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Cancer Therapy: Preclinical

A Novel Fusion Toxin Derived from an EpCAM-Specific Designed Ankyrin Repeat Protein Has Potent Antitumor Activity

Patricia Martin-Killias [1], Nikolas Stefan[1], Sacha Rothschild[2,4], Andreas Plückthun[1], and Uwe Zangemeister-Wittke[1,3]

Abstract

Purpose: Designed ankyrin repeat proteins (DARPins) hold great promise as a new class of binding molecules to overcome the limitations of antibodies for biomedical applications. Here, we assessed the potential of an epithelial cell adhesion molecule (EpCAM)–specific DARPin (Ec4) for tumor targeting as a fusion toxin with *Pseudomonas aeruginosa* exotoxin A.

Experimental design: DARPin Ec4 was genetically fused to a truncated form of *Pseudomonas aeruginosa* exotoxin A (ETA″) and expressed in *Escherichia coli*. The cytotoxicity of Ec4-ETA″ was measured against tumor cell lines of various histotypes *in vitro*. Tumor localization and antitumor activity were determined in mice bearing 2 different EpCAM-positive tumor xenografts.

Results: Ec4-ETA″ expressed very well in soluble form in the cytoplasm of *E. coli* and yielded up to 40 mg after purification per liter of culture. The protein was monomeric and the disulfides of ETA″ formed spontaneously. Ec4-ETA″ bound to EpCAM with low nanomolar affinity, similar to free Ec4. Furthermore, it was highly cytotoxic against various EpCAM-positive tumor cell lines *in vitro* with IC$_{50}$ values less than 0.005 pmol/L. This effect was competed by free Ec4, but not by unspecific DARPins. Upon systemic administration in athymic mice, Ec4-ETA″ efficiently localized to EpCAM-positive tumors to achieve maximum accumulation 48 to 72 hours after injection, whereas an irrelevant control fusion toxin did not accumulate. Tumor targeting with Ec4-ETA″ resulted in a strong antitumor response including complete regressions in some animals.

Conclusions: Our data show for the first time the potential of DARPins for the generation of protein therapeutics for tumor targeting, and that Ec4-ETA″ deserves attention for clinical development. *Clin Cancer Res; 17(1); 100–10. ©2010 AACR.*

Introduction

The concept of tumor-targeted therapy is based on the use of conjugates consisting of ligands binding to tumor-associated antigens or growth factor receptors that deliver cytotoxic agents selectively to tumors, while sparing normal tissues from destruction (1). These agents include radio-isotopes, small organic compounds, antisense oligonucleo-tides, and protein toxins. All of them exert different modes of action, compared with standard chemotherapy, and thus might be particularly useful to combat drug-resistant cancer. Nonetheless, it remains to be shown in every single case whether a tumor-specific localization actually occurs and what profile of antitumor action compared with side effects is seen.

Immunotoxins are a class of conjugates in which antibodies or antibody fragments are chemically linked to protein toxins, whereas in the more advanced constructs targeting ligand and toxin are genetically fused (2, 3). The most popular toxins used for this purpose are diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A (ETA; ref. 4), both of which act by irreversibly inhibiting protein synthesis in cells. In ETA-based fusion toxins a truncated variant lacking the N-terminal cell binding domain and carrying a C-terminal Lys-Asp-Glu-Leu (KDEL) peptide (denoted here ETA″) is commonly used (5). Currently, several of these fusion toxins are in clinical trials for the treatment of lymphomas, leukemias (6, 7), mesothelioma, and cancers of the ovary, pancreas, and bladder (8, 9).

On solid tumors, well-investigated targets for antibody-based therapies are members of the epidermal growth factor receptor family, such as EGFR itself and ErbB2,

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Translational Relevance

Antibodies or antibody fragments are widely used as targeting moiety for the delivery of cytotoxic drugs to tumors. However, many of these constructs have limitations due to their poor expression and aggregation tendency. To overcome these limitations, we used for the first time designed ankyrin repeat proteins (DAR-Pins) as non-IgG scaffolds with favorable biophysical properties and much higher expression yield in *E. coli* and systematically tested their potential for tumor-targeted delivery of a highly potent biotoxin in preclinical studies. The fusion toxin Ec4-ETA″ recognizing the carcinoma-associated antigen EpCAM was potently cytotoxic against carcinoma cell lines of various histotypes *in vitro*. In athymic mice targeting of human carcinoma xenografts with Ec4-ETA″ resulted in a strong antitumor response including complete regressions. This shows that DARPins are well suited for tumor targeting and that the fusion toxin Ec4-ETA″ holds promise for clinical development.

