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Orthogonal Assembly of a Designed Ankyrin Repeat Protein–Cytotoxin Conjugate with a Clickable Serum Albumin Module for Half-Life Extension

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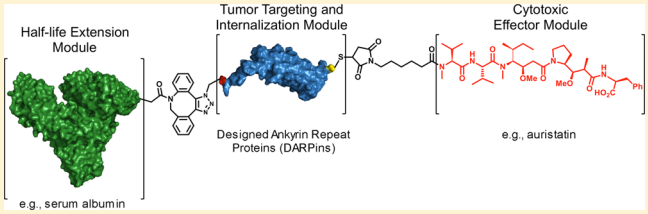
Supporting Information

ABSTRACT: The generation of drug conjugates for safe and effective tumor targeting requires binding proteins tolerant to functionalization by rational engineering. Here, we show that Designed Ankyrin Repeat Proteins (DARPins), a novel class of binding proteins not derived from antibodies, can be used as building blocks for facile orthogonal assembly of bioconjugates for tumor targeting with tailored properties. DARPin Ec1, which targets the Epithelial Cell Adhesion Molecule (EpCAM), was genetically modified with a C-terminal cysteine for conjugation of the small molecule cytotoxin monomethylauristatin F (MMAF). In addition, it was N-terminally functionalized by metabolic introduction of the non-natural amino acid azidohomoalanine to enable linkage of site-specifically dibenzocyclooctyne-modified mouse serum albumin (MSA) for half-life extension using Cu(I)-free click chemistry. The conjugate MSA-Ec1-MMAF was assembled to obtain high yields of a pure and stable drug conjugate as confirmed by various analytical methods and in functional assays. The orthogonality of the assembly led to a defined reaction product and preserved the functional properties of all modules, including EpCAM-specific binding and internalization, FcRn binding mediated by MSA, and cytotoxic potency. Linkage of MMAF to the DARPin increased receptor-specific uptake of the drug while decreasing nonspecific uptake, and further coupling of the conjugate to MSA enhanced this effect. In mice, albumin conjugation increased the serum half-life from 11 min to 17.4 h, resulting in a more than 22-fold increase in the area-under-the-curve (AUC). Our data demonstrate the promise of the DARPin format for facile modular assembly of drug conjugates with improved pharmacokinetic performance for tumor targeting.



INTRODUCTION

The use of antibody drug conjugates (ADCs) for cancer therapy is appealing, as it combines tumor targeting of cytotoxic payloads and selective destruction of tumor cells.[1−3] Several ADCs empowered with potent microtubule-disrupting agents have been brought to clinical trials, and the first promising data were reported recently.[4] Nonetheless, the use of antibodies for engineering safe and effective cancer therapeutics is limited by their inherent disulfide-stabilized multidomain complexity and the molecular size of the IgG format, and its divalent nature, which may cause unintended receptor activations. Furthermore, homogeneous antibody products with predictable conjugation ratios and attachment sites are difficult to obtain.[1] These downsides of current ADCs have motivated the search for alternative non-IgG binding scaffolds, which may be better suited to translate intelligent drug design by protein engineering into clinical practice.

In principle, less complex antibody fragments comprising only the minimum protein sequence required for target binding might overcome these limitations, and small size also favors tumor penetration and homogeneous tissue distribution of proteins.[5] Since recently, alternative small non-IgG scaffolds have emerged as a novel class of binding proteins which, because of their robustness, provide more opportunities for engineering and more efficient manufacturing.[6−8] Designed Ankyrin Repeat Proteins (DARPins) are derived from naturally occurring ankyrin repeats, which together form one contiguous binding site in a rigid protein. These novel scaffold proteins are characterized by high biophysical stability[8,9] and protease-resistance,[10] which makes DARPins ideal for engineering disease-targeting biomedicines.

For tumor targeting and receptor function analysis we combined rational library design and powerful selection techniques[7] to generate DARPins against various tumor-associated antigens, including members of the epidermal growth factor receptor (Her) family[11,12] and the Epithelial Cell Adhesion Molecule (EpCAM).[6,13] EpCAM is appealing for tumor targeting as it shows widespread distribution in carcinomas, and is expressed on circulating tumor cells and cancer stem cells.[14] We previously reported the generation and preclinical investigation of drug and gene delivery systems based on high-affinity anti-EpCAM DARPins as cell binding

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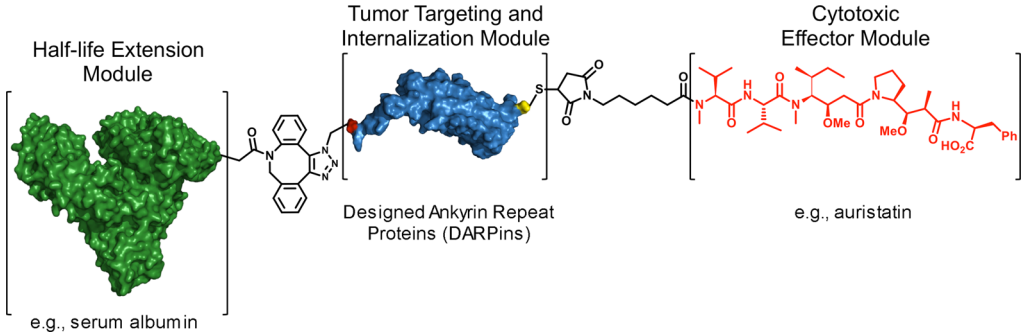

Figure 1. Schematic illustration of a DARPin conjugated to a half-life extension module (e.g., serum albumin) and a small molecule cytotoxin (e.g., MMAF) for tumor targeting. DARPins containing an azide (red) and a free thiol (yellow) enable orthogonal assembly of a modular bioconjugate retaining all properties of the components. The bifunctional linker between albumin and DARPin allows unique site-specific coupling.

moiety.[13,15,16] Furthermore, we found that DARPins are ideally suited for one step bio-orthogonal modification using strain-promoted azido-alkyne cycloaddition (also known as Cu(I)-free click chemistry) after introduction of a unique azide functionality at any desired position in the protein by replacing the sole native Met by the non-natural amino acid azidohomoalanine.[17,18] In addition, DARPins are devoid of Cys, which can thus be introduced by design to enable a site-specific reaction with the maleimide group of an additional effector function. In principle, this intriguing property, which is unique to the DARPin scaffold, enables engineering of drug conjugates comprising a highly potent cytotoxic payload like monomethylauristatin at one position and another module, e.g., designed to improve pharmacokinetics and tumor targeting of DARPins, at another position of choice.

The efficiency of tumor targeting with anticancer agents correlates with their circulation half-life. The unacceptably low percentage of injected dose per gram tumor tissue mainly results from rapid elimination by renal clearance.[5,19] Several strategies have been investigated to increase the serum half-life of classical anticancer agents and protein therapeutics, e.g., by derivatization with a biocompatible polymer like polyethylene glycol[20,21] or by fusion to antibody Fc domains.[22,23] Association or covalent conjugation with serum albumin is another attractive approach to serum half-life extension,[24−28] which may further improve tumor targeting by increasing endothelial transcytosis and tissue penetration.[29,30]

Here we demonstrate that DARPins can be readily converted to a cytotoxic bioconjugate with high potency and improved pharmacokinetic performance using orthogonal chemistries for modular assembly with a cytotoxin and a half-life modifier. As an example, the anti-EpCAM DARPin Ec1 was engineered for functionalization with the small molecule cytotoxin monomethylauristatin F (MMAF) at the C-terminus and serum albumin at the N-terminus using thiol and click chemistry, respectively (Figure 1).

MATERIALS AND METHODS

Materials. Unless otherwise stated all chemicals were obtained from Sigma Aldrich (Buchs, Switzerland). *N*-(3-Aminopropionyl)-5,6-dihydro-11,12-didehydrodibenzo-[b,f]-azocine (amino-*aza*-dibenzo-cyclooctyne, DBCO-NH_2) and *aza*-dibenzo-cyclooctyne-PEG_4-maleimide (DBCO-PEG_4-Mal) were purchased from Click Chemistry Tools (Scottsdale, Arizona, USA). Sulfhydryl-activated MSA was obtained from Albumin Bioscience Inc. (Huntsville, Alabama, USA). Maleimidocaproyl-monomethylauristatin F (mcMMAF) was purchased from Concortis Biosystems Corp. (San Diego, California, USA).

