

Increasing the Antitumor Effect of an EpCAM-Targeting Fusion Toxin by Facile Click PEGylation

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Abstract

Fusion toxins used for cancer-related therapy have demonstrated short circulation half-lives, which impairs tumor localization and, hence, efficacy. Here, we demonstrate that the pharmacokinetics of a fusion toxin composed of a designed ankyrin repeat protein (DARPin) and domain I-truncated *Pseudomonas* Exotoxin A (PE40/ETA^{''}) can be significantly improved by facile bioorthogonal conjugation with a polyethylene glycol (PEG) polymer at a unique position. Fusion of the anti-EpCAM DARPin Ec1 to ETA^{''} and expression in methionine-auxotrophic *E. coli* enabled introduction of the nonnatural amino acid azidohomoalanine (Aha) at position 1 for strain-promoted click PEGylation. PEGylated Ec1-ETA^{''} was characterized by detailed biochemical analysis, and its potential for tumor targeting was assessed using carcinoma cell lines of various histotypes *in vitro*, and subcutaneous and orthotopic tumor xenografts *in vivo*. The mild click reaction resulted in a well-defined mono-PEGylated product, which could be readily purified to homogeneity. Despite an increased hydrodynamic radius resulting from the polymer, the fusion toxin demonstrated high EpCAM-binding activity and retained cytotoxicity in the femtomolar range. Pharmacologic analysis in mice unveiled an almost 6-fold increase in the elimination half-life (14 vs. 82 minutes) and a more than 7-fold increase in the area under the curve (AUC) compared with non-PEGylated Ec1-ETA^{''}, which directly translated in increased and longer-lasting effects on established tumor xenografts. Our data underline the great potential of combining the inherent advantages of the DARPin format with bioorthogonal click chemistry to overcome the limitations of engineering fusion toxins with enhanced efficacy for cancer-related therapy. *Mol Cancer Ther*; 13(2); 375–85. ©2013 AACR.

Introduction

Tumor targeting with naked antibodies and antibody drug conjugates (ADC) has become an established strategy for cancer-related therapy, particularly if conventional therapies have failed (1, 2). Recent advances in antibody engineering and linker technology, together with a growing arsenal of potent anticancer agents, have paved the way for the development of drug conjugates targeting tumors with exquisite efficacy and specificity (1–3). In theory, many different types of payloads can be linked to antibodies; in practice, however, engineering ADC with high stability and efficacy has been hampered by technical limitations and unfavorable properties inherent to the antibody format (3–6).

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Designed ankyrin repeat proteins (DARPin) are derived by consensus engineering from naturally occurring repeat proteins and are composed of internal repeat modules responsible for binding and a N- and C-terminal capping repeat providing solubility (4, 5, 7). Their robust nature and high expression yield in soluble form in *E. coli* make them ideal candidates for many biomedical applications (5, 8, 9). Importantly, DARPins lack cysteines, which can thus be introduced for site-specific conjugation of effector functions. Recently, we reported the use of bioorthogonal click chemistry as a further strategy of DARPin functionalization (8, 9). The DARPin scaffold was found to well tolerate the replacement of its N-terminal methionine by a nonnatural clickable L-azidohomoalanine (Aha; and, if necessary, the replacement of any other Met residues), resulting in a fully functional binder, which can be site-specifically conjugated with polyethylene glycol (PEG) or other conjugation partners in a one-step reaction (8, 9, 10).

We previously generated high-affinity DARPins targeting various tumor-associated antigens, including members of the EGF receptor family (11, 12) and the epithelial cell adhesion molecule (EpCAM; ref. 13). EpCAM, also known as CD326, is a 40 kDa type I membrane glycoprotein frequently expressed in human carcinomas, and involved in cell proliferation by linking to components of the Wnt signaling pathway and regulators of the cell cycle (14, 15). It initially attracted attention as a target for cancer-related immunotherapy due to its abundant

expression in solid tumors, although expression in normal epithelia is low (14–17), and recent studies further unveiled its association with cancer stem cells (15–19) and circulating tumor cells (16, 18, 19). Currently, several anti-EpCAM antibodies are under clinical development (15), with the human antibody adecatumumab being the most advanced candidate (20, 21).

In addition to immunotherapy with naked antibodies, EpCAM has also been successfully evaluated for tumor targeting with drug conjugates owing to its high rate of receptor-mediated endocytosis (22–26). Domain I truncated variants of *Pseudomonas* Exotoxin A (PE40/PE38) have been most frequently used for this purpose in the form of recombinant fusion toxins with antibody fragments or cytokines (27, 28). Although so far clinical responses have been limited to hematologic malignancies (29), there is hope that recent advances in protein engineering may eventually provide novel fusion toxin generations with efficacy also against solid tumors. Recently, we demonstrated for the first time the compatibility of the DARPin format with PE40 (here denoted ETA^{''}) to produce high yields of a potent anti-EpCAM fusion toxin (25). Because, however, these small recombinant proteins have an elimination half-life of hardly more than 10 minutes (25, 30, 31), which limits tumor localization, pharmacologic improvements are mandatory. PEGylation offers several advantages for biomedical compounds, including an increased hydrodynamic radius and serum stability, resulting in increased blood residence time due to decreased proteolysis, renal filtration, and liver clearance, and delayed recognition by the immune system (32, 33). So far, nine PEGylated protein products have been marketed, which could demonstrate improved efficacy for various diseases (34).

To increase the circulation half-life of the anti-EpCAM fusion toxin Ec1-ETA^{''}, we used strain-promoted click chemistry for bioorthogonal PEGylation upon introduction of a unique Aha at the N-terminus of DARPin Ec1. The new generation fusion toxin retained EpCAM-binding affinity in the pmol/L range and demonstrated enhanced antitumor efficacy *in vivo* as a result of its improved pharmacokinetic performance.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich. *E. coli* strain B834 (DE3; F⁻ *ompT gal hsdS_B (r_B⁻ m_B⁻) met dcm lon (lacI, lacUV5-T7 gene 1, ind1, sam7, nin5)* was from EMD Chemicals Inc. Aza-dibenzocyclooctyne-PEG (20 kDa; DBCO-PEG_{20kDa}) was a kind gift from Click Chemistry Tools.

Tumor cell lines

All cell lines were obtained from and authenticated by the American Type Culture Collection. The EpCAM-positive breast carcinoma cell lines MDA-MB-468 (HTB-132) and MCF7 (HTB-22) were purchased in 2011 and 2008, respectively. The EpCAM-positive colorectal carcinoma cell line HT29 (HTB-38) and the EpCAM-negative non-

Hodgkin lymphoma cell line RL (CRL-2261) were both purchased in 2006. Cells were cultured in humidified incubators (37°C, 5% CO₂) in Dulbecco's Modified Eagle Medium or RPMI-1640 (Invitrogen) medium supplemented with 10% fetal calf serum (Amimed) and 1% penicillin/streptomycin (Invitrogen). All cells were tested negative for mycoplasma.