and certain tumor-associated carbohydrates (2, 10). The epithelial cell adhesion molecule (EpCAM) has also emerged as a promising structure for targeted therapy of solid tumors. One reason is that its efficient internalization promotes access of surface bound effector molecules to intracellular targets (11–14). EpCAM is a homophilic cell adhesion molecule of 39 to 42 kDa, consisting of an extracellular domain with an epidermal growth factor–like and a human thyroglobulin–like domain, and a short cytoplasmic domain. Its processing by regulated intramembrane proteolysis releases a cytoplasmic tail that activates the *wnt* signaling pathway and induces transcription of c-myc and cyclins (15, 16). How this mechanism contributes to tumor progression *in vivo* is unclear. EpCAM is expressed at low levels on basolateral cell surfaces of some normal epithelia (17). In contrast, high levels of homogenously distributed EpCAM are detectable on cells of epithelial tumors (15, 18), and its overexpression represents an independent prognostic marker for reduced survival in patients with breast and ovarian cancer (19, 20). Recently, EpCAM was also identified as a marker of cancer-initiating cells in colon (21), breast (22), pancreatic (23), and hepatocellular carcinomas (24) providing the opportunity to target cancer stem-like cells, which usually respond poorly to standard therapy. The favorable properties of EpCAM for cancer therapy are currently exploited in phase II clinical trials with a scFv-ETA″ immunotoxin (9, 11–13), which we developed previously (13).

The tumor-targeting moiety for the delivery of cytotoxic agents including protein toxins is usually derived from antibodies or antibody fragments, which, however, have practical limitations due to their poor expression yield and aggregation tendencies, at least for some constructs (25, 26). For fusion toxins no other feature of the antibody than

antigen binding is required, so a solution might come from the use of alternative non-IgG binding scaffolds as targeting moieties (27). These can be engineered for improved specificity, affinity, and stability to increase the production yield. One such protein class are designed ankyrin repeat proteins (DARPins; refs. 28, 29). The ankyrin repeat motif consists of 33 amino acids forming a loop, a β-turn, and 2 antiparallel α-helices connected by a tight turn. Their high stability and favorable biophysical properties provide proteins that tolerate engineering procedures usually not applicable to antibodies. Moreover, they contain no cysteine, which can instead be introduced for site-specific modifications. Thus, DARPins fulfill the requirement of almost ideal candidates for many biomedical applications including tumor targeting. Using combinatorial libraries of DARPins along with selection by ribosome and phage display, we recently generated several binders specific for EpCAM (P. Martin-Killias, N. Stefan, S. Wyss-Stoeckle, A. Honegger, U. Zangemeister-Wittke, and A. Plückthun, in preparation), and showed their potential for efficient delivery of therapeutic siRNA into tumor cells (14).

Here we describe for the first time the use of a high-affinity DARPin (Ec4) specific for EpCAM to generate a fusion toxin with ETA″. Ec4-ETA″ expressed very well in *Escherichia coli*, was easily purified to high yields, and proved to be specifically cytotoxic against various EpCAM-positive tumor cell types *in vitro*. In vivo, fluorescence imaging and therapy studies in athymic mice showed its ability to efficiently localize to subcutaneously growing tumors upon intravenous administration, and induce strong antitumor effects including complete regressions.

Material and Methods

Tumor cells

The squamous cell carcinoma cell line of the tongue CAL27 and the colorectal carcinoma cell line HT29 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen). The breast carcinoma cell line MCF7 and the non-Hodgkin's lymphoma cell line RL were obtained from ATCC (American Type Culture Collection). The small cell lung carcinoma cell line SW2 was maintained in our laboratory. All cells were cultured in Dulbecco's modified medium (Sigma), supplemented with 10% fetal calf serum (Amimed, Bioconcept), 100 U/mL of penicillin and 100 μg/mL of streptomycin (Sigma). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. All cells were tested negative for mycoplasma using MycoAlert (Lonza).