Expression and Purification of Clickable DARPins. Chemically competent *E. coli* B834 (DE3) (F^- *ompT gal* $hsdS_B$ (r_B^- m_B^-) *met dcm lon* (*lacI, lacUV5-T7 gene 1, ind1, sam7, nin5*)) were transformed with plasmid pQIq-Ec1c encoding anti-EpCAM DARPin Ec1c and plasmid pQIq-Off7ΔMc encoding a mutant of the DARPin Off7 (as negative control, not binding to EpCAM) in which all internal ATG codons had been exchanged by alanine codons.[31]

As described recently,[17] a single colony was used to inoculate an overnight culture in 2 × YT medium containing 1% glucose and 100 mg/L ampicillin. For the pre-expression cultures, M9 minimal medium was supplemented with glucose-free nutrient mix containing 19 amino acids (Glucose-Free SelenoMethionine Medium Complete, Molecular Dimensions Ltd.), 40 mg/L methionine, 50 mg/L ampicillin, and 1% glucose, as well as 0.4% glycerol. The medium was inoculated at an OD_{600} of 0.06 and incubated in baffled flasks at 37 °C for about 3.5 h until an OD_{600} of 1.0−1.2 was reached. The cultures were centrifuged (5000 × *g*, 12 min, 4 °C) and washed 3 times with ice-cold 0.9% NaCl solution by resuspending and centrifugation. The washing step was performed to remove any residual methionine. The washed cell pellets were used to inoculate fresh M9 minimal medium supplemented with glucose-free nutrient mix, 40 mg/L L-azidohomoalanine (Bapeks, Riga, Latvia), 50 mg/L ampicillin, and 0.4% glycerol. The expression cultures were incubated at 37 °C for 10 min followed by induction with 1 mM IPTG. Expression was stopped after 4 h and the cultures were centrifuged (5000 × *g*, 12 min, 4 °C). The supernatant was discarded and the pellet snap-frozen and stored at −80 °C.

Prior to lysis, pellets were thawed on ice and suspended in 25 mL HBS_W (50 mM HEPES, 150 mM NaCl, 20 mM imidazole, pH 8.0) containing 20 mM *β*-mercaptoethanol (*β*-ME) and 1 mg/mL lysozyme. The cells were then lysed 4 times using a French Press (Aminco) at 1200 psi. The supernatant was separated from cell debris by centrifugation (13 000 × *g*, 1 h, 4 °C) and purified by immobilized metal ion affinity chromatography (IMAC) with buffers containing 20 mM *β*-ME to keep a reducing milieu. Endotoxin was removed by washing with 300 column volumes (CV) PBS (pH 7.5) containing 0.1% Triton X114. The resin was thoroughly washed with PBS (pH 7.5) prior to elution. The proteins, termed *Az*Ec1cand *Az*Off7, were dialyzed overnight against PBS containing 20 mM *β*-ME, analyzed by SDS-PAGE (15%), snap-frozen, and stored at −80 °C until use.

Generation of DARPin-MMAF Conjugates. The DARPins *Az*Ec1c and *Az*Off7c as negative control were loaded on a PD-10 column (GE Healthcare Europe GmbH, Switzerland) and eluted with PBS to remove β-ME. Immediately after desalting, 26.9 μL mcMMAF (100 mM in DMSO, 2.0 equiv) was added to 5 mL *Az*Ec1c or *Az*Off7 (384 μM in PBS). After incubation at 37 °C for 1 h, the reaction was quenched by adding 10 eq β-ME. DARPin-MMAF conjugates were desalted on a PD-10 column (elution with PBS) and analyzed by SDS-PAGE (22%). The conjugates were further purified by anion exchange chromatography with a MonoQ GL 5/50 column connected to an Äkta Explorer FPLC device (GE Healthcare Europe GmbH, Switzerland), diluted with Buffer A (20 mM HEPES, 20 mM NaCl, pH 8.0) and loaded onto the column. A linear gradient of 0.75% per min Buffer B (20 mM HEPES, 1 M NaCl pH 8.0) was applied to remove unreacted DARPins from the products. Fractions containing *Az*Ec1-MMAF or *Az*Off7-MMAF were pooled and the buffer was exchanged to PBS using ultrafiltration (Amicon Ultra-4 Centrifugal Filter Unit, MWCO 3 kDa, Millipore, U.S.A.) according to the manufacturer's protocol. Purity was controlled by SDS-PAGE (22%); the mass was determined by ESI-MS.

Modification of Mouse Serum Albumin with DBCO-PEG$_4$-Mal. RP-HPLC (Hewlett-Packard Series 1100 device, Agilent Technology) with a C18 column (EC 250/4 Nucleosil 120−5, Macherey-Nagel) was used to follow the reaction of sulfhydryl-activated MSA (Albumin Bioscience Inc., USA) with *aza*-dibenzocyclooctyne-PEG$_4$-maleimide (DBCO-PEG$_4$-Mal). To this end, 1 μL DBCO-PEG$_4$-Mal (0.01 M in DMF, 1.0 equiv) was added to 100 μL mouse serum albumin (MSA) (100 μM in PBS), and after 1 h at 37 °C 20 μL of the reaction mixture were injected into the HPLC device. Chromatography conditions were as follows: flow rate 1 mL/min, mobile phase A ddH_2O + 0.1% TFA, mobile phase B MeCN + 0.1% TFA, gradient 25%−75% mobile phase B in 25 min. The specific absorption of dibenzocyclooctyne (DBCO) and DBCO-modified proteins at 309 nm was used for detection. In addition, DBCO-NH_2 was used as a control to assess nonspecific binding to MSA. To follow the time course of the reaction, 2 μL DBCO-PEG$_4$-Mal (0.01 M in DMF, 2.0 equiv) was incubated with 100 μL MSA (100 μM in PBS) at 37 °C. A sample of 20 μL was taken after 5, 10, 20, 30, and 60 min, quenched with 10 equiv β-ME and subjected to HPLC separation using the same chromatographic conditions as above. To obtain MSA-conjugates on a preparative scale, 67.5 mg sulfhydryl-activated MSA was dissolved in 2.25 mL PBS and 10.1 μL DBCO-PEG$_4$-Mal (0.1 M in DMF, 1.2 equiv) was added. Subsequently, the reaction mixture was incubated at 37 °C for 1 h followed by desalting on a PD-10 column. Aliquots of the resulting MSA-DBCO were stored at −80 °C.

Assembly of MSA-DARPin-MMAF Conjugates. *Az*Ec1-MMAF or *Az*Off7-MMAF (0.72 mL, 768 μM in PBS) were added to 2.3 mL MSA-DBCO (323 μM in PBS, 1.3 equiv) and the reaction mixture was stored at 4 °C, slightly shaken for 72 h, and finally analyzed by SDS-PAGE (4−12%) (NuPAGE, Life Technologies, Zug, Switzerland). Anion exchange chromatography and subsequent size exclusion chromatography were used for purification of the MSA-DARPin-MMAF conjugates. The reaction mixture was filtered using a 0.22 μm sterile filter and diluted 5-fold with Buffer A (20 mM HEPES, 20 mM NaCl, pH 8.0) before solutions were loaded onto a MonoQ GL 5/50 column connected to an Äkta Explorer FPLC device (GE Healthcare Europe GmbH, Switzerland). Unreacted MSA was separated from MSA-DARPin-MMAF by elution with a gradient of Buffer B (20 mM HEPES, 1 M NaCl, pH 8.0) at a flow rate of 1 mL/min. All eluted species were analyzed by SDS-PAGE (4−12%, NuPAGE) to assess purity. Fractions containing MSA-DARPin-MMAF conjugate were pooled, concentrated, and further purified on a HiLoad Superdex 200 26/60 column using PBS as running buffer (flow rate: 2 mL/min). Purity of the MSA-conjugates was controlled by SDS-PAGE (4−12%, NuPAGE) and the mass was determined by ESI-MS. Proteins were snap-frozen and stored at −80 °C until further use.

