 ml) and DCM (4 × 10 min × 10 ml), the compound was cleaved from the resin by agitating with a mixture of TFA (6 ml), TIS (1.1 ml), H_2O (300 µl), m-Cresol (300 µl) and Thioanisol (300 µl) at room temperature for 1 h. Cleavage solution was added drop-wise to ice cold diethyl ether (50 ml) obtaining a white precipitate. The pellet was collected by centrifugation, dried under vacuum, redissolved in Millipore water and added with an excess of Tris(2-carboxyethyl)phosphine hydrochloride (30 eq). The product was purified by reversed-phase HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). After lyophilization compound **12** was collected as a white powder (27 mg, 21.2 µmol, 11% yield).

MS (ESI) m/z calcd. for $[C_{54}H_{75}N_{14}O_{20}S]^{1+}$: 1271.4924 $[M+H]^{1+}$, found: 1271.5669; m/z calcd. for $[C_{54}H_{76}N_{14}O_{20}S]^{2+}$: 636.2462 $[M+2H]^{2+}$, found: 636.2623.



Supplementary Figure 17 Analytical UPLC trace of compound **12** on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml min^{-1}, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.

Compound 13 - AAZ^{+}-IRdye680RD

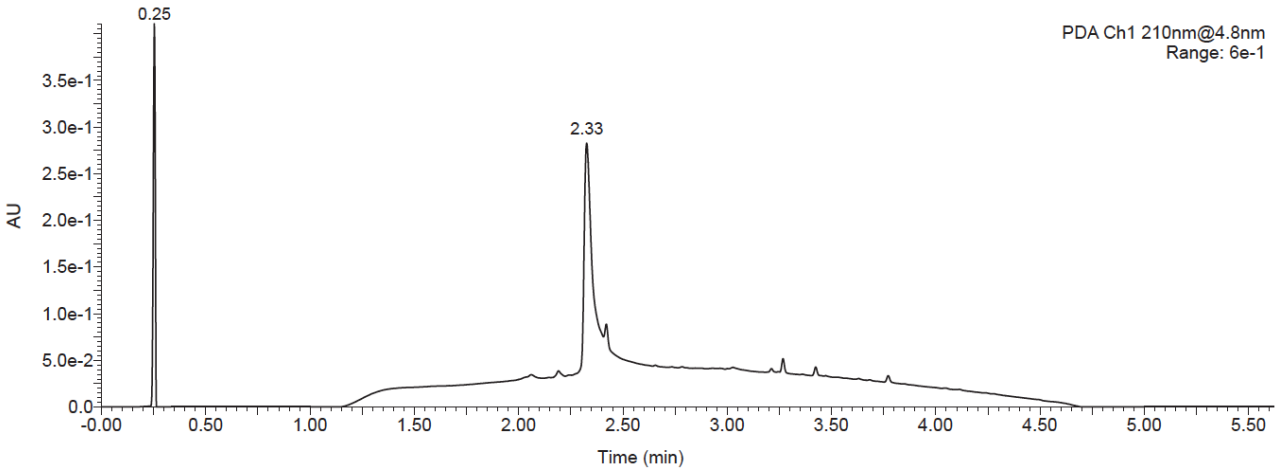


Chemical Formula: $C_{101}H_{126}ClN_{22}O_{34}S_6$

Molecular Weight: 2420.05

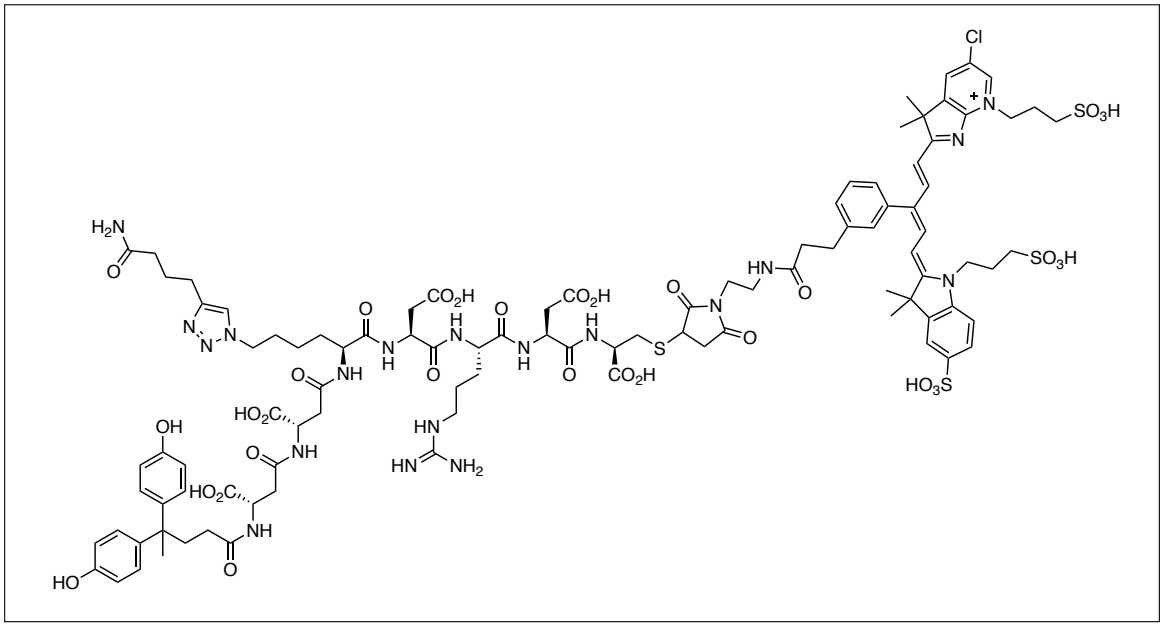