Construction, expression, and purification of DARPin-ETA″ fusion toxins

The EpCAM-specific high-affinity DARPin Ec4 was selected from a DARPin library as described (P. Martin-Killias, et al., in preparation), the control DARPins off7 (targeting the maltose binding protein) and E3_5 (an unselected member of the N3C library) have been described elsewhere (29, 30). The sequences encoding the DARPins

were inserted via *Bam*HI and *Hin*dIII upstream into an expression vector derived from pQE30, containing a 12 amino acid linker $(GSG_4)_2$ and the 40-kDa truncated form of $ETA_{252-608}$KDEL (ETA″), which was cloned as described (11, 31). ETA comprises residues Glu252-Pro608 (numbering of the mature protein), fused to a C-terminal His_6 tag followed by KDEL (denoted $ETA_{252-608}$KDEL or ETA″).

For purification and detection the construct in addition contains an MRGS-His_6 tag at the N terminus. The DARPin-ETA″ fusion proteins were expressed in soluble form in the *E. coli* strain BL21(DE3) (Stratagene). Cultures were harvested 4 hours after induction with 1 mmol/L isopropyl-β-D-thio-galactopyranoside. For purification the bacteria were resuspended in TBS_{400} (50 mmol/L of Tris, 400 mmol/L of NaCl, pH 7.4, at 4°C) with 20 mmol/L of imidazole and lysed with a TS 1.1-kW cell disruptor (Constant Systems Ltd.). On centrifugation (48,000 × *g*, 30 minutes at 4°C) and filtration (pore size 0.22 μm), the fusion toxins present in the clear supernatant were purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA superflow (Qiagen).

Endotoxin removal

For *in vivo* application, the DARPin-ETA″ fusion toxins were further purified to eliminate endotoxin. To this end, an additional washing step with 150 column volumes phosphate buffered saline (PBS) containing 20 mmol/L imidazole and 0.1% Triton-X-114 was performed during Ni-NTA purification, followed by size exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare). Monomeric fractions were further depleted of residual endotoxin by passage over an EndoTrap Red column (Hyglos), and the final endotoxin content was determined using a Limulus amebocyte lysate endochrome kit (Charles River).

Measurement of EpCAM-binding affinity

Surface plasmon resonance. The EpCAM-binding affinities of Ec4 and Ec4-ETA″ were measured by surface plasmon resonance using a ProteOn XPR36 (Bio-Rad Laboratories) instrument. For ProteOn measurements, 1 ligand channel of a neutravidin sensor chip was coated with 500 resonance units of the extracellular domain of EpCAM (residues 1 to 242 of the mature protein) biotinylated using a C-terminal AviTag. Kinetic data were obtained by parallel injection of different concentrations of Ec4 or Ec4-ETA″ ranging from 0.32 to 31.6 nmol/L at a buffer flow of 60 μL/min in PBS at pH 7.4 containing 3 mmol/L EDTA and 0.005% Tween-20. Data evaluation was performed using the ProteOn Manager software (Bio-Rad).

Flow cytometry. The binding affinity of Ec4 to EpCAM expressed on cells was measured by flow cytometry, and the equilibrium dissociation constant K_D was determined as the ratio of dissociation rate constant over association rate constant. MCF-7 cells were preincubated for 30 minutes at 37°C in PBS supplemented with 1% bovine serum albumin (BSA; Sigma) and 0.2% sodium azide (Fluka) to inhibit

internalization. For dissociation experiments, 3×10^5 cells were saturated for 1 hour at 4°C with 100 nmol/L Ec4 labeled with Alexa Fluor 488 C_5 maleimide (Molecular Probes, Invitrogen) via a C-terminal cysteine (Ec4cA488). Cells were centrifuged and resuspended in 1 μmol/L Ec4 to prevent rebinding. The mean fluorescence intensities were recorded at different time points ranging from 0 to 4 hours. For association experiments, 3×10^5 cells were incubated with 2.5, 7.5, or 22.5 nmol/L Ec4cA488 and measured at time points ranging from 1 to 60 minutes without prior washing. To correct for the small amount of nonspecific binding, probably caused by Alexa Fluor 488, the association of a nonbinding DARPin labeled with Alexa Fluor 488 was measured and subtracted. Data evaluation was performed with Prism (Graphpad).