Determination of EpCAM Binding by SPR. The ProteOn XPR36 instrument (BioRad AG, Reinach, Switzerland) was used to measure the binding activity of the DARPin conjugates to the purified extracellular domain of EpCAM (hEpEX). hEpEX was expressed in HEK cells and enzymatically biotinylated using a C-terminal AviTag as described recently.[13]

For affinity determination, 1000 RU of biotinylated hEpEX in PBS-TE (PBS pH 7.4, 0.005% Tween, 3 mM EDTA) were coated on a neutravidin chip (NLC). After baseline stabilization by buffer run, a serial dilution of the conjugates (31.6 nM − 316 pM) was used to determine the association rate constant (k_a) and the dissociation rate constant (k_d) in duplicates on separate ligand channels (flow rate: 60 μL, association time 417 s, dissociation time: 5000 s). hEpEX was regenerated on the chip using two bursts of 25 μL 100 mM H_3PO_4 for 25 s between analyte runs. Interspot signals and a blank analyte channel were used for referencing. Data were fitted to a 1:1 Langmuir model and analyzed using the ProteOn Manager Software (BioRad).

Determination of Mouse Neonatal Fc-Receptor (mFcRn) Binding by SPR. mFcRn-GST (Inven2 Biologics, Norway) was chemically biotinylated using NHS-LC-LC-Biotin (Pierce, Rockford, USA). Briefly, 2 mg of the linker were dissolved in H_2O at a concentration of 10 mM and immediately added in 4-fold excess to 20 μL of mFcRn at a protein concentration of 2 mg/mL in PBS pH 7.4. The reaction mixture was kept on ice for 2 h, followed by quenching with a 10-fold excess ethanolamine for 20 min. Excess biotin was quantitatively removed using Amicon Ultra Centrifugal Filters (MWCO 10 kDa, Millipore) and repeated washing steps with PBS.

For affinity determination of MSA conjugates to mFcRn, PBS-T (0.005% Tween20) at pH 6.0 or PBS-T at pH 7.4 was used as running buffer or dilution buffer and all measurements were performed at 25 °C. Briefly, biotinylated mFcRn-GST was immobilized on a neutravidin chip (NLC) by injecting a solution of the protein (10 μg/mL) for 60 s at a flow rate of 25 μL/min to give a response of 300 RU. Serial dilution series of MSA-Ec1-MMAF (20, 10, 5, 2.5, 1.25 μM) were injected at pH 6.0 or pH 7.4 (flow rate: 60 μL/min, contact time: 200 s). Subsequently, running buffer at either pH was injected to determine the dissociation rate constant (flow rate: 60 μL/min, dissociation time: 1000 s). Duplicated measurements on separate ligand channels were used to assess the kinetic data, and interspot signals and a blank run were used for referencing. Data were fitted to a 1:1 Langmuir model or fitted with an equilibrium model and analyzed using the ProteOn Manager Software (BioRad).

Tumor Cell Cultures. The colorectal carcinoma cell line HT29, the breast carcinoma cell line SKBR3, the embryonic kidney cell line HEK293T, and the breast carcinoma cell line MCF7 were obtained from ATCC (American Type Culture

Collection, LGC Standards S.à.r.l., Molsheim Cedex, France). HT29 and HEK293T cells were cultured in Dulbecco modified Eagle's medium (DMEM), SKBR3 cells were grown in Advanced RPMI 1640 medium (Gibco, Life Technologies Europe BV, Zug, Switzerland), MCF7 cells were cultured in a mixture of DMEM (50% v/v) and Ham F12 medium (50% v/v). All media were supplemented with 10% fetal calf serum (Amimed, Bioconcept, Allschwil, Switzerland), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in tissue culture flasks at 37 °C in a humidified atmosphere at 5% CO_2.

Analytical Flow Cytometry. Clickable *Az*Ec1c and, as a negative control, *Az*Off7c were conjugated with Alexa Fluor 488 C5 maleimide (Alexa488) (Invitrogen, Life Technologies Europe BV) as described above for MMAF-Mal. The Alexa488-labeled DARPins were directly used for the click conjugation to MSA-DBCO as prepared above without further purification. MSA-DARPin-Alexa488 conjugates were subsequently purified by anion exchange followed by size exclusion chromatography (see above). Binding of Alexa488-labeled conjugates on EpCAM-positive HT29 and MCF7 cells was assessed by flow cytometry (CyFlow Space, Partec GmbH, Münster, Germany). Briefly, cells were harvested from a confluent culture flask, washed with PBS, and diluted in FACS buffer (PBS, 1% BSA, 0.2% NaN_3) to a concentration of 1×10^6 cells/mL and 0.8×10^6 cells/mL for HT29 and MCF7 cells, respectively. The conjugates (*Az*Ec1-Alexa488, *Az*Off7ΔM-Alexa488, MSA-Ec1-Alexa488, and MSA-Off7ΔM-Alexa488, where ΔM denotes the removal of internal methionines) were added at a concentration of 100 nM to allow binding on cells for 1 h at 4 °C on a rocker. For epitope blocking (competition) experiments, cells were preincubated at 4 °C for 1 h with a 20-fold excess (2 μM) of unmodified DARPin Ec1 prior to addition of the DARPin-conjugates. Cells were centrifuged (500 × *g*, 3 min, 4 °C) and washed with FACS buffer followed by pelleting. Pellets were stored on ice and resuspended in 1.5 mL FACS buffer right before use for flow cytometry. For each experiment, 1×10^4 cells were counted (gated for viable and single cells). Data was assessed using FlowJo (Tree Star Inc., Ashland, Oregon, U.S.A.) software.

Cytotoxicity Assay. The cytotoxic potency of the conjugates *Az*Ec1-MMAF and MSA-Ec1-MMAF, and the corresponding Off7 control conjugates on HT29, SKBR3, and HEK293T cells was assayed as described[13] using the standard colorimetric XTT cell proliferation kit II (Roche Diagnostics GmbH). Cells were grown to a confluency of 80−90%, detached, and resuspended in the corresponding medium. The concentration was determined with a CASY cell counter (Roche Diagnostics GmbH). Cells were seeded in 96-well plates in a volume of 50 μL per well and at a density of 5000, 2600, and 2200 cells per well for HT29, SKBR3, and HEK293T, respectively. After 24 h at 37 °C (5% CO_2) the conjugates (DARPin-MMAF and MSA-DARPin-MMAF) and MMAF or unmodified DARPin Ec1 were added in serial dilutions in a total volume of 100 μL per well. All samples were analyzed in triplicates.

To demonstrate that the cytotoxicity of the Ec1 conjugates was dependent on EpCAM binding and internalization, a receptor blocking (competition) experiment was performed. To this end, unconjugated DARPin Ec1 was preincubated with a 10-fold excess for 20 min prior to addition of the DARPin conjugates. After incubation for 72 h, plates were decanted, followed by addition of 50 μL XTT reagent (Roche Diagnostics, Rotkreuz, Switzerland). This incubation time was chosen to have the most reliable readout, following the manufacturer's recommendation. After further incubation for 1−3 h at 37 °C, the absorbance was measured at 480 nm and at 650 nm used as reference wavelength by an ELISA plate reader (infinite M1000, Tecan, Männedorf, Switzerland). Untreated cells were used for normalization. Prism 5.0 (GraphPad Software, San Diego, USA) and Excel (Microsoft, Redmond, USA) were used for data evaluation. Dose−response curves with variable slope were fitted to all data points, using the following equation

$$Y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + 10^{(\log IC_{50} - X)\cdot HillSlope}} \quad (1)$$

where X is the drug concentration, Y is the measured XTT readout after normalization, with the fitting parameters Y_{max} and Y_{min} (upper and lower plateau values), IC_{50} (the concentration at which cell viability was reduced by 50%), and the *HillSlope*.

Analysis of *in Vitro* Serum Stability. MSA-Ec1-MMAF was diluted in mouse serum (PAA laboratories GmbH) to a concentration of 1 μM and incubated at 37 °C. Samples of 40 μL were taken after 0, 1, 2, 3, 4, 5, 6, 24, and 48 h, and frozen at −20 °C. For assessment of serum stability, samples were thawed on ice and the His-tagged DARPin conjugates in the serum were isolated. To this end, 15 μL of Ni-NTA magnetic agarose beads (Qiagen) and 55 μL HBS_HS (50 mM HEPES, 1 M NaCl, pH 8.0) supplemented with 20 mM imidazole were added to each sample. After pulldown at 4 °C for 1 h, magnetic beads were captured using a magnet rack and immobilized protein samples were washed twice with 500 μL HBS_HS (supplemented with 50 mM imidazole, pH 8.0). Finally, supernatants were discarded and Laemmli buffer containing 1 M imidazole was directly added to the beads for elution of His-tagged proteins. Samples were loaded on a SDS-PAGE gel (15%), which was subsequently used for semidry Western blotting. The PVDF membrane was blocked overnight with 5% nonfat milk powder (Migros, Zurich, Switzerland) in PBS-T and His-tagged DARPin conjugates were directly detected by mouse anti-RGSHis$_6$-HRP antibody conjugate (Qiagen, Hilden, Germany) at a dilution of 1:1000 for 1 h at room temperature. The blot was thoroughly washed and developed with a chemiluminescent HRP substrate (Immobilon Western, Millipore Corporation, Billerica, U.S.A.).