Site-directed mutagenesis of DARPins

All internal methionine codons (ATG) of control DARPin Off7 (binding to maltose-binding protein) were exchanged to alanine codons using site-directed mutagenesis. The mutation M34L was first introduced as described (8, 9) before the primers 5'-CGCTGCGG-ACTCTGATGGTGGACTCCACTGCACCTGGC-3' and 5'-GTCGCACCATCAGAGTCCGCAGCGTTAACGTCA-GCACCG-3' were used to remove all internal ATG codons from the DARPin sequence. The resulting DARPin was sequenced and designated Off7ΔM. The anti-EpCAM DARPin Ec1 (12) has no internal methionine codons.

Expression and purification of Aha-modified fusion toxins

The DARPins Ec1 and Off7ΔM were subcloned into pQIq vectors using *Bam*HI and *Hind*III, for fusion to ETA^{''} via a Gly-Ser linker (24). All constructs were sequenced and the methionine-auxotrophic *E. coli* B-strain B834 (DE3) was transformed. A single colony was taken to inoculate 2 × YT medium supplemented with 1% glucose and 100 μg/mL ampicillin and grown overnight. Both Aha-Ec1-ETA^{''} and Aha-Off7ΔM-ETA^{''} were expressed using a modified medium exchange method to substitute methionine by Aha during expression (8, 9). Methionine-containing Ec1-ETA^{''} was expressed from the same plasmid, pQIq-Ec1-ETA^{''}, using *E. coli* BL21 (DE3) with TB medium and purified via IMAC as described previously (24). For endotoxin removal, 300 to 500 column volumes of PBS-T (PBS, pH 7.4, 0.1% Triton-X-114) were used during the IMAC purification procedure. All proteins were eluted in PBS_E (PBS, 300 mmol/L imidazole, pH 7.4) and the protein yield was determined with a Nanodrop 1000 photometer (Thermo Scientific AG).

PEGylation of fusion toxins using click chemistry

A stock of 5 mmol/L DBCO-PEG_{20kDa} (Click Chemistry Tools) was used to PEGylate the azido-modified DARPin-ETA^{''} fusion proteins. DBCO-PEG_{20kDa} was added in a 2-fold molar excess to the IMAC purified proteins in PBS_E (1 × PBS, 300 mmol/L imidazole), mixed gently and left for up to 24 to 72 hours at 4°C for bioorthogonal mono-PEGylation using Cu(I)-free click chemistry. PEGylation was monitored by 12% SDS-PAGE before further purification.

To generate a reversibly PEGylated fusion toxin as control, a 3C protease cleavage site was introduced between the N-terminal MRGSH₆-tag and DARPin. This was encoded by insertion of a double-stranded oligonucleotide at a *Bam*HI site. The protein was expressed,

PEGylated, and purified as described above. Proteolytic removal of the N-terminal PEG_{20kDa} was achieved by coincubation of 0.1 eq. 3C protease with PEG_{20kDa}-3C-DARPin-ETA'' at 5 μmol/L for 2 hours on ice. De-PEGylation was detected by SDS-PAGE.

Purification of fusion toxins

The fusion toxins were diluted in buffer A (50 mmol/L Hepes, 20 mmol/L NaCl, pH 8.0) and loaded on an anion exchange column (Mono Q GL 5/50, GE Healthcare) connected to an ÄKTA Explorer FPLC (fast protein liquid chromatography; GE Healthcare) for separation of the conjugate PEG_{20kDa}-Ec1-ETA'' from the reactants DBCO-PEG_{20kDa} and Aha-Ec1-ETA''. The proteins were separated with a step gradient of buffer B (50 mmol/L Hepes, 1 mol/L NaCl, pH 8.0), and the peak fraction corresponding to PEG_{20kDa}-Ec1-ETA'' was pooled. The peak of non-PEGylated protein was also pooled, concentrated (Amicon Ultra-4 Centrifugal Unit, MWCO 30 kDa) and the buffer was exchanged to PBS. The concentration was determined with a Nanodrop 1000 and the non-PEGylated protein was again subjected to PEGylation using a 2-fold excess of DBCO-PEG_{20kDa}, followed by separation via anion exchange as mentioned above. The PEGylated protein fractions were further concentrated to a small volume followed by gel filtration on a Superdex 200 prep grade 16/60 column (GE Healthcare) using PBS pH 7.2 as running buffer. The concentrations of the resulting mono-PEGylated fusion toxins were measured with a Nanodrop 1000, diluted, aliquoted, snap-frozen, and stored at -80°C until use.

All proteins were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue and iodine to confirm PEGylation according to Kurfürst (34). Briefly, gels previously stained with Coomassie were incubated for 10 minutes in water followed by 15 minutes in 20 mL 0.1 mol/L perchloric acid. Then, 5 mL 5% BaCl₂ in 1 mol/L HCl and 2 mL 0.05 mol/L iodine solution was added, and the gels were incubated briefly until PEG staining became visible. Gels were finally destained with water.

Analytical gel filtration

Ec1-ETA'' and PEG_{20kDa}-Ec1-ETA'' were analyzed by analytical size exclusion chromatography using an ÄKTA Micro FPLC device (GE Healthcare). A volume of 50 μL of each protein solution (final concentration 5 μmol/L) was separated on a Superdex 200 PC3.2/30 column (GE Healthcare) using PBS pH 7.2 as running buffer. A standard containing β-amylase, BSA (bovine serum albumin), and cytochrome *c* was applied in a separate run to determine the apparent molecular weight of the fusion toxins.

Surface plasmon resonance measurements

The affinity of non-PEGylated Ec1-ETA'', N-terminally modified Aha-Ec1-ETA'' (containing the N-terminal Aha instead of methionine), and the PEGylated fusion toxin PEG_{20kDa}-Ec1-ETA'' was determined using surface plasmon resonance (SPR) measurements on a ProteOn XPR36

(Bio-Rad Laboratories AG). For all measurements, a medium to high density (1,000 RU) of biotinylated extracellular domain of EpCAM (EpEX-bio) was immobilized on a NLC chip (Bio-Rad Laboratories AG) and thoroughly equilibrated with sterile-filtered running buffer (PBS, 3 mmol/L EDTA, 0.005% Tween-20) with a flow rate of 60 μL/min. For association with EpCAM, different concentrations prepared in a serial dilution (100, 31.6, 10, 3.16, and 1 nmol/L) were applied in parallel on separate analyte channels and in duplicates for 417 seconds. Dissociation of proteins from the chip was monitored for 10,000 seconds. Data were normalized using interspot referencing and subtraction of a separate analyte channel run with buffer only. All sensograms were fitted using a 1:1 Langmuir model provided by the ProteOn Manager Software (Bio-Rad Laboratories AG), and the association (k_a) and dissociation (k_d) rate constants were used to determine the equilibrium dissociation constants (K_D).