Disulfide assays

The formation of the 2 disulfide bonds in Ec4-ETA″ after purification of protein expressed in the cytoplasm was quantified according to ref. 32. Briefly, 1.25 nmol of fusion protein was treated with 4,4′-dithiodipyridine (4-DPS) in the presence of 8 mol/L urea and compared with an equally treated sample reduced with sodium borohydride, quantified by HPLC and evaluated according to a standard curve made with known amounts of cysteine.

Cytotoxicity assays

The specific cytotoxic activities of the DARPin-ETA″ fusion toxins were assessed by measuring cell viability in standard colorimetric XTT assays; XTT = (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tretrazolium-5-carboxanilide) sodium salt (Roche). Briefly, EpCAM-positive or -negative tumor cells were seeded at 5,000 cells per well in a 96-well plate and incubated overnight at 37°C under standard cell culture conditions as described earlier. The fusion proteins were added to the cells at the indicated concentrations to a final volume of 100 μL. After 72 hours, 50 μL of XTT reagent was added as specified by the manufacturer's protocol and cells were further incubated for 2 hours. The absorbance at 450 nm was measured with a HTS 7000 plus microplate reader (PerkinElmer) and cell viability was calculated after subtraction of blanks (wells without cells) as the percentage of living cells in treated wells relative to untreated cells (cells without DARPin-ETA″ toxin).

For competition analysis of specificity, cells were first preincubated for 10 minutes with unconjugated DARPins at the concentrations indicated before Ec4-ETA″ was added and viability was determined as described earlier.

Fluorescence labeling of DARPin-ETA″ fusion proteins with Cy5.5

Ec4-ETA″ and off7-ETA″ were incubated with a 3-fold molar excess of Cy5.5 NHS ester (GE Healthcare) at pH 7.4 for 1.5 hours at room temperature. At this relatively low pH labeling of the N-terminal amino group is favored over labeling of lysine residues at short incubation times and modest excess of dye. The samples were passed over a PD-10 column (GE Healthcare) to remove unreacted dye and

exchange the buffer to 100 mmol/L sodium bicarbonate with 20 mmol/L of NaCl at pH 8. Monolabeled fusion proteins were separated from unlabeled and multiply labeled proteins by anion exchange chromatography on a MonoQ column (GE Healthcare) in 100 mmol/L sodium bicarbonate and 1 mol/L of NaCl at pH 8 with isocratic elution.

Animals and tumor xenografts

For *in vivo* experiments, 8- to 10-week-old female athymic mice (NMRI nu/nu, Harlan Laboratories or CD1 nu/nu, Charles River) were used. Mice were housed and maintained under specific pathogen-free conditions according to the guidelines of the veterinary offices of the Kanton Zürich and Bern. Tumors were raised by subcutaneous injection into the lateral flank of HT29 or SW2 cells (10^7 cells in 100 μL of PBS).

In vivo serum half-life of Ec4-ETA″

Female nude mice were intravenously injected with a single dose of 30 μg Ec4-ETA″ in 100 μL of PBS. Blood samples were collected at different time points (3, 5, 10, 20, 30, and 60 minutes) from the time of the injection. Serum was harvested from the blood samples and the concentration of Ec4-ETA″ was determined by ELISA in comparison to a standard curve of the corresponding pure immunotoxin.

Briefly, microtiter plates were coated with anti-Pseudomonas Exotoxin A antibody (Sigma) diluted 1:2,000 in PBS overnight at 4°C. Plates were blocked with PBS containing 1% BSA for 2 hours at room temperature followed by washing 4 times in PBS containing 0.05% Tween-20. A standard curve was made with pure Ec4-ETA″, whereas serum samples were diluted 1:100 and 1:1,000 in PBS. One hundred microliters of standards or samples were applied and incubated for 1 hour at room temperature. After washing, anti–RGS-His antibody conjugated with horseradish peroxidase (Qiagen) diluted 1:2,000 was incubated for 1 hour at room temperature. After washing, plates were developed using 3,3′-5,5′-tetramethylbenzidine for 15 minutes and the absorbance was measured at 450 nm.

In vivo fluorescence imaging

Ten days after subcutaneous tumor cell injection, mice were intravenously injected with 30 μg of Ec4-ETA″ or off7-ETA″ conjugated with the fluorescent dye Cy5.5 ($n = 3$ for each group). *In vivo* imaging was performed 6, 24, 48, 72, and 96 hours after injection. During imaging mice were anaesthetized by intraperitoneal injection of body-weight–adapted doses of 10% ketamine and 2% xylazine. In addition, 48 hours after intravenous injection, 1 mouse of each group was euthanized and fluorescence images of dissected organs were obtained. Images were acquired using the NightOwl II NC100, Type LB 893, 2006 imaging system (Berthold Technologies) with an exposure time of 60 seconds. For colocalization of the fluorescence image on the animal body, gray scale and pseudocolor images were merged. Quantification of signal intensity in all animals was performed with WinLight32 Software.