Analysis of Blood Clearance in Mice. Female CD-1 nude mice (6−8 weeks of age) were obtained from Charles River (Sulzfeld, Germany). Mice were injected into the tail vein with equimolar amounts of either *Az*Ec1-MMAF (1.2 nmol, 22 μg protein) or MSA-Ec1-MMAF (1.2 nmol, 100 μg protein) in 150 μL PBS, and blood samples were drawn from the tail tip at various time points (for *Az*Ec1-MMAF 3, 10, 20, 30, and 60 min and for MSA-Ec1-MMAF at 3, 60, and 120 min, and again after 6, 24, and 48 h post injection).

After blood coagulation at room temperature for 30 min, serum was obtained by two centrifugation steps (20 min, 20 000 × *g*, 4 °C) and stored at −20 °C until quantification of serum concentrations of conjugates by ELISA. Briefly, MaxiSorp 96-well plates (Nunc GmbH & Co. KG, Langenselbold, Germany) were coated with anti-RGS-His$_6$ mAb at a dilution of 1:2000 in PBS at 4 °C overnight. The wells were blocked with 300 μL PBS-TB (PBS, 0.2% BSA, 0.1% Tween20) for 1 h at room temperature. Serum samples were diluted 1:50, 1:100, or 1:300 in PBS-TB and applied in

duplicate to the coated wells. A control well per sample coated with BSA was used for background subtraction. For quantification, a serial dilution of *Az*Ec1-MMAF ranging from 2000 pM to 25 pM and MSA-Ec1-MMAF ranging from 14 000 pM to 62.5 pM was included on each plate. All standard dilutions were prepared in PBS-TB supplemented with commercially available mouse serum to ensure an identical composition compared to the serum samples. Incubation of the samples was for 1 h at room temperature. After stringent washes with PBS-T (PBS, 0.1% Tween20), rabbit anti-DARPin polyclonal antibody (custom-made, Eurogentec, Seraing, Belgium) was added as the primary antibody at a dilution of 1:5000 in PBS-TB (1 h at room temperature). Washing with PBS-T was followed by incubation with the secondary antibody (goat anti-rabbit-HRP conjugate mAb) at a dilution of 1:10 000 in PBS-TB for 1 h at room temperature. After a final wash with PBS-T, the assay was developed using the Amplex Red ELISA detection kit (Invitrogen, Life Technologies, Europe B.V., Zug, Switzerland). Linear regression was used to fit the data points from serial dilutions to a standard curve and used to quantify *Az*Ec1-MMAF and MSA-Ec1-MMAF from serum samples.

RESULTS

Conjugation, Purification, and Biochemical Analysis of *Az*Ec1-MMAF. DARPins can be readily functionalized by orthogonal conjugation using thiol chemistry (upon introduction of a Cys) and strain-promoted cycloaddition (upon introduction of an azide by incorporating the non-natural amino acid azidohomoalanine).[17] Here we generated an anti-EpCAM DARPin, *Az*Ec1c for orthogonal N- and C-terminal conjugation. After IMAC purification and removal of endotoxins, the purity of the protein was assessed by 15% SDS-PAGE (SI Figure S1). For payloading the binder with a cytotoxic anticancer agent, maleimidocaproyl-linked monomethylauristatin F (mcMMAF) was conjugated to the unique cysteine introduced at the C-terminus of *Az*Ec1c and purified by anion exchange chromatography (SI Figure S3). Keeping the binders under reducing conditions until conjugation with MMAF resulted in quantitative conversion even at low molar excess of mcMMAF, as detected by a representative band-shift in 22% SDS-PAGE (SI Figure S2). The mass of *Az*Ec1-MMAF (19 490 Da) was confirmed by ESI MS (SI Figure S4).

The cytotoxic potency of the conjugate on HT29 cells was determined in XTT assays, using unconjugated Ec1 and free MMAF as controls. As shown in SI Figure S5, *Az*Ec1-MMAF was potently cytotoxic, with an IC_{50} of 600 pM. The activity was indeed mediated by binding to EpCAM, since upon blocking of EpCAM with unconjugated Ec1, cytotoxicity was decreased to the approximate level of the negative control *Az*Off7ΔM-MMAF (IC_{50} of 231 nM and 436 nM, respectively). Free MMAF tested as a control (IC_{50}: 6 nM) was 10-fold less potent than *Az*Ec1-MMAF, whereas non-modified DARPin Ec1 did not show any effect in the concentration range tested.

Preparation of Mouse Serum Albumin Modified for SPAAC (MSA-DBCO). Site-specific introduction of a single reactive cyclooctyne functionality into MSA was achieved by conjugation of DBCO-PEG_4-Mal to the free Cys34 present in domain I of MSA. First, we tested the quality of sulfhydryl-activated MSA by addition of 2 equiv of DBCO-PEG_4-Mal and monitored the conjugation kinetics of the DBCO linker. Sulfhydryl-activated MSA rapidly reacted with DBCO-PEG_4-Mal, resulting in a 1:1 coupling ratio in less than 5 min (SI Figure S6). Monitoring at later time points showed that for the product MSA-PEG_4-DBCO a plateau was reached quickly, indicating only limited additional reactivity toward other nucleophilic residues within MSA when using this low molar linker excess.

To generate DBCO-containing albumin monomodified at Cys34 (MSA-DBCO), we thus reacted the DBCO cross-linker with MSA and monitored the reaction with RP-HPLC. Figure 2 shows that addition of 1.0−1.2 equiv of DBCO-PEG_4-Mal over MSA resulted in a quantitative reaction and excess DBCO cross-linker could be readily removed by desalting.

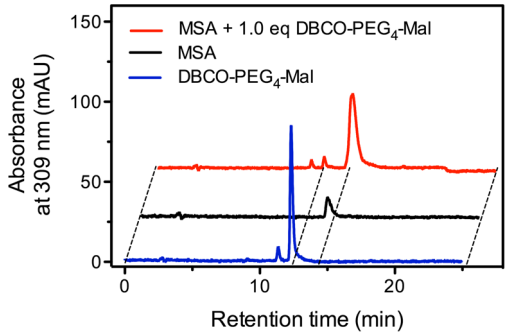

Figure 2. RP-HPLC elution profiles from samples before and after modification of MSA (100 μM) with 1.0 equiv DBCO-Mal (reacted for 1 h in PBS at 37 °C). To detect the conjugation of DBCO-PEG_4-Mal to MSA, educts and product were monitored using the DBCO-specific absorption at 309 nm.

Analysis of Nonspecific Interaction of DBCO with Serum Albumin. Cyclooctyne variants, structurally different from the one used here, have been reported to nonspecifically interact with endogenous serum albumin *in vivo* via unknown mechanisms,[32] but no data for the cyclooctyne DBCO employed in the present study are available. We therefore probed DBCO for nonspecific interaction with mouse serum albumin using DBCO-NH_2 (not carrying a reactive group) at different stoichiometric ratios, using concentrations up to the high micromolar range, and determined the amount of remaining educts by RP-HPLC using the DBCO-specific absorption at 309 nm. As shown in SI Figure S7, no complexation of DBCO with MSA could be detected even at high micromolar concentrations.

Assembly of MSA-Ec1-MMAF Using SPAAC. To increase the serum half-life, *Az*Ec1-MMAF was reacted with DBCO-modified MSA as depicted in Figure 1. Simple mixing of *Az*Ec1-MMAF with 1.3 equiv MSA-DBCO and incubation for up to 72 h at 4 °C resulted in assembly of MSA-Ec1-MMAF by bio-orthogonal click chemistry as detected by a single band-shift toward the expected molecular weight of the reaction product (MW = 86 142 Da) in SDS-PAGE (SI Figure S8). MSA-Ec1-MMAF was further purified to homogeneity using anion exchange and size exclusion chromatography (Figure 3 and SI Figure S9). Analysis of MSA-Ec1-MMAF by ESI-MS also confirmed the expected mass of the conjugate ($MW_{app.}$ = 86 143.5 Da, $MW_{calc.}$ = 86 141.9 Da) (SI Figure S10 and S11).