Limulus amoebocyte lysate assay

Contamination of the fusion toxins with endotoxin was measured using the limulus amoebocyte lysate (LAL) assay (Charles River Laboratories) following the manufacturer's protocol.

Cytotoxicity assay

Serial dilutions of the fusion toxins were used to determine the IC₅₀ (concentration at which cell viability was decreased by 50%) of the constructs in XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]H-tetrazolium-5-carboxanilide inner salt) assays (the Cell Proliferation Kit II; Roche Diagnostics GmbH). Briefly, 5,000 cells were seeded into a 96-well plate, incubated overnight in a standard humidified cell culture incubator (37°C, 5% CO₂), and treated with the fusion toxins in quadruplicates the following day. The medium was discarded after 96 hours, 50 μL of XTT reagent were added and cells were incubated for 1 to 3 hours at 37°C. The cytotoxicity of the reversibly PEGylated fusion toxin was measured in 72 hours XTT assays using HT29 cells in the presence of 3C protease.

Cell viability was analyzed in an Infinite M1000 Pro plate reader (Tecan) at 480 nm. Untreated cells were used for normalization. The data were analyzed using Excel (Microsoft) and Prism (v 5.04, GraphPad Software Inc.). Dose-response curves with variable slope were fitted to all data points (10).

In vitro serum stability of fusion toxins

The fusion toxins Ec1-ETA'' and PEG_{20kDa}-Ec1-ETA'' were diluted to a concentration of 2 μg/mL in nonheat-inactivated mouse serum (PAA Laboratories GmbH) and incubated at 37°C to mimic the situation *in vivo* after intravenous injection. At different time points (0, 0.5, 1, 2, 3, and 6 hours), 100 μL of the samples were snap-frozen and stored at -20°C. For analysis, samples were thawed on ice and incubated with magnetic protein G beads (Dynabeads Protein G; Life Science Technologies) previously coated with rabbit anti-*Pseudomonas* Exotoxin

polyclonal serum (P2318; Sigma-Aldrich) for 30 minutes. The fusion toxin was pulled down from serum using a magnetic rack and washed according to the manufacturer's protocol. Magnetic beads were directly mixed with nonreducing 1 × SDS loading buffer and proteins were eluted by boiling for 10 minutes. The nonpurified samples were loaded directly on a 12% SDS-PAGE gel and subjected to semidry Western blotting. Membranes were blocked overnight with milk powder using PBS-TM (1 × PBS, 0.1% Tween-20, 5% skimmed nonfat milk powder) and the membrane was incubated for 1 hour at room temperature with a mouse anti-His6 IgG₁ horseradish peroxidase (HRP) conjugate (#11965085001; Roche Diagnostics AG) diluted 1:500 in PBS-TM. A chemiluminescent HRP substrate was used for detection (Immobilon Western Millipore Corporation).

Blood clearance of fusion toxins

Elimination half-life ($t_{1/2}$) and area under the curve (AUC) of Ec1-ETA'' and PEG_{20kDa}-Ec1-ETA'' were determined in serum from female 8- to 10-week-old CD1 nude mice (Charles River Laboratories) with an average body weight of 25 g. Groups of 4 mice received a single intravenous dose of 170 pmol/mouse and blood samples were drawn from the tail tip at various time points (3, 10, 30, 60, and 120 minutes for Ec1-ETA'' or 3, 60, 180, 360, 720 minutes for PEG_{20kDa}-Ec1-ETA''). Collected blood samples were left for 30 minutes at room temperature followed by two centrifugation steps (3,000 × g, 20 minutes and 10,000 × g, 20 minutes) to allow the separation of serum. The serum samples were snap-frozen and stored at -20°C until use.

The amount of Ec1-ETA'' and PEG_{20kDa}-Ec1-ETA'' in serum was measured using a quantitative ELISA. Briefly, MaxiSorp 96-well plates (Nunc GmbH and Co. KG) were coated for 1 hour at room temperature with mouse anti-tetra-His antibody at a dilution of 1:1,000 in PBS (Qiagen) or with BSA for normalization. The wells were blocked overnight at 4°C with PBS-B (PBS, 0.2% BSA). For detection of fusion toxins in serum, a dilution of 1:100 or 1:300 of the samples was applied in duplicates on the precoated plate. A serial dilution of both Ec1-ETA'' and PEG_{20kDa}-Ec1-ETA'' was included on each plate as standard for quantification. All samples were incubated for 1 hour at room temperature followed by stringent washes with PBS-T (PBS, 0.1% Tween-20). A dilution of 1:5,000 of rabbit anti-PE polyclonal serum (Sigma-Aldrich) was added as primary antibody (1 hour at room temperature) in PBS-TB (PBS, 0.2% BSA, 0.1% Tween-20) followed by washing with PBS-T and incubation with goat-anti-rabbit IgG-HRP conjugate (Sigma-Aldrich) diluted 1:10,000 in PBS-TB for 1 hour at room temperature. The plates were washed with PBS-T and the fusion toxins were assayed using the Amplex Red ELISA detection Kit (Invitrogen; Life Technologies Europe B.V.). All wells were normalized with the appropriate BSA control wells and protein was quantified using the standard curves for Ec1-ETA'' or PEG_{20kDa}-Ec1-ETA''.

The measured concentrations were plotted using GraphPad Prism Software and curves were fitted with a monoexponential decay function. Prism was used to determine the serum half-life of the fusion toxins and the corresponding AUC. Furthermore, data were used to calculate the clearance, excretion constant rate (k_e), and volume of distribution (V_D).

Examination of antitumor effects

Subcutaneous tumor xenografts were raised by injection of 1×10^7 EpCAM-positive HT29 cells in a volume of 100 μ L in the right lateral flank of 6- to 8-week-old female CD1 nude mice (Charles River Laboratories). Tumors were measured with calipers and the volume was calculated with the formula (short² × long diameter) × 0.4. After 5 to 7 days, when tumors reached an average size of 50 to 60 mm³, mice were randomized to groups of 5 per cohort and treatment was started the following day (day 0) by intravenous injection of 340 pmol (equal to 20 μ g protein) Ec1-ETA'', PEG_{20kDa}-Ec1-ETA'', PEG_{20kDa}-Off7 Δ M-ETA'', or PBS in a volume of 100 μ L. The treatment was repeated on days 4, 8, and 12, and mice were monitored for 28 days.