Antitumor activity *in vivo*

Mice bearing established HT29 tumors of 50 to 100 mm³ in size were intravenously injected on days 1, 3, and 5 with either 30 μg or 20 μg Ec4-ETA″ or with 30 μg off7-ETA″ in 100 μL of PBS. Treatment with the lower dose (20 μg) of Ec4-ETA″ was repeated on days 9, 13, and 15. Mice bearing established SW2 tumors of 40 to 80 mm³ in size were intravenously injected on days 1, 3, 5, 8, 10, and 12 with 20 μg Ec4-ETA″ or 20 μg off7-ETA″ in 100 μL PBS. For both tumor models mice treated with PBS at the same time points were used as control.

Animals were monitored for tumor growth by caliper measurement of the shortest diameter and the longest perpendicular diameter. Tumor volume was calculated according to the formula: (short diameter)² × (long diameter) × 0.4. Mice were euthanized when tumors reached a volume of 1,500 mm³ or when tumors showed skin ulcerations.

In vivo toxicity determination

During treatment with the fusion toxins animals were controlled daily for weight loss or other signs of toxicity and discomfort (apathy, ungroomed appearance, dehydration, etc.). Liver toxicity was assessed post mortem by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in plasma samples. Blood samples from 4 mice treated with PBS and 4 mice treated with 30 μg Ec4-ETA″ 3 times every second day were collected 24 hours after the final injection. On plasma separation, ALT and AST activities were measured photometrically.

Statistical analysis

All data represent the mean ± SD. Statistical analyses of *in vivo* tumor growth were performed using the Kruskal–Wallis test. A value of $P < 0.05$ was considered statistically significant.

Results

Expression and purification of DARPin-ETA″ fusion proteins

The EpCAM-specific high-affinity DARPin Ec4 (P. Martin-Killias et al., in preparation) and the control DARPins off7 (29) and E3_5 (30) were fused via a $(GSG_4)_2$ linker to a truncated form of *Pseudomonas aeruginosa* exotoxin A (residues Glu252-Pro608, numbering of the mature protein), containing a C-terminal KDEL sequence (denoted $ETA_{252–608}KDEL$ or ETA″) to increase cytotoxicity in mammalian cells (33). For purification and detection, the constructs further contained a His_6 tag at the C terminus in front of the KDEL sequence and an $RGSHis_6$ tag at the N terminus.