We further used analytical size exclusion chromatography (aSEC) to determine the increase in molecular weight and hydrodynamic radius of MSA-Ec1-MMAF compared to its building blocks *Az*Ec1-MMAF and MSA-DBCO. The retention volumes in aSEC were used to extrapolate the apparent molecular weight ($MW_{app.}$) for comparison with the calculated

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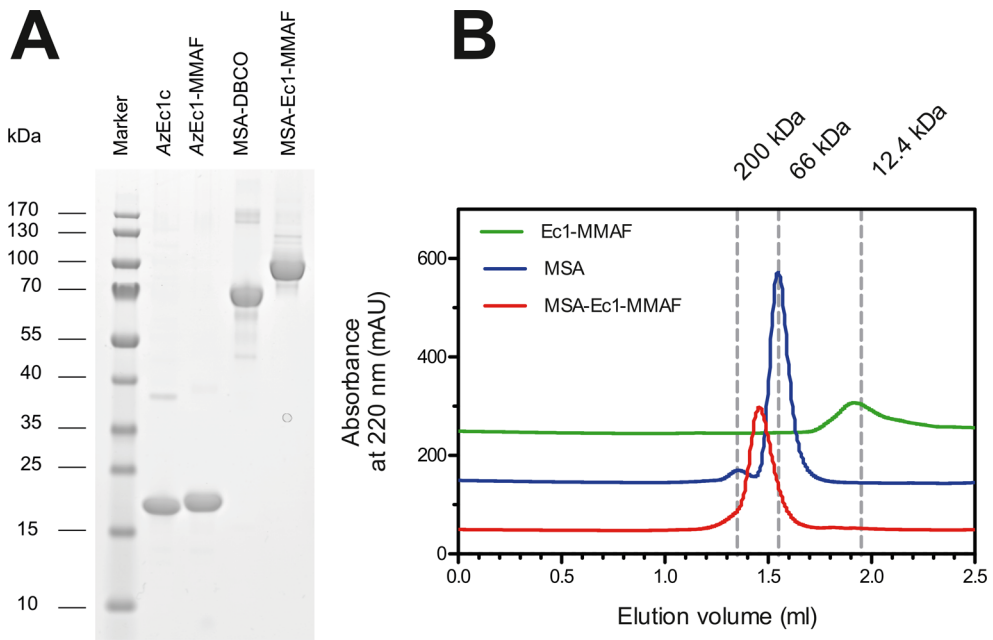

Figure 3. (A) SDS-PAGE of purified MSA-Ec1-MMAF and its building blocks. (B) Analysis of MSA-Ec1-MMAF and its educts by analytical size exclusion chromatography.

molecular weights ($MW_{calc.}$) derived from the amino acid sequence. As shown in Figure 3B, *Az*Ec1-MMAF eluted with an apparent molecular weight (MW_{app}) of 14 kDa ($MW_{calc.}$ = 19.5 kDa). Both MSA and MSA-DBCO eluted with a $MW_{app.}$ of 64.7 kDa ($MW_{calc.}$ (MSA) = 66.0 kDa, $MW_{calc.}$ (MSA-DBCO) = 66.6 kDa) (data not shown). The final product MSA-Ec1-MMAF showed an elevated $MW_{app.}$ of 93.7 kDa ($MW_{calc.}$ = 86.1 kDa) in solution as a result of stable triazole ring formation after linkage of MSA-DBCO and *Az*Ec1-MMAF.

Binding of MSA-Ec1-MMAF to purified EpCAM. The affinity of MSA-Ec1-MMAF on EpCAM was determined by surface plasmon resonance (SPR) measurement using the purified extracellular domain of human EpCAM (hEpEX) (SI Figure S15). DARPin Ec1 and its MMAF conjugate *Az*Ec1-MMAF showed very similar binding characteristics with an identical dissociation rate constant (k_d) of 6.0×10^{-6} s^{-1} and comparable association rate constant (k_a) for the conjugate (1.4×10^5 $M^{-1} \times s^{-1}$ versus 9.5×10^4 $M^{-1} \times s^{-1}$, respectively). In contrast, MSA-Ec1-MMAF displayed a k_a decreased by a factor of 2 (Table 1).

Table 1. Affinity of DARPin Ec1 and its Conjugates to Purified hEpEX Determined by SPR

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (pM)
Ec1[a]	1.4×10^5	5.8×10^{-6}	42
*Az*Ec1-MMAF[a]	9.5×10^4	6.1×10^{-6}	64
MSA-Ec1-MMAF[a]	5.3×10^4	6.8×10^{-6}	129

[a]Data were fitted to a 1:1 Langmuir model using ProteOn Manager Software (Bio-Rad) used to calculate K_D.

Cell Binding of MSA-Ec1-Alexa488. To assess the binding of MSA-Ec1-MMAF to EpCAM-positive cells by flow cytometry, a fluorescently labeled conjugate was prepared by replacing the C-terminal mcMMAF molecule with Mal-C5-Alexa488 (SI Figure S12–S14). Both *Az*Ec1-Alexa488 and MSA-Ec1-Alexa488 efficiently bound to HT29 and MCF7 cells with comparable median fluorescence intensities (MFI) in a strictly EpCAM-dependent manner. The MFI of MSA-Ec1-Alexa488 was 87% and 80% of the MFI determined for *Az*Ec1-Alexa488 on HT29 and MCF7 cells, respectively (Figure 4), indicating that albumin conjugation only slightly reduced the cell binding activity of the MSA conjugate. Furthermore, EpCAM-specificity of binding was confirmed in receptor-blocking experiments by preincubation of cells with a 20-fold excess of nonlabeled Ec1 DARPin, which decreased the signals for *Az*Ec1-Alexa488 and MSA-Ec1-Alexa488 close to background levels. No binding was detectable for the Off7 control constructs.

Cytotoxicity of MSA-Ec1-MMAF. The cytotoxic potency of the conjugate was determined in XTT assays using EpCAM-positive HT29 and SKBR3 tumor cells and EpCAM-negative HEK293T control cells (Figure 5, Table 2). As expected from its nonspecific mode of action, free unconjugated MMAF showed comparable IC_{50} values for all tested cell lines, ranging from 2.3 to 6.3 nM. In contrast, *Az*Ec1-MMAF was markedly more potent than the free drug on EpCAM-positive cells with IC_{50} values in the subnanomolar range (350–440 pM). N-terminal conjugation with albumin (MSA-Ec1-MMAF) slightly decreased the potency of the conjugate 2- to 4.5-fold, depending on the EpCAM-positive cell line tested. For comparison, the nonspecific MMAF conjugate MSA-Off7ΔM-MMAF showed a much lower cytotoxicity with high IC_{50} values in the upper nanomolar to micromolar range.

To better illustrate the ratio of EpCAM-dependent and independent cytotoxicity, we calculated a specificity index for the DARPin-MMAF and MSA-DARPin-MMAF conjugates by comparing EpCAM-specific with EpCAM-independent IC_{50} values (i.e., with Off7Δ*M* control constructs). On HT29 and SKBR3 cells the index for DARPin-MMAF was 500 and 251-fold, respectively. Interestingly, conjugation to albumin even increased the specificity index to 1200- and 3400-fold in the two cell lines, respectively (Table 2).