Compound **11** (2.5 mg, 1.70 µmol, 1.6 eq) was dissolved in degassed TBS (pH 7.6; 800 µl). Commercially available Maleimidocaproyl-IRDy680RD (1.1 mg, 1.10 µmol, 1.0 eq) was added as a DMF solution (200 µl) and the mixture was stirred at room temperature. After UPLC-MS indicated completion, the solvents were removed under vacuum and the crude was diluted in a 1:1 H_2O/MeCN mixture (1 ml). The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve **13** as a purple solid (1.3 mg; 0.85 µmol; 48.1% yield).

MS (ESI) m/z calcd. for $[C_{101}H_{126}ClN_{22}O_{34}S_6]^{2+}$: 1208.8410 $[M]^{2+}$, found: 1208.8380.



Supplementary Figure 18 Analytical UPLC trace of compound **13** on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml min^{-1}, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.

Compound 14 - NH_2^+-IRdye680RD

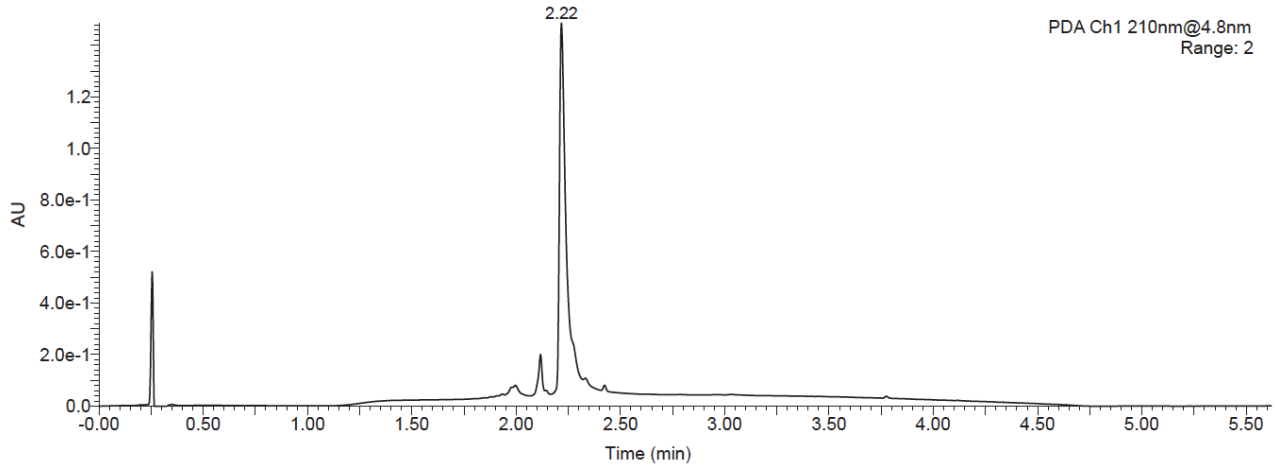


Chemical Formula: $C_{99}H_{125}ClN_{19}O_{32}S_4$

Molecular Weight: 2256.88

Compound **12** (1.7 mg, 1.30 μmol, 1.6 eq) was dissolved in degassed TBS (pH 7.6; 800 μl). Commercially available Maleimidocaproyl-IRDy680RD (1.1 mg, 1.10 μmol, 1.0 eq) was added as a DMF solution (200 μl) and the mixture was stirred at room temperature. After UPLC-MS indicated completion, the solvents were removed under vacuum and the crude was diluted in a 1:1 H_2O/MeCN mixture (1 ml). The mixture was purified by RP-HPLC (Synergi RP Polar, 5% MeCN in 0.1% aq. TFA to 80% over 20 min). Pure fractions were collected and lyophilized overnight to achieve **14** as a purple solid (1.5 mg; 0.66 μmol; 60.1% yield).