All DARPin-ETA″ fusion proteins were expressed in soluble form in *E. coli* at 37°C. The protein yield was up to 40 mg/L of bacterial culture. Purification was achieved using IMAC, which for *in vivo* experiments was followed by extensive Triton X-114 washing, size exclusion chromatography, and an EndoTrap column to remove endotoxin.
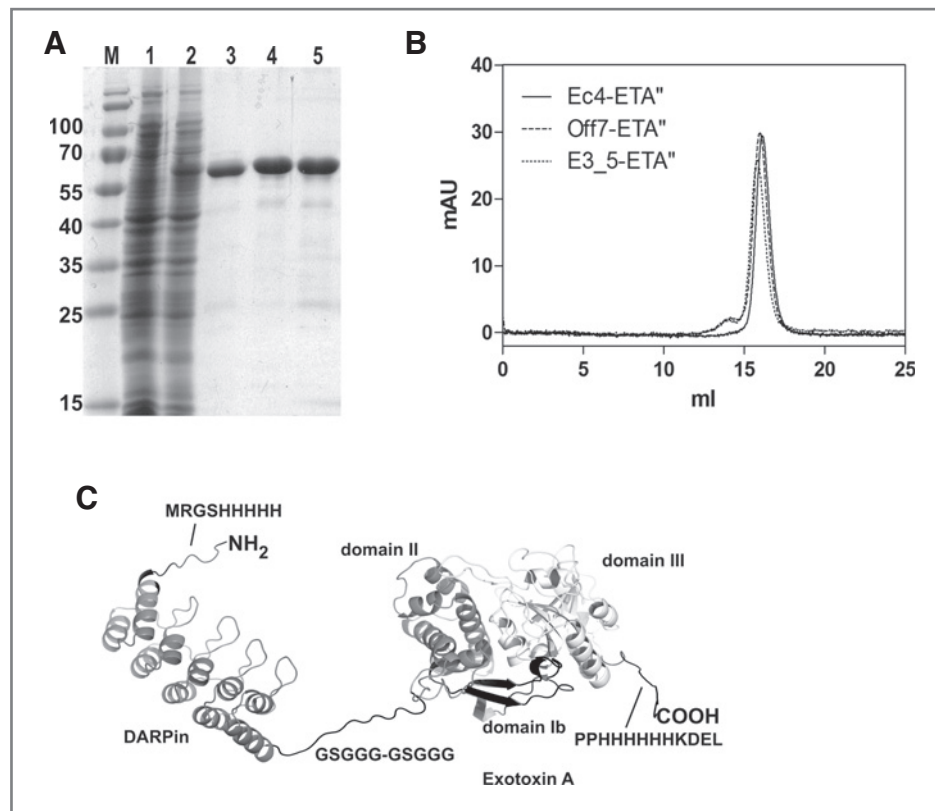
Figure 1. Biochemical characterization of the DARPin-ETA″ fusion toxins. A, for SDS-PAGE analysis, fractions were loaded onto a 10% polyacrylamide gel and proteins were detected by staining with Coomassie brilliant blue: lane M, molecular weight marker (kDa); lane 1, uninduced bacterial cell lysate; lane 2, cell lysate 4 hours after induction; lane 3, purified Ec4-ETA″; lane 4, purified off7-ETA″; and lane 5, purified E3_5-ETA″. B, size exclusion chromatography of DARPin-ETA″ fusion toxins analyzed on a Superdex 200 10/300 GL column. C, model of the DARPin-toxin construct was built on the basis of the X-ray structures of a consensus DARPin (PDB entry 2QYJ) and *Pseudomonas aeruginosa* exotoxin A (ETA; 1IKQ) using InsightII (Accelrys) and the ROSETTA suite programs. The figure shows the DARPin targeting moiety and ETA domains. The flexible N-terminal RGS-His$_6$-tag, the C-terminal His$_6$ tag and KDEL ER-retention signal and the linker connecting the DARPin to the toxin were modeled in order to visualize their sizes relative to those of the protein domains. The figure was generated using the program PyMol (DeLano Scientific LLC).

The fusion toxins showed a band at the predicted molecular weight of approximately 59 kDa when analyzed on SDS-PAGE (Fig. 1A), and size exclusion chromatography revealed a mainly monomeric fraction (Fig. 1B). Figure 1C shows a structural model of Ec4-ETA″. ETA″ contains 2 disulfide bonds, and although the fusion toxins were expressed in the bacterial cytoplasm, and no redox shuffle system was used during purification, quantitative disulfide assays (see Materials and Methods) revealed that more than 90% of the protein had both disulfide bonds formed after purification, most probably by spontaneous air oxidation (data not shown). We conclude from these findings that DARPins are well suited for the easy and cost-effective production of fusion toxins for tumor targeting.

EpCAM-binding affinity of Ec4-ETA″

To investigate whether the fusion to ETA″ impaired the binding activity of DARPin Ec4 to EpCAM, the association (k_a) and dissociation rate constants (k_d) of both Ec4 and Ec4-ETA″ were measured by surface plasmon resonance (Supplementary Fig. S1A and B). The measurements revealed that Ec4 and Ec4-ETA″ have comparable rate constants of associa-

tion (1.1×10^5 and 6.2×10^4 L/mol/s, respectively) and dissociation (1.8×10^{-4} and 1.3×10^{-4} 1/s, respectively), resulting in similar equilibrium dissociation constants of 1.7 and 2.2 nmol/L. In addition, the equilibrium dissociation constant of fluorescently labeled Ec4 to EpCAM-positive MCF-7 cells was measured on a flow cytometer, again from the ratio k_d/k_a. A slightly higher K_D of 5.8 nmol/L was obtained from association ($k_a = 5.5 \times 10^4$ L/mol/s) and dissociation ($k_d = 3.2 \times 10^{-4}$ 1/s) rate constants (Supplementary Fig. S1C and D).