In control experiments with EpCAM-negative HEK293T cells, a 14-fold increased IC_{50} value was measured for *Az*Ec1-MMAF (IC_{50} = 88 nM) compared to free MMAF (IC_{50} = 6.3

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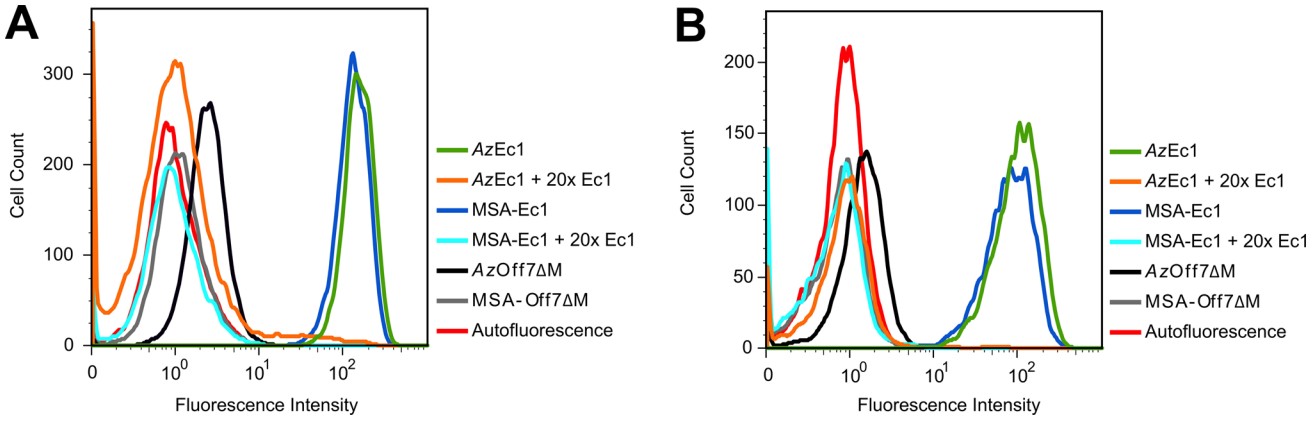

Figure 4. EpCAM-specific binding of *Az*Ec1 and MSA-Ec1 on EpCAM-positive cell lines (A) HT29 and (B) MCF7, analyzed by analytical flow cytometry. For detection, DARPins were conjugated with Alexa488 (instead of mcMMAF) via their C-terminal cysteine.

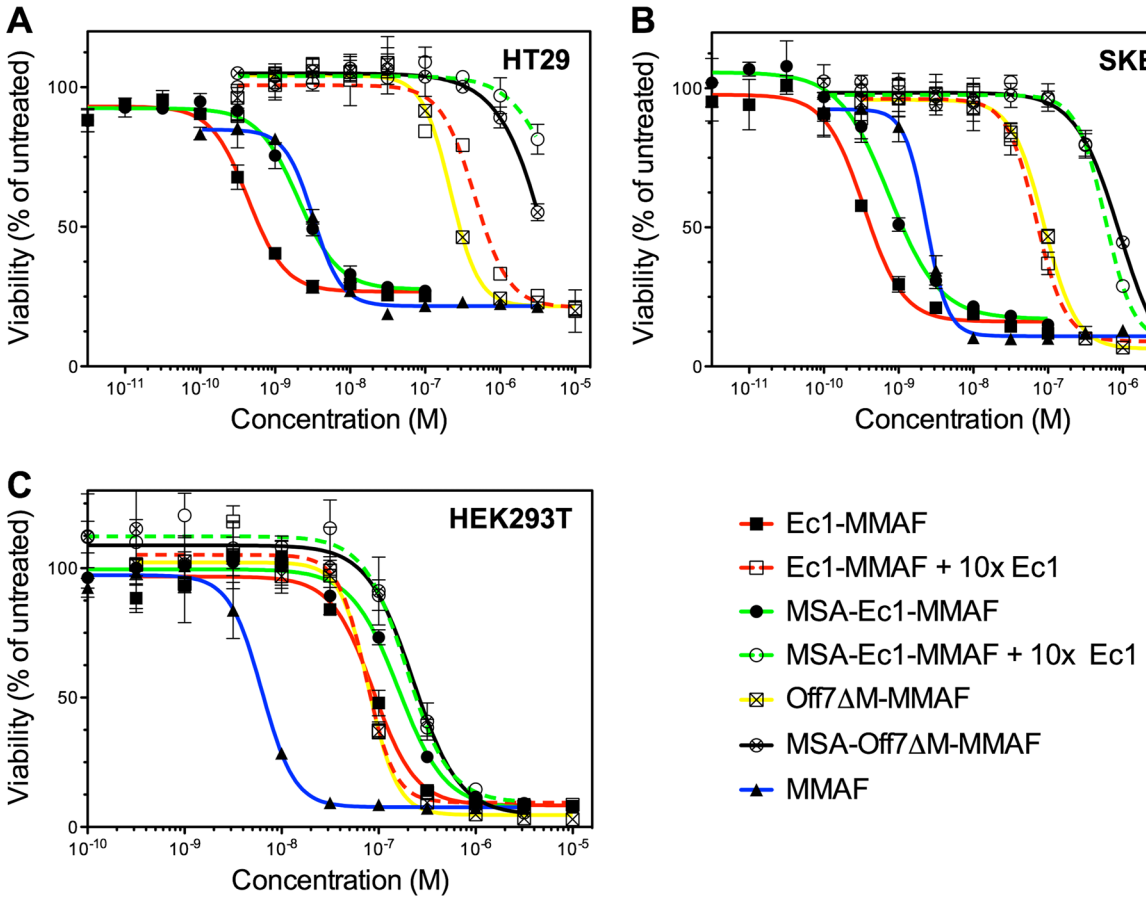

Figure 5. Cytotoxicity of assembled conjugates determined by XTT assays. The EpCAM-specific constructs MSA-Ec1-MMAF and *Az*Ec1-MMAF were compared to control conjugates MSA-Off7ΔM-MMAF and *Az*Off7ΔM-MMAF and to free MMAF on the EpCAM-positive tumor cell lines (A) HT29 and (B) SKBR3. Furthermore, receptor-blocking experiments with excess of unconjugated Ec1 prior to addition of conjugates were performed. (C) The EpCAM-negative cell line HEK293T was used as a control. All cells were incubated with serial dilutions of conjugates or free MMAF and incubated for 72 h before cell viability was determined. Each data point represents the mean of triplicates ± SD.

nM). This effect was even more pronounced for MSA-Ec1-MMAF (IC_{50} = 160 nM) with a 25-fold difference compared to free MMAF (Table 2). Our data confirm the high EpCAM-specificity of the conjugates mediated by Ec1.

Binding of MSA-Ec1-MMAF to Mouse FcRn. The extraordinary serum half-life of albumin is due to salvage by a recycling process involving binding to the neonatal Fc receptor (FcRn). The FcRn−albumin interaction is highly pH dependent, with no detectable interaction at neutral pH and increased binding (resulting in an affinity in the micromolar range) upon acidification of the endolysosomal compartment *in vivo*.[33] We used SPR with immobilized recombinant mouse FcRn (mFcRn) to detect a potential pH-dependent interaction of the MSA-Ec1-MMAF conjugate (Figure 6). The affinity was measured, and data points were fitted to a kinetic 1:1 Langmuir model resulting in an association rate constant (k_a) of 4.8 × 10^3

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Table 2. Cytotoxicity of the Various Conjugates against Various Tumor Cell Lines

						specificity indices	
Cell line	**1** mol/L	**2** mol/L	**3** mol/L	**4** mol/L	**5** mol/L	$(IC_{50}(\mathbf{3}))/(IC_{50}(\mathbf{1}))$	$(IC_{50}(\mathbf{4}))/(IC_{50}(\mathbf{2}))$
HT29	4.4×10^{-10}	2.0×10^{-9}	2.2×10^{-7}	6.8×10^{-6}	3.2×10^{-9}	500	3400
SKBR3	3.5×10^{-10}	7.5×10^{-10}	8.8×10^{-8}	9.1×10^{-7}	2.3×10^{-9}	251	1213
HEK293T	8.8×10^{-8}	1.6×10^{-7}	7.9×10^{-8}	2.3×10^{-7}	6.3×10^{-9}	0.9	1.4

1: *Az*Ec1-MMAF, **2**: MSA-Ec1-MMAF, **3**: *Az*Off7ΔM-MMAF, **4**: MSA-Off7ΔM-MMAF, **5**: MMAF.