To raise orthotopic tumors, 1×10^7 EpCAM-positive MDA-MB-468 cells in a volume of 100 μ L were mixed on ice 1:1 with Matrigel (Becton Dickinson AG) and 100 μ L of the mixture (5×10^6 cells) were injected into the mammary fat pad of 6- to 8-week-old female CD1 nude mice. Tumor growth was measured as described above. After 6 weeks, when tumors had reached an average size of approximately 50 mm³, mice were randomized in groups of 5 to 7 per cohort and treatment was started the following day (day 0) by intravenous injection of 340 pmol (equal to 20 μ g protein) Ec1-ETA'', PEG_{20kDa}-Ec1-ETA'', PEG_{20kDa}-Off7 Δ M-ETA'', or PBS in a volume of 100 μ L. The treatment was repeated on days 4, 8 and 12, and 16, and mice were monitored for 40 days.

Statistical analysis

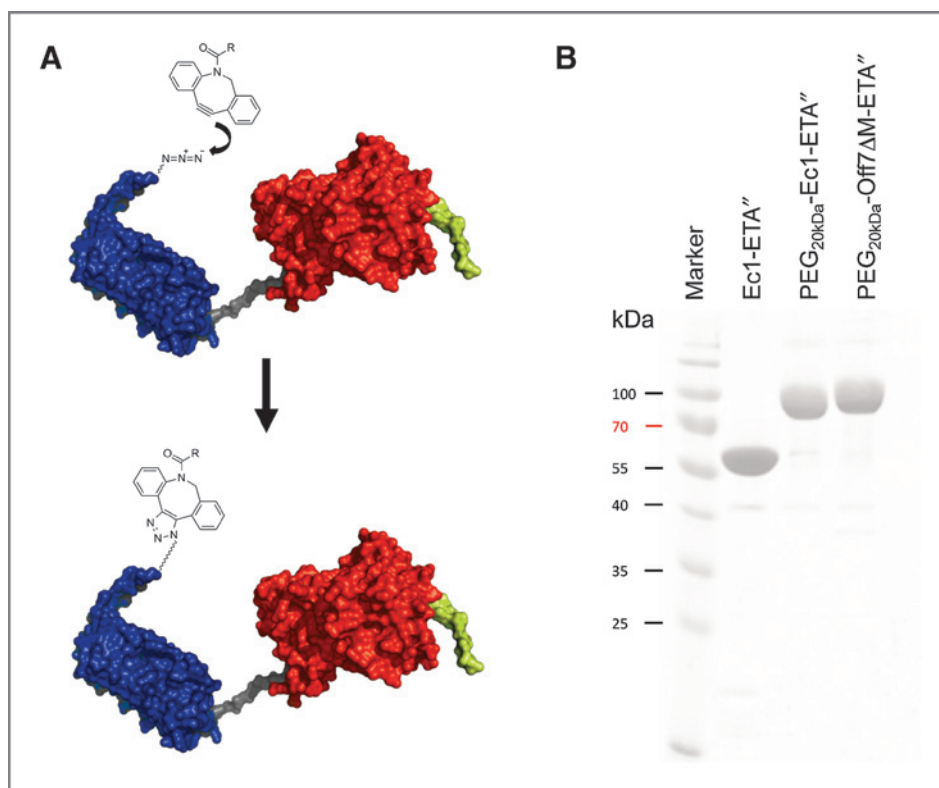
All data are presented as mean ± SD or SEM. The *in vitro* serum stability of the various fusion toxin preparations was compared using a paired *t* test. The differences in tumor growth were analyzed using the Kruskal-Wallis test. As post test, a Dunn multiple comparison test was used to compare PEG_{20kDa}-Ec1-ETA'' and Ec1-ETA''. *P* < 0.05 was considered statistically significant.

Results

Generation of fusion toxins containing a unique Aha

To enable site-specific modification using click chemistry, the anti-EpCAM DARPIn Ec1 was subcloned into the respective vector as described (25) to generate a fusion toxin with domain I-truncated Exotoxin A (PE40, here denoted ETA''); Fig. 1A). In parallel, internal methionine codons were removed from the nontargeted control DARPIn Off7 by two-step site-directed mutagenesis, and then fused to ETA''.

Figure 1. A, schematic illustration of the bioorthogonal modification of Ec1-ETA'' by Cu(I)-free click chemistry. The nonnatural amino acid Aha is introduced uniquely at amino acid position 1 (or any other position in the polypeptide chain). Addition of DBCO-activated compounds such as PEG (shown as "R") results in site-specific modification at the desired position by formation of a stable covalent triazole linkage. B, SDS-PAGE analysis of PEGylated fusion toxins. Of note, 5 µg of each protein was loaded on a reducing 12% gel and mono-PEGylation was detected as a band-shift toward higher molecular weight.



The DARPin-ETA'' fusion toxins were expressed in the methionine-auxotrophic *E. coli* strain B834 (DE3), followed by a medium exchange strategy for metabolic introduction of the nonnatural amino acid Aha at the N-terminus (8, 9). Purification was done by IMAC, including extensive washing with Triton-X114 to remove endotoxins. The proteins were analyzed by SDS-PAGE and showed the expected molecular weight of 58.7 kDa (Fig. 1B). This resulted in purified soluble Aha-containing fusion toxins at yields up to 4 mg/L in shake flasks.

Bioorthogonal PEGylation of fusion toxins using click chemistry

For subsequent PEGylation at the N-terminus, the fusion toxins were mixed with a 2-fold excess of DBCO-PEG_{20kDa} in PBS at 4°C. As described previously (8), PEG reacted in a time-dependent manner, leading to exclusively mono-PEGylated protein as detected by a single band-shift toward higher molecular weight (about 90–100 kDa) by SDS-PAGE (Fig. 1B). The PEGylated proteins were separated from unconjugated PEG by anion exchange chromatography (Supplementary Fig. S1A and S1B). Subjecting the nonreacted fraction to a second round of PEGylation with a 2-fold excess of DBCO-PEG_{20kDa} again provided PEGylated products, indicating that the reactivity of the N-terminal azide of Aha was retained (Supplementary Fig. S2). The pooled fractions of PEGylated protein were further purified using preparative gel filtration (Supplementary Fig. S1C and S1D), and the final

products were analyzed by SDS-PAGE using Coomassie and iodine staining of PEG (Supplementary Fig. S1E). As shown in Fig. 2, this yielded exclusively mono-PEGylated protein without side products. Analysis of endotoxin contamination in the LAL assay showed only trace amounts of lipopolysaccharide (Ec1-ETA'' 3.5 EU/mg, PEG_{20kDa}-Ec1-ETA'' 1.3 EU/mg, and PEG_{20kDa}-Off7ΔM-ETA'' 13.2 EU/mg).