Cytotoxicity of Ec4-ETA″ against various tumor cell lines *in vitro*

The cytotoxic effect of Ec4-ETA″ and the control fusion proteins off7-ETA″ and E3_5-ETA″ on various EpCAM-positive tumor cell lines and EpCAM-negative control cells was determined in colorimetric XTT cell viability assays on a 72-hour incubation. As shown in Fig. 2A, Ec4-ETA″ was potently cytotoxic against all EpCAM-positive cell lines tested, MCF7, SW2, CAL27, and HT29. The IC$_{50}$ values (concentration at which cell viability was reduced by 50%) ranged from less than 0.005 pmol/L to 0.7 pmol/L. In
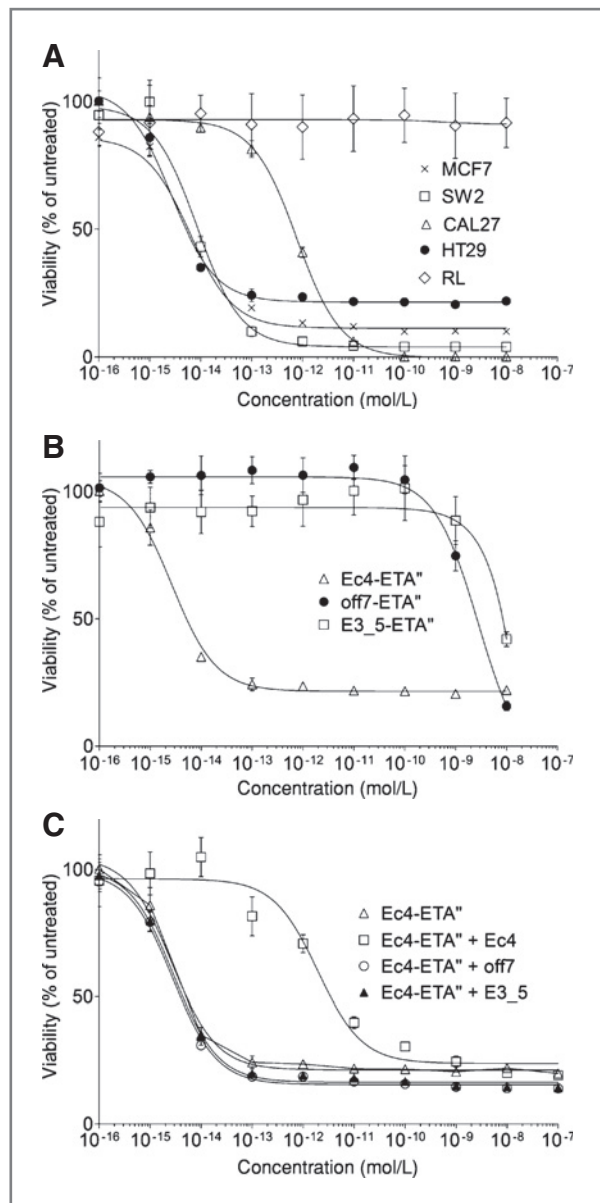
Figure 2. *In vitro* cytotoxicity of Ec4-ETA″ and the control fusion toxins off7-ETA″ and E3_5-ETA″ tested on various tumor cell lines. A, the EpCAM-positive cell lines MCF7, SW2, CAL27, and HT29, and the EpCAM-negative cell line RL, were incubated for 72 hours with different concentrations of Ec4-ETA″ before cell viability was determined in colorimetric XTT assays. B, HT29 cells were incubated with the unspecific fusion proteins E3_5-ETA″ or off7-ETA″ for 72 hours in XTT assays, and viability was compared with cells treated with Ec4-ETA″. C, in competition assays, EpCAM-positive HT29 cells were incubated with DARPin Ec4, E3_5, and off7 (500 nmol/L) 10 minutes before Ec4-ETA″ was added and cell viability determined as described. All determinations were done in triplicates, data represent the mean ± SD.

contrast, the IC_{50} value on EpCAM-negative RL cells was more than 100,000-fold higher (IC_{50} > 10 nmol/L). Similarly, the unspecific fusion proteins off7-ETA″ and E3_5-ETA″ showed cytotoxic effects only at much higher con-

centrations (IC_{50} > 1 nmol/L) than the EpCAM-specific ones when tested on EpCAM-positive HT29 cells (Fig. 2B).