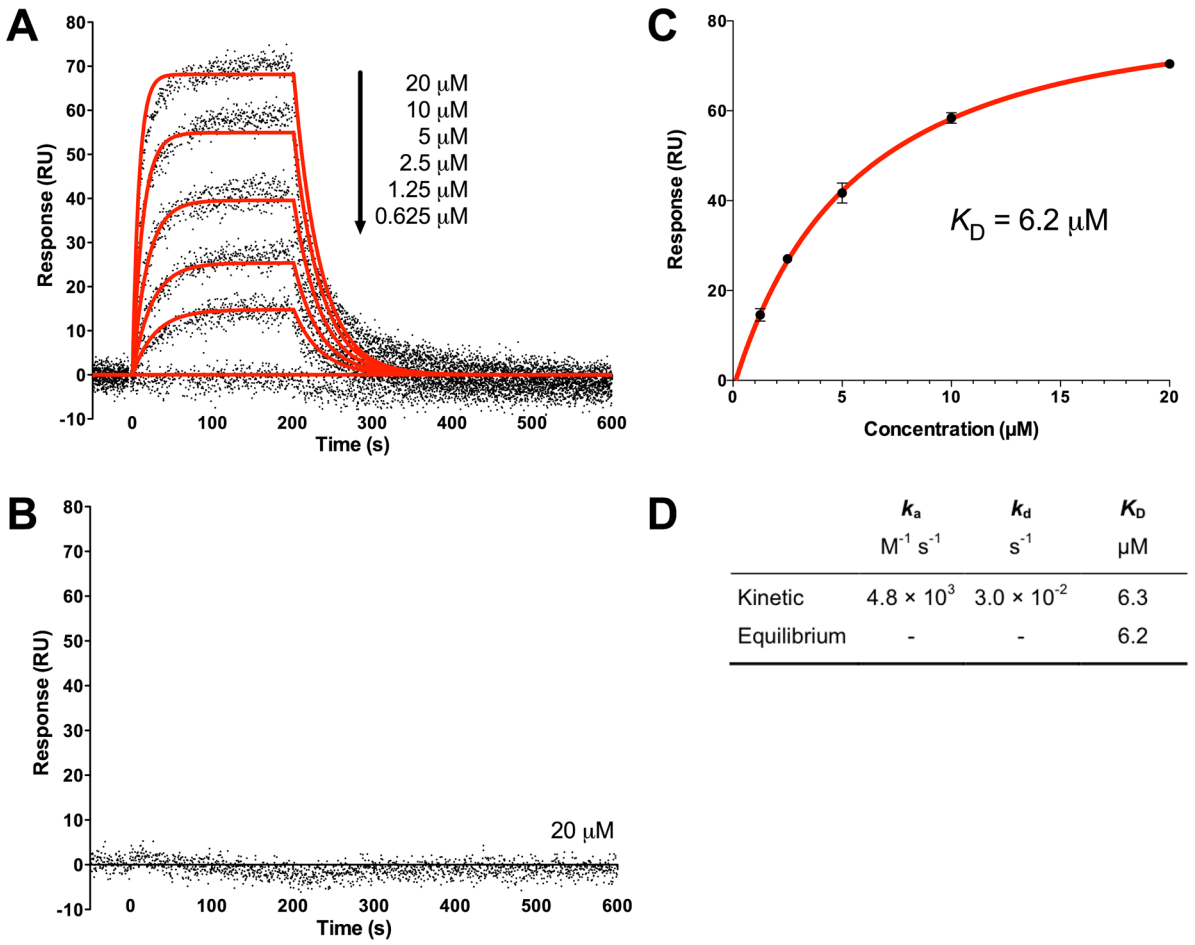

Figure 6. SPR analysis of the pH-dependent interaction of MSA-Ec1-MMAF with mouse FcRn (mFcRn) at pH 6.0 and 7.4 (25 °C). For all experiments, biotinylated GST-tagged mFcRn was immobilized to give 300 RU. A serial dilution of the conjugate was injected at a flow-rate of 60 μL/min and the interaction was measured either at (A) pH 6.0 or at (B) pH 7.4. The resulting sensograms at pH 6.0 were fitted with a (A) 1:1 Langmuir model and (C) an equilibrium model. (D) The determined affinity parameters k_a, k_d, and K_D for the FcRn: MSA-Ec1-MMAF interaction at pH 6.0 are shown. No K_D could be determined at pH 7.4.

M^{-1} s^{-1} and a dissociation rate constant (k_d) of 3×10^{-2} s^{-1}, giving an equilibrium dissociation constant ($K_{D(kinetic)}$) of 6.3 μM (Figure 6A and D). Complementarily, the K_D was extrapolated from the equilibrium data of the plateaus from the same sensogram resulting in a comparable K_D ($K_{D\ (equil.)}$ = 6.2 μM) (Figure 6C and D). As expected, MSA-Ec1-MMAF showed no detectable affinity for mFcRn at pH 7.4 (Figure 6B).

Serum Stability of MSA-Ec1-MMAF. To determine the stability of MSA-Ec1-MMAF under physiological conditions, the conjugate was incubated with mouse serum *in vitro*. As shown in SI Figure S16, analysis of the integrity of the conjugate by pull down assays and Western blotting did not reveal signs of degradation or instability of the conjugate for up to 48 h.

Blood Clearance of *Az*Ec1-MMAF and MSA-Ec1-MMAF. To investigate the ability of serum albumin to increase the circulation half-life of the conjugates in mice, we measured the serum concentration of MSA-Ec1-MMAF and *Az*Ec1-MMAF in blood samples drawn at different time points after intravenous injection. As shown in Figure 7, *Az*Ec1-MMAF was rapidly cleared from the blood, resulting in a serum half-life of 10.6 min and an area-under-the-curve (AUC) of 26.85 (% of 3 min value) × *h*. In contrast, conjugation to serum albumin significantly increased the half-life of the conjugate MSA-Ec1-MMAF, which now unveiled a biphasic elimination kinetics with a fast α-phase ($t_{1/2}(\alpha)$ = 109 min) and a slow β-phase ($t_{1/2}(\beta)$ = 17.36 h), similar to free albumin. This resulted in a 22-fold increase in the AUC ($AUC_{(MSA\text{-}Ec1\text{-}MMAF)}$ = 599.2 (% of 3 min value) × *h*).

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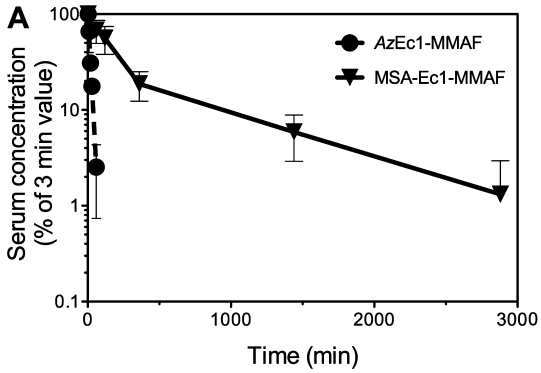

B

	$t_{1/2}$ (α) (min)	$t_{1/2}$ (β) (min)	AUC (% of 3 min value × h)
*Az*Ec1-MMAF[a]	10.6	-	26.85
MSA-Ec1-MMAF[b]	108.8	1042	599.21

[a] one-phase exponential decay fit, [b] two-phase exponential decay fit

Figure 7. (A) Blood clearance of *Az*Ec1-MMAF and MSA-Ec1-MMAF in mice. The conjugates *Az*Ec1-MMAF and MSA-Ec1-MMAF were injected *i.v.* in CD-1 nude mice and serum concentrations were quantified by ELISA from blood samples drawn at different time points (3, 10, 20, 30, and 60 min for *Az*Ec1-MMAF and 3 min, 1, 2, 6, 24, and 48 h for MSA-Ec1-MMAF). For comparison, data are shown relative to the value of the first measured time point (at 3 min), whose average was set to 100%. Each data point represents the mean of 3 mice ± SD. (B) Fitted curves were used to determine the serum half-lives ($t_{1/2}$) and the area-under-the-curve (AUC) for both conjugates.

DISCUSSION

The manufacture of protein therapeutics with full control over stoichiometries of their components is desirable for the construction of next generation anticancer agents with increased therapeutic indices. DARPins represent a particularly well-suited platform of highly stable binding proteins with extraordinary potential for engineering robust tailor-made bioconjugates for tumor targeting. We recently reported the bio-orthogonal modification of DARPins using thiol and click chemistry in the same molecule.[17] The free choice of the number and position of the reaction sites in the DARPin enables the generation of bioconjugates with defined coupling ratios and desired pharmacokinetic and cytotoxic properties.