In addition, we determined the increase in the hydrodynamic radii of the fusion toxins as a result of PEGylation by analytical gel filtration. As shown in Supplementary Fig. S3, non-PEGylated Ec1-ETA'' eluted with the expected molecular weight of approximately 60 kDa, whereas mono-PEGylated Ec1-ETA'' eluted at smaller volumes, which is comparable with a molecular weight of more than 250 kDa typical for a mono-PEGylated protein (36).

Effect of PEGylation on fusion toxin binding to EpCAM

The binding activity of PEGylated and non-PEGylated fusion toxin was compared using SPR. To monitor potential intermolecular inhibition effects that might be derived from N-terminal PEGylation (9, 37), we immobilized EpCAM on the chip with medium to high density, thereby mimicking the membrane surface of EpCAM-positive target cells with high antigen density. Ec1-ETA'' and Aha-Ec1-ETA'' (which differ only in the first amino acid, by containing Met or Aha, respectively) showed very similar data for k_a or k_d (Supplementary Fig. S4 and Table S1). After PEGylation, however, the association rate

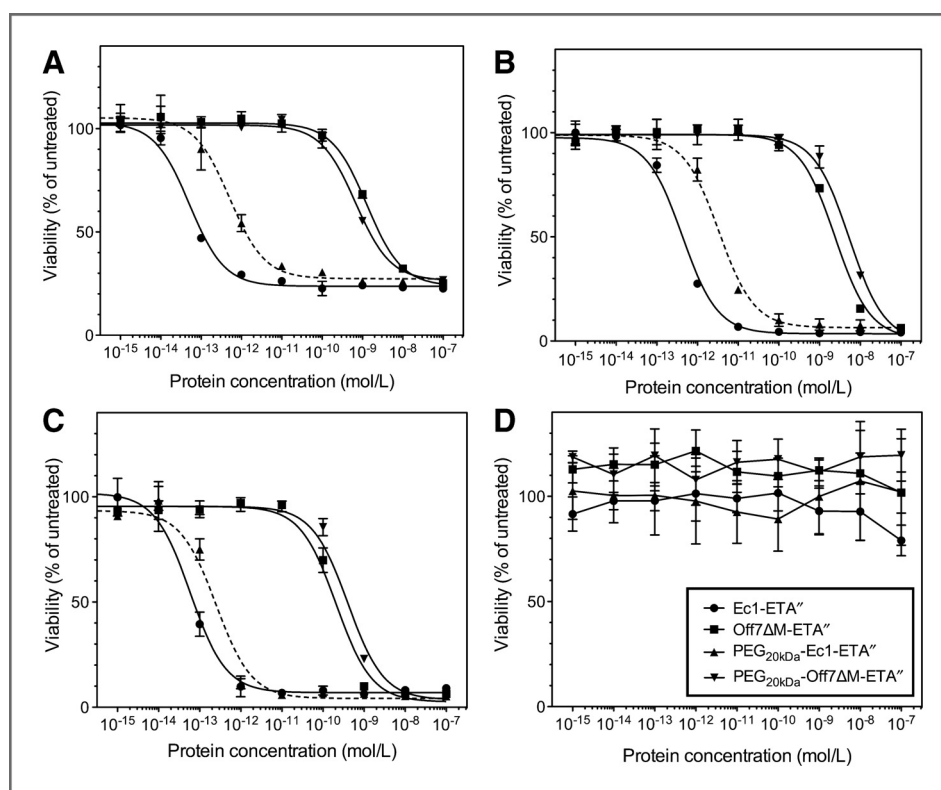


Figure 2. Cytotoxicity of PEGylated and non-PEGylated fusion toxins against various tumor cell lines determined in 96-hour XTT assays. Serial dilutions of the proteins were added to the EpCAM-positive cell lines (A) HT29, (B) MDA-MB-468, and (C) MCF7. D, the EpCAM-negative cell line RL was used as a nontarget control. Bars, SD.

constant of $\text{PEG}_{20\text{kDa}}\text{-Ec1-ETA}''$ was 2-fold lower than the non-PEGylated fusion toxin, whereas no difference was found for k_d . This resulted in an overall reduction in K_D by a factor of two (139 pmol/L before and 290 pmol/L after PEGylation), possibly due to intramolecular blocking effects (37). In addition, the maximal response on the chip (which is proportional to the number of fusion toxin molecules able to interact with the surface) showed a reduction for the PEGylated fusion toxin, compared with its non-PEGylated counterpart, probably due to intermolecular blocking effects resulting from steric hindrance by the $\text{PEG}_{20\text{kDa}}$ polymer (Supplementary Fig. S4 and Table S1; ref. 36).

Cytotoxicity of fusion toxins

Tumor cell lines of different histotypes were used to determine the cytotoxicity of PEGylated and non-PEGy-

lated $\text{Ec1-ETA}''$ and the respective nontargeted control fusion toxins $\text{Off7}\Delta\text{M-ETA}''$ and $\text{PEG}_{20\text{kDa}}\text{-Off7}\Delta\text{M-ETA}''$. Figure 2 shows the cell viability curves determined in XTT assays, the IC_{50} values (concentrations at which cell viability was decreased by 50%) are depicted in Table 1. With all EpCAM-positive cell lines, $\text{Ec1-ETA}''$ and $\text{PEG}_{20\text{kDa}}\text{-Ec1-ETA}''$ showed IC_{50} values in the femtomolar range, which was up to 10^4 -fold more potent than the nontargeted control fusion toxins (Fig. 2A–C). Depending on the cell line, $\text{PEG}_{20\text{kDa}}\text{-Ec1-ETA}''$ was 4- to 10-fold less potent than the non-PEGylated variant (Supplementary Table S2). Decreased cytotoxicity upon PEGylation, albeit to a smaller extent and at much higher absolute concentrations, was also observed for the control fusion toxin $\text{Off7}\Delta\text{M-ETA}''$ (Fig. 2). On EpCAM-negative RL cells, the potency was four orders of magnitude lower, indicating the strong EpCAM specificity of the effect (Fig. 2D).

Table 1. Cytotoxicity of fusion toxins against various tumor cell lines

Cell line	$\text{Ec1-ETA}''$ (mol/L)	$\text{PEG}_{20\text{kDa}}\text{-Ec1-ETA}''$ (mol/L)	$\text{Off7}\Delta\text{M-ETA}''$ (mol/L)	$\text{PEG}_{20\text{kDa}}\text{-Off7}\Delta\text{M-ETA}''$ (mol/L)
HT29	5.1×10^{-14}	5.1×10^{-13}	1.3×10^{-9}	6.8×10^{-10}
MDA-MB-468	4.2×10^{-13}	3.4×10^{-12}	2.4×10^{-9}	5.4×10^{-9}
MCF7	5.9×10^{-14}	2.5×10^{-13}	2.1×10^{-10}	4.0×10^{-10}
RL	$>1 \times 10^{-8}$	$>1 \times 10^{-8}$	$>1 \times 10^{-8}$	$>1 \times 10^{-8}$

NOTE: Data represent IC_{50} values calculated from the curves depicted in Fig. 3 after fitting by nonlinear regression.