MS (ESI) m/z calcd. for $[C_{99}H_{125}ClN_{19}O_{32}S_4]^{2+}$: 1217.3655 $[M]^{2+}$, found: 1217.3931.



Supplementary Figure 19 Analytical UPLC trace of compound **14** on a BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm at a flow rate of 0.6 ml min^{-1}, 5% MeCN in 0.1% aq. FA to 80% MeCN in 6 min.

Statistical analysis of therapy experiments

Differences in tumor volume between therapeutic groups were compared using the two-way ANOVA analysis with Bonferroni post-test of Graphpad Prism 6 (La Jolla, CA, USA). Days are counted after tumor implantation.

Therapy experiment 1 – Comparison between AAZ$^+$-ValCit-MMAE and AAZ-ValCit-MMAE in SKRC-52 bearing nude mice [Figure 2]

Tumor Size (mg)

*Vehicle vs Compound **2** (AAZ$^+$-ValCit-MMAE):*

day 15 $p < 0.05$

day 16 $p < 0.01$

day 17 $p < 0.001$

from day 18 $p < 0.0001$

Vehicle vs Compound **3** *(negative control):*

non-significant differences

Vehicle vs "presaturation" group:

day 16 $p < 0.01$

day 17 $p < 0.01$

from day 18 $p < 0.0001$

Vehicle vs Compound **1** *(AAZ-ValCit-MMAE):*

day 14 $p < 0.05$

day 15 $p < 0.05$

day 16 $p < 0.001$

from day 17 $p < 0.0001$

Compound **2** *(AAZ$^+$-ValCit-MMAE) vs Compound* **3** *(negative control):*

day 18 $p < 0.05$

day 19 $p < 0.05$

day 20 $p < 0.01$

from day 21 $p < 0.0001$

Compound **2** *(AAZ^+-ValCit-MMAE) vs "presaturation" group:*

day 21 $p < 0.01$

day 22 $p < 0.001$

day 23 $p < 0.001$

day 24 $p < 0.001$

from day 25 $p < 0.0001$

Compound **1** *(AAZ-ValCit-MMAE) vs Compound* **2** *(AAZ^+-ValCit-MMAE):*

day 28 $p < 0.01$

Compound **3** *(negative control) vs "presaturation" group:*

from day 24 $p < 0.05$

Compound **1** *(AAZ-ValCit-MMAE) vs compound* **3** *(negative control):*

day 20 $p < 0.05$

day 21 $p < 0.01$

day 22 $p < 0.01$

day 24 $p < 0.001$

from day 25 $p < 0.0001$

Compound **1** *(AAZ-ValCit-MMAE) vs "presaturation" group:*

day 25 $p < 0.01$

day 26 $p < 0.0001$

day 28 $p < 0.01$

Body Weight Change (%)

*Vehicle vs Compound **2** (AAZ^+-ValCit-MMAE):*

day 24 $p < 0.01$

from day 25 $p < 0.001$

*Vehicle vs Compound **3** (negative control):*

non-significant differences

Vehicle vs "presaturation" group:

day 24 $p < 0.05$

day 25 $p < 0.05$

day 26 $p < 0.0001$

*Vehicle vs Compound **1** (AAZ-ValCit-MMAE):*

day 25 $p < 0.05$

day 26 $p < 0.001$

*Compound **2** (AAZ^+-ValCit-MMAE) vs Compound **3** (negative control):*

day 24 $p < 0.05$

day 25 $p < 0.0001$

*Compound **2** (AAZ^+-ValCit-MMAE) vs "presaturation" group:*

non-significant differences

*Compound **1** (AAZ-ValCit-MMAE) vs Compound **2** (AAZ^+-ValCit-MMAE):*

day 26 $p < 0.05$

day 28 non-significant difference

*Compound **3** (negative control) vs "presaturation" group:*

day 25 $p < 0.05$

Compound **1** *(AAZ-ValCit-MMAE) vs compound* **3** *(negative control):*

non-significant differences

Compound **1** *(AAZ-ValCit-MMAE) vs "presaturation" group:*

non-significant differences

Therapy experiment 2 – Effect of the combination AAZ$^+$-ValCit-MMAE/L19-IL2 in SKRC-52 bearing nude mice [Figure 3]