Here, we exploited the favorable properties of the DARPin format for conjugation with the potent microtubule-disrupting agent monomethylauristatin F (MMAF). Using a nonhydrolyzable maleimidocaproyl linker, antibodies have been successfully payloaded with the charged auristatin analog MMAF to generate potent drug conjugates with reduced nonspecific toxicity.[34] We employed maleimidocaproyl-modified MMAF (mcMMAF) for site-specific, covalent linkage to the well-internalizing anti-EpCAM DARPin Ec1[6] at the C-terminus upon genetic introduction of a unique Cys residue. Prior reduction of the DARPin with an excess of reducing agent completely reduces all Cys in the protein preparation and enables maximum coupling efficiency by maleimide conjugation.[18] As expected, the Ec1-MMAF conjugate retained picomolar affinity for EpCAM as determined on EpCAM-immobilized SPR chips, and conserved binding on target cells. Thus, the site-specific and terminal modification of Ec1 (and therefore at a position in the protein remote from the EpCAM binding site) is advantageous and should provide a generic solution because of the conserved structure of the binding interface of the DARPins.

As shown for antibody−MMAF conjugates, upon internalization and lysosomal degradation the cysteine-linked mcMMAF is released into the cytosol to achieve target cell killing.[34] In the present study, Ec1-MMAF demonstrated high EpCAM-specific cytotoxicity in the picomolar range, and both specificity and potency were significantly higher compared to free MMAF. This is expected, as the charged nature of free MMAF limits cellular uptake, which in case of the conjugate was efficiently facilitated by receptor-mediated endocytosis upon EpCAM-binding by the DARPin Ec1. In addition, we found that conjugation to Ec1 decreased the nonspecific uptake of MMAF, probably mediated by pinocytosis, thereby further increasing the therapeutic index. In principle, increasing the number of genetically introduced Cys in the DARPin, which can be readily accomplished, would enable higher payloading, while still maintaining site-specificity and thus integrity of the protein.

Small antibody fragments and alternative scaffold proteins are characterized by improved tumor penetration compared to intact IgGs. On the other hand, their molecular size below the renal filtration threshold generally leads to rapid clearance from the circulation, which limits the percentage of injected dose delivered to tumors. Based on studies investigating the role of affinity and size of binding proteins for tumor targeting,[19] we concluded that the therapeutic potential of Ec1-MMAF might benefit from additional conjugation to a long-circulating macromolecule, which can be readily accomplished using bio-orthogonal click chemistry after installation of an N-terminal azide.[17,18] For half-life extension, conjugation to serum albumin is particularly appealing, as it not only increases the molecular weight above the renal filtration threshold but also uses a salvage pathway mediated by interaction with the neonatal Fc receptor (FcRn) on endothelial cells.[35,36] Moreover, serum albumin and hence also albumin-conjugated biomedicines show preferential localization to tumors and other inflamed tissues by complex interaction mechanisms,[26,28,37] a desired property with the potential to increase treatment efficacy. While the Fc portion of an antibody also provides a similar salvage pathway through the FcRn molecule, its dimeric nature is not always ideal for targeting of receptors, as some might become activated, thus making serum albumin a more attractive alternative.

Most of the cysteines present in serum albumin form disulfide bonds involved in protein stabilization. In fact, serum albumin from most mammalian species exhibits only one free cysteine at position 34 (Cys34), which can be specifically reacted with a maleimide.[24,38,39] Importantly, Cys34 is located at a position remote from domain III, which is responsible for binding to the FcRn required for the albumin salvage pathway.[36,40,41] Here, we modified Cys34 of mouse serum albumin (MSA) with a heterospecific linker, compatible with thiol and click chemistry. Using recombinant MSA selectively reduced at position 34, we could also exclude targeting of a second free Cys at position 579 in the mouse protein, but

which is absent in the human homologue. Thus, an exactly analogous conjugate can be produced with human serum albumin. The resulting building block MSA-DBCO was conjugated to an N-terminal azide introduced into DARPin Ec1 using bioorthogonal strain-promoted azido-alkyne cycloaddition (SPAAC), resulting in well-defined and homogeneous products.

This conjugation strategy using a heterobifunctional linker avoids many side-products, which would become an issue if maleimide chemistry were to be used both on the serum albumin as well as on the targeting protein, here a DARPin. Furthermore, it allows the DARPin to be separately coupled in a cysteine−maleimide reaction with a drug (or a dye) without interfering with the reaction of Cys34 on serum albumin. The current strategy also permits the rapid switching to the serum albumin of the animal species to be treated, and of course also the human variant, thereby exploiting the optimal interaction with the species' own FcRn. With fusion proteins, a new protein would have to be manufactured and characterized every time, or compromises in FcRn interactions would have to be accepted.

Although cyclooctynes of a different chemical composition were reported to nonspecifically react with albumin by unknown mechanisms,[32,42,43] we could not find evidence that the cyclooctyne DBCO used in this study interacted nonspecifically with MSA or underwent a thiol-yne coupling with cysteines (or any side reactions with other residues) of MSA in a detectable manner. In contrast, the linker DBCO-PEG$_4$-Mal reacted rapidly and almost exclusively with Cys34 of MSA at low molar excess via Michael addition, allowing for full conversion and control of the reaction. It is worth mentioning that the use of DBCO-modified serum albumin as described here (or its human homologue HSA-DBCO, which can be manufactured in the same way) is generic in nature and in principle applicable to improving the pharmacokinetic performance of various other biomedicines upon introduction of an azide functionality for click chemistry.

We found that conjugation of Ec1-MMAF carrying a single N-terminal azide functionality (here denoted as *Az*Ec1-MMAF) with MSA slightly decreased its cytotoxic potency *in vitro*. Results from EpCAM-independent cytotoxicity assays, where the albumin conjugate is also less toxic, suggest that EpCAM binding is not the rate-limiting factor for cytotoxicity, and rather points to impaired internalization. The effect of the MSA conjugation on EpCAM binding is only very small, and due to a moderately (2-fold) decreased association rate constant (k_a) with an identical dissociation rate constant (k_d)).

Furthermore, we compared the specific, EpCAM-dependent and the nonspecific activity of all conjugates (Table 2) from cytotoxicity assays with different EpCAM-positive cell lines by using the ratio of IC_{50} values of MMAF coupled to EpCAM-specific DARPin vs to a nonspecific DARPin. The same comparison was made after coupling both molecules to MSA. Importantly, whereas *Az*Ec1-MMAF showed an IC_{50} 2 orders of magnitude lower than the nontargeting DARPin, conjugation to MSA even enhanced this therapeutic index to 3 orders of magnitude. This suggests that linkage to albumin significantly diminishes the nonspecific cellular uptake (probably due to its increased molecular size), while retaining EpCAM-specific binding with picomolar affinity and efficient EpCAM-mediated internalization characteristics.

Interaction of serum albumin with FcRn is required to initiate the salvage pathway of recycling from the endosomes of endothelial cells back to the blood circulation and is strictly pH-dependent.[33,35,36] In fact, when measuring the binding of MSA-Ec1-MMAF to mouse FcRn by SPR at different pH, mimicking the extra- and intracellular milieu of endothelial cells, the affinity was exclusively detectable at low pH (pH 6.0) with a K_D of 6 μM, which is comparable to the K_D of native MSA.[35] In contrast, receptor binding was fully abrogated at pH 7.4, demonstrating preserved pH-dependent properties of the MSA after conjugation. As a result, the intact FcRn binding capacity of the MSA module in MSA-Ec1-MMAF resulted in considerably improved pharmacokinetic performance with the blood clearance curve, now showing a biphasic kinetics very similar to native MSA, and a 22-fold increased AUC compared to *Az*Ec1-MMAF. Future experiments are on the way to investigate in detail the toxicity profile of the albumin conjugate and its antitumor activity.

Taken together, site-specific conjugation to MSA-DBCO underlines the great potential to entirely alter the pharmacokinetics by simply clicking this module to the drug of choice, which is not merely limited to the utility of clickable DARPins. With the introduction of clickable albumin, we have added yet another building block to tailor the half-life of many drugs with bio-orthogonal compatibility.

CONCLUSIONS

We describe here the generation of a novel drug conjugate with high specificity and potency for tumor targeting by modular assembly of an anti-EpCAM DARPin with the potent cytotoxin MMAF and modified serum albumin using click and thiol chemistry. The modular design and orthogonality of this approach opens new possibilities for engineering tailor-made biomedicines for more effective treatments.

ASSOCIATED CONTENT

Supporting Information

SDS-PAGE and ESI-MS analysis, elution profiles, colorimetric cell viability assays, and reaction kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): AP is a co-founder and shareholder of Molecular Partners AG, who are commercializing the DARPin technology.

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