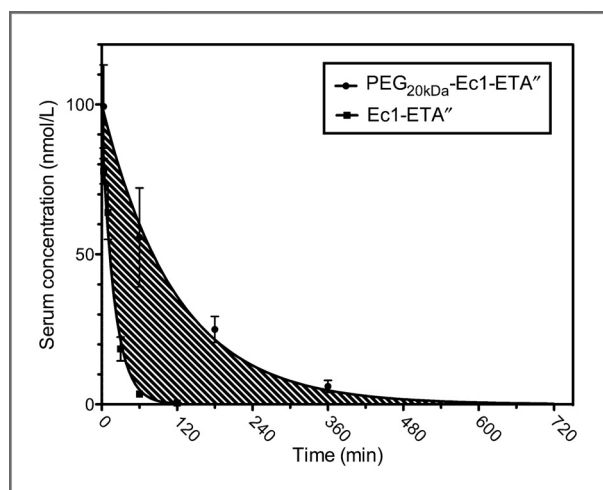


Figure 3. Blood clearance of Ec1-ETA'' and PEG_{20kDa}-Ec1-ETA'' determined in CD1 nude mice upon a single intravenous injection of 170 pmol protein. Serum titers were calculated from blood samples of 4 mice per group drawn at different time points after injection. Bars, SD

We compared both the Aha and Met containing version of non-PEGylated Ec1-ETA'' to exclude the possibility that the results were affected by using different *E. coli* strains for expression [B834 (DE3) vs. BL21 (DE3)], different expression media (M9 vs. 2 × YT) and/or different expression temperatures (30°C vs. 37°C). Both fusion toxin variants were found to be equally potent in XTT assays, indicating that exchanging methionine by Aha did not affect cytotoxicity and that the reduced effect of the PEGylated variant indeed resulted from polymer conjugation (data not shown).

To analyze this phenomenon further, we generated a reversibly PEGylated variant of Ec1-ETA'' from which the PEG tail can be removed. To this end, a 3C protease site was genetically introduced at the N-terminus between the His-tag and the DARPin to generate PEG_{20kDa}-3C-Ec1-ETA'' (Supplementary Fig. S5A). The protein was expressed, PEGylated, purified as described above and analyzed by SDS-PAGE (Supplementary Fig. S5B). Cleavage of PEG_{20kDa}-3C-Ec1-ETA'' with 3C protease was quantitative under the conditions described and yielded a product with a lower molecular weight, as expected for non-PEGylated Ec1-ETA'' (Supplementary Fig. S5B). The

cytotoxicity of fusion toxins was determined in XTT assays with and without N-terminal PEG cleavage. As shown in Supplementary Fig. S5C, on HT29 cells PEG_{20kDa}-3C-Ec1-ETA'' showed a 10-fold lower potency (calculated as IC₅₀), compared with Ec1-ETA'', but completely regained its activity upon removal of the polymer.

Serum stability and *in vivo* blood clearance of fusion toxins

We first compared the serum stability of Ec1-ETA'' and PEG_{20kDa}-Ec1-ETA'', as PEGylation was previously shown to increase the resistance of proteins to proteolytic degradation (38). Interestingly, Ec1-ETA'' and the PEGylated variant were found to be similarly stable in serum at physiologic conditions and showed no signs of degradation or loss of integrity as analyzed by Western blotting (Supplementary Fig. S6).

Serum levels of PEG_{20kDa}-Ec1-ETA'' and the non-PEGylated Ec1-ETA'' control were determined by ELISA using blood samples from mice drawn at different time points after a single tail vein injection. Figure 3 illustrates the serum concentration profiles; the pharmacokinetic parameters calculated from the data by noncompartmental analysis are summarized in Table 2. The non-PEGylated fusion toxin was rapidly cleared from the blood, resulting in a terminal half-life ($t_{1/2}$) of only 14 minutes. In contrast, mono-PEGylation decreased the blood clearance 6-fold and increased the half-life to 82 minutes. Correspondingly, the AUC significantly increased 7.5-fold from 29 to 217 nmol/L × h. Interestingly, the apparent volume of distribution (V_d) was similar for both fusion toxin variants, indicating that tissue distribution was not further reduced by the hydrophilic PEG_{20kDa} polymer. The increased elimination half-life of the mono-PEGylated fusion toxin correlated well with a 5-fold slower clearance (Table 2), which was likely due to reduced renal excretion of PEG_{20kDa}-Ec1-ETA'' with an apparent molecular weight of approximately 250 kDa.

Antitumor effects of fusion toxins

To assess the effect of PEGylation and half-life extension on the therapeutic efficacy of the non-PEGylated and PEGylated Ec1-ETA'' fusion toxins, antitumor effects were compared in models of subcutaneous HT29 and orthotopic MDA-MB-468 tumor xenografts in nude mice.

Table 2. Pharmacokinetics of Ec1-ETA'' and PEG_{20kDa}-Ec1-ETA'' in nude mice upon a single intravenous injection

	AUC _{0-last} (nmol/L · h)	$t_{1/2}$ (min)	Cl (L · h ⁻¹ · kg ⁻¹)	V_d (L · kg ⁻¹)	k_{el} (h ⁻¹)
Ec1-ETA''	45	14.1	0.215	0.073	2.95
PEG _{20kDa} -Ec1-ETA''	217	81.6	0.035	0.068	0.51

NOTE: Data were calculated from the blood clearance curves shown in Fig. 4.

Abbreviations: AUC_{0-last}, area under the serum concentration curve; Cl, clearance; k_{el} , first-order elimination rate constant; $t_{1/2}$, elimination half-life; V_d , apparent volume of distribution.