Tumor Size (mg)

Vehicle vs L19-IL2:

day 13 $p < 0.05$

from day 14 $p < 0.0001$

Vehicle vs Compound **2** *(AAZ$^+$-ValCit-MMAE):*

day 11 $p < 0.01$

from day 12 $p < 0.0001$

Vehicle vs combination group:

day 11 $p < 0.001$

from day 12 $p < 0.0001$

L19-IL2 vs Compound **2** *(AAZ$^+$-ValCit-MMAE):*

day 21 $p < 0.01$

day 22 $p < 0.001$

L19-IL2 vs combination group:

day 16 $p < 0.05$

day 17 $p < 0.001$

from day 18 $p < 0.0001$

Compound **2** *(AAZ$^+$-ValCit-MMAE) vs combination group:*

day 14 $p < 0.05$

from day 15 $p < 0.0001$

Body Weight Change (%)

Vehicle vs L19-IL2:

day 16 $p < 0.05$

from day 17 non-significant difference

Vehicle vs Compound **2** *(AAZ^+-ValCit-MMAE):*

non-significant differences

Vehicle vs combination group:

non-significant differences

L19-IL2 vs Compound **2** *(AAZ^+-ValCit-MMAE):*

non-significant differences

L19-IL2 vs combination group:

non-significant differences

Compound **2** *(AAZ^+-ValCit-MMAE) vs combination group:*

non-significant differences

Therapy experiment 3 – Effect of the combination AAZ$^+$-ValCit-MMAE/L19-IL2 in CT26.3E10 bearing immunocompetent mice [Figure 6]

Tumor Size (mg)

Vehicle vs L19-IL2:

day 18 $p < 0.05$

from day 20 $p < 0.0001$

Vehicle vs Compound **2** *(AAZ$^+$-ValCit-MMAE):*

day 18 $p < 0.05$

day 20 $p < 0.0001$

day 21 $p < 0.001$

from day 22 $p < 0.0001$

Vehicle vs combination group:

day 17 $p < 0.01$

day 18 $p < 0.001$

from day 20 $p < 0.0001$

L19-IL2 vs Compound **2** *(AAZ$^+$-ValCit-MMAE):*

day 30 $p < 0.01$

Compound **2** *(AAZ$^+$-ValCit-MMAE) vs combination group:*

day 29 $p < 0.05$

day 30 $p < 0.05$

day 31 $p < 0.01$

L19-IL2 vs combination group:

day 28 $p < 0.001$

day 29 $p < 0.001$

day 30 $p < 0.0001$

Body Weight Change (%)

Vehicle vs L19-IL2:

non-significant differences

Vehicle vs Compound **2** *(AAZ$^+$-ValCit-MMAE):*

non-significant differences

Vehicle vs combination group:

day 22 $p < 0.05$

from day 23 $p < 0.0001$

L19-IL2 vs Compound **2** *(AAZ$^+$-ValCit-MMAE):*

non-significant differences

L19-IL2 vs combination group:

day 22 $p < 0.05$

day 23 $p < 0.01$

day 24 $p < 0.01$

day 25 $p < 0.01$

from day 28 $p < 0.0001$

Compound **2** *(AAZ^+-ValCit-MMAE) vs combination group:*

day 29 $p < 0.01$

References

[1] Cazzamalli S, Dal Corso A, Neri D. Acetazolamide serves as selective delivery vehicle for dipeptide-linked drugs to renal cell carcinoma. Mol Cancer Ther 2016.

[2] Cazzamalli S, Corso AD, Neri D. Linker stability influences the anti-tumor activity of acetazolamide-drug conjugates for the therapy of renal cell carcinoma. J Control Release 2017;246:39-45.

[3] Wichert M, Krall N, Decurtins W, Franzini RM, Pretto F, Schneider P, et al. Dual-display of small molecules enables the discovery of ligand pairs and facilitates affinity maturation. Nat Chem 2015;7(3):241-9.

[4] Krall N, Pretto F, Mattarella M, Muller C, Neri D. A technetium 99m-labeled ligand of carbonic anhydrase IX selectively targets renal cell carcinoma in vivo. J Nucl Med 2016.

[5] Krall N, Pretto F, Decurtins W, Bernardes GJ, Supuran CT, Neri D. A small-molecule drug conjugate for the treatment of carbonic anhydrase IX expressing tumors. Angew Chem Int Ed Engl 2014;53(16):4231-5.