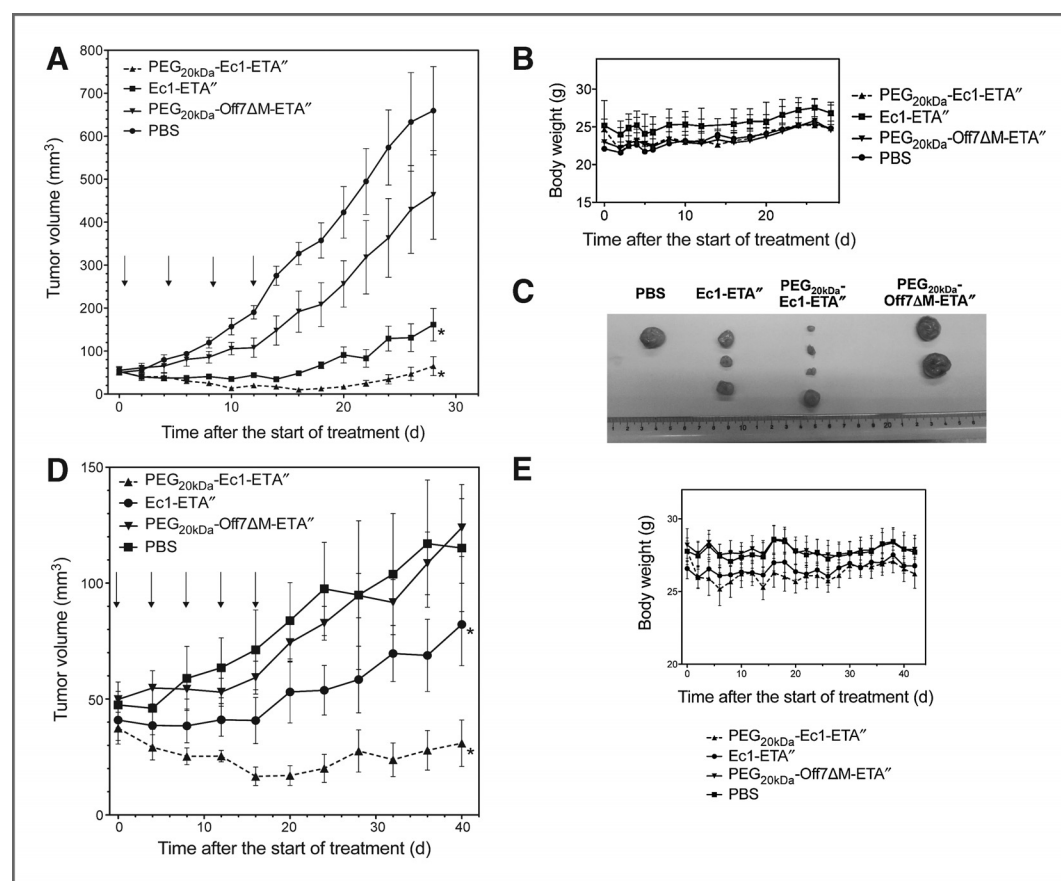


Figure 4. A, effect of PEGylated and non-PEGylated fusion toxins on subcutaneously growing HT29 tumors. Seven to 10 days after subcutaneous tumor inoculation (10^7 cells in $100 \mu\text{L}$), nude mice were injected four times intravenously with $100 \mu\text{L}$ of a 340 pmol solution (equivalent to $20 \mu\text{g}$ protein) of either EpCAM-targeted $\text{PEG}_{20\text{kDa}}\text{-Ec1-ETA}''$, $\text{Ec1-ETA}''$, or the nontargeted control fusion toxin $\text{PEG}_{20\text{kDa}}\text{-Off7}\Delta\text{M-ETA}''$. Control mice received equal volumes of PBS. Tumor growth was monitored for 28 days by caliper measurements. B, body weight of mice during treatment with the fusion toxins or PBS. C, size of representative HT29 tumors from each treatment group. Mice were euthanized on day 40 after the start of the treatment and tumors were excised for comparison. D, effect of PEGylated and non-PEGylated fusion toxins on orthotopically growing MDA-MB-468 tumors. Six weeks after tumor cell inoculation into the mammary fat pad (5×10^6 cells in $100 \mu\text{L}$), nude mice were injected five times intravenously with $100 \mu\text{L}$ of a 340 pmol solution of the fusion toxins, control mice received equal volumes of PBS. Tumor growth was monitored for 6 weeks by caliper measurements. E, body weight of mice during treatment with the fusion toxins or PBS. Arrows, injection time points; bars, SEM; *, $P < 0.05$.

In the subcutaneous model, mice bearing established HT29 tumors were treated intravenously with 4 doses of 340 pmol $\text{Ec1-ETA}''$ or $\text{PEG}_{20\text{kDa}}\text{-Ec1-ETA}''$. To minimize the nonspecific systemic toxicity of ETA'' , we chose a 4-day treatment interval. As shown in Fig. 4A, tumors of mice treated with PEGylated and non-PEGylated $\text{Ec1-ETA}''$ responded immediately, resulting in a steady decrease in tumor volume during the course of the treatment (until day 14). However, whereas tumors of mice treated with non-PEGylated $\text{Ec1-ETA}''$ started to regrow to a mean size of 161 mm^3 on day 28, treatment with $\text{PEG}_{20\text{kDa}}\text{-Ec1-ETA}''$ resulted in a more pronounced and longer-lasting antitumor effect ($P < 0.05$) with longer injection-free intervals. The mean tumor volume measured on day 28 was only 65 mm^3 and in 1 mouse remained stable even after discontinuation of the treatment. In contrast, tumors of mice treated with PBS or the nontargeted $\text{PEG}_{20\text{kDa}}\text{-Off7}\Delta\text{M-ETA}''$ rapidly progressed to a mean size of 660 mm^3 and 464 mm^3 , respectively, on

day 28 (Fig. 4A). The lack of antitumor activity of the nontargeted fusion toxin suggests that significant passive tumor localization by the enhanced permeability and retention (EPR) effect (32, 39) can be excluded. All treatments were well tolerated by the animals with only very marginal weight loss (Fig. 4B). In Fig. 4C, the sizes of excised tumors are shown for comparison. Repetitive treatment of regrowing HT29 tumors with the anti-EpCAM fusion toxins on days 28, 32, and 36 unveiled that they were still responsive (data not shown).

In the orthotopic tumor model, nude mice bearing established MDA-MB-468 tumors in the mammary fat pad were treated intravenously with 5 doses of 340 pmol $\text{Ec1-ETA}''$ or $\text{PEG}_{20\text{kDa}}\text{-Ec1-ETA}''$. As shown in Fig. 4D, compared with the PBS control, treatment with non-PEGylated $\text{Ec1-ETA}''$ resulted in a transient tumor growth inhibition without decreasing the tumor size (82 mm^3 on day 42). In contrast, tumors of mice treated with $\text{PEG}_{20\text{kDa}}\text{-Ec1-ETA}''$ responded more strongly, resulting in significant shrinkage to an

average size of 31 mm³ on day 40 with 1 animal showing complete tumor regression ($P > 0.05$). Again, as demonstrated for subcutaneous HT29 tumors, the control fusion toxin PEG_{20kDa}-Off7ΔM-ETA'' had no effect on the growth of orthotopic MDA-MB-468 tumors (Fig. 4D). Similar to the subcutaneous tumor model treatments were well tolerated by the animals (Fig. 4E).

Discussion

EpCAM is abundantly expressed in solid tumors and its rapid internalization upon ligand binding makes it ideal for tumor targeting with antibodies or alternative binding proteins payloaded with anticancer agents acting on intracellular targets (22, 23, 26). We previously reported on a fusion toxin composed of the high affinity anti-EpCAM DARPin Ec4 and domain I-truncated ETA (ETA'', also known as PE40), which could be expressed at very high yields and demonstrated promising antitumor effects (25). Fusion toxins of this rather small size, however, have very short elimination half-lives of hardly more than 10 minutes, which negatively affects tumor targeting (6). To increase the therapeutic index, the half-life must be extended without increasing systemic toxicity. Here, we improved the pharmacokinetic and therapeutic performance of Ec1-ETA'' by bioorthogonal conjugation to a 20 kDa PEG at a desired position in the protein backbone.

Conjugation of proteins to PEG is an established strategy that has been used for various marketed protein therapeutics and other types of medicines (40, 41). Conventional PEGylation procedures for proteins have used polymers activated by amine-reactive succinimidyl esters or thiol-reactive maleimides. However, lysines with their primary amines and cysteines are commonly present in proteins, which results in an unwanted mixture of mono- and multi-PEGylated positional isomers with random conjugation sites (40–42). To avoid heterogeneity of the PEGylated product, a single cysteine was introduced in the linker sequence of the fusion toxin for subsequent conjugation to maleimide-activated PEG (31). Unfortunately, this strategy may impair protein folding due to incorrect disulfide formation in ETA'' (27, 28, 31), and stability due to possible maleimide exchange with cysteine-rich serum proteins (43). In a different approach, which is likely not tolerated by many proteins, Onda et al. (44) engineered a Lys-free immunotoxin in which a unique Lys was then introduced for site-specific conjugation.

DARPins contain no cysteines and most importantly only a single N-terminal methionine at position 1 (ATG) in addition to another Met in the backbone, which can be conveniently replaced by leucine without loss of biophysical performance (8, 9, 10). The anti-EpCAM DARPin Ec1 contains even only the methionine at position 1, which we replaced by Aha to enable bioorthogonal strain-promoted cycloaddition of a DBCO-activated PEG_{20kDa} (9, 10). We demonstrate that this strategy is perfectly suited to modify DARPin-ETA'' fusion toxins beyond the classical way of protein engineering by genetic alterations. PEGylation of Ec1-ETA'' was accomplished by simply mixing the pro-

tein with DBCO-PEG_{20kDa}, which provides a strained alkyne for covalent conjugation exclusively with the N-terminal azide of the fusion toxin. Maximum PEGylation was obtained with a low molar excess (2 eq.) of DBCO-PEG_{20kDa} over protein and yielded more than 60% of biologically active PEG_{20kDa}-Ec1-ETA'', which could be conveniently purified by anion exchange chromatography. The PEGylation yield was not 100% even in the presence of a high molar excess of DBCO-PEG_{20kDa}; however, the remaining unreacted protein fraction could be readily recycled from the column and successfully subjected to a second conjugation round, indicating that the N-terminal azide was still reactive. To our knowledge, PEG_{20kDa}-Ec1-ETA'' is the first protein therapeutic engineered for tumor targeting by click chemistry. Beyond PEGylation, the azide-directed conjugation of DARPins or DARPin fusion proteins described here is generic in nature and in principle applicable to various other conjugation partners designed to improve tumor targeting (45, 46).

Even if the bulky PEG polymer is conjugated to sites remote from the active center, some loss of activity is commonly observed with therapeutic proteins (32). We found that PEGylation of Ec1-ETA'' decreased its cytotoxicity 4- to 10-fold. Although the on-rate decreased 2-fold after PEGylation, the loss in potency cannot be simply explained by the reduced binding affinity as the fusion toxin was permanently present in the assays. It is, however, plausible that steric hindrance by the polymer decreased the absolute quantity of fusion toxin capable of associating with EpCAM on the cell surface (a phenomenon previously described as intermolecular blocking; ref. 9, 37) as a first and crucial step of the cellular intoxication process of ETA''. This conclusion is supported by SPR experiments and previous binding studies with PEGylated Ec1 (9). Recently, from studies with the plant toxin gelonin, Pirie and colleagues (47) reported the existence of a near-universal threshold for the amount of internalized toxin that is required for induction of cell death. It is thus conceivable that cell death induction by PEG_{20kDa}-Ec1-ETA'' obeys the same rules and internalization is significantly more effective after unveiling of PEG once the fusion toxin is localized in the tumor.

In contrast with non-PEGylated Ec1-ETA'', which at the tested dose schedule only inhibited tumor growth, PEG_{20kDa}-Ec1-ETA'' induced tumor shrinkage with one long-lasting response and one complete regression. This suggests that the shortcomings of PEGylation measured *in vitro* could be fully offset *in vivo* by half-life extension. Although the remaining tumors resumed growth after discontinuation of treatment, they were still responsive to a second treatment cycle, indicating that engineering the fusion toxin for further enhanced pharmacologic performance is warranted. Because nontargeted PEG_{20kDa}-Off7ΔM-ETA'' did not affect tumor growth, a role of passive tumor targeting by the EPR effect (39), as described for other PEGylated nanomedicines (38, 40, 48, 49), can be excluded.

Cellular intoxication by ETA'' is a complex process (27, 50, 51), several steps of which might be negatively affected by a bulky polymer. On the basis of this consideration, PEGylation may improve tumor localization of the fusion toxin and reduce its uptake by the reticulo-endothelial system (RES), but once it encounters its target cell becomes dispensable or even unwanted. Therefore, a construct engineered for de-PEGylation under specific conditions in the tumor microenvironment, for example, by tumor proteases, might be advantageous. That such a design is in principle possible is suggested by control experiments with a reversibly PEGylated Ec1-ETA'' construct for which cell binding and cytotoxic potency could be entirely restored after proteolytic de-PEGylation.

In conclusion, we describe a novel anti-EpCAM fusion toxin for tumor targeting engineered by PEGylation, using bioorthogonal click chemistry, and report its improved pharmacokinetic and therapeutic performance. In addition to standard protein engineering techniques, the high compatibility of DARPin and drug conjugates derived thereof with click chemistry opens new perspectives for more effective cancer-related therapy.

Disclosure of Potential Conflicts of Interest

Andreas Plückthun has ownership interest (including patents) in Molecular Partners AG. No potential conflicts of interest were disclosed by the other authors.

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