Furthermore, the cytotoxicity of Ec4-ETA″ was markedly decreased when cells were preincubated with an excess of unfused DARPin Ec4 as competitor (Fig. 2C). This decrease was specific for EpCAM blocking, as preincubation with the nonspecific DARPins off7 and E3_5 did not diminish Ec4-ETA″ cytotoxicity. Moreover, the use of the unfused DARPins did not affect cell viability (data not shown). Taken together, these data show that the cytotoxicity is mediated by EpCAM-specific uptake and background cytotoxicity by unspecific uptake of the DARPin-ETA″ fusion proteins was in general very low.
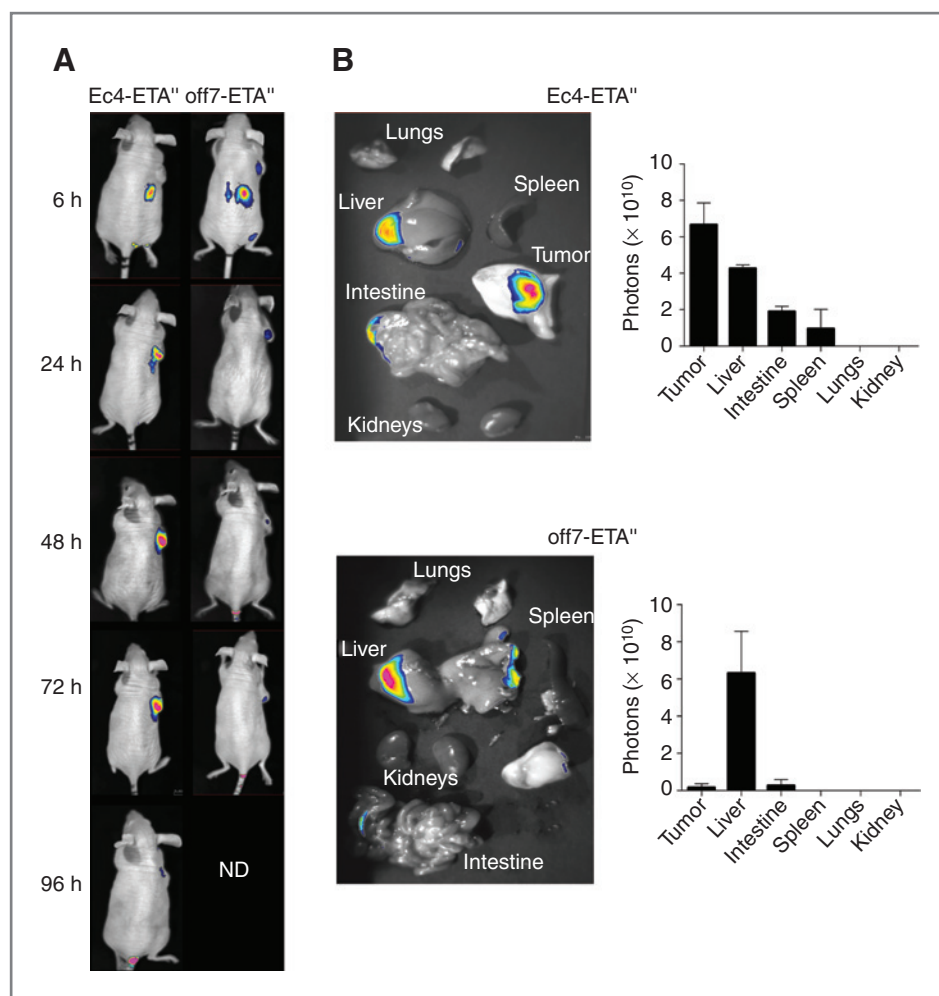
Tumor localization of Ec4-ETA″

To show that Ec4-ETA″ efficiently localizes to tumors upon systemic administration and that this effect is EpCAM-dependent, *in vivo* fluorescence imaging was performed in athymic mice bearing subcutaneous HT29 tumor xenografts. Ec4-ETA″ and off7-ETA″ control were N-terminally labeled with the fluorescent dye Cy5.5 (emission maximum 680 nm). After the coupling reaction, the mono-labeled proteins were purified by anion exchange chromatography to eliminate unlabeled protein, free dye, and other labeled protein species. Mice were injected intravenously with 30 μg Ec4-ETA″_Cy5.5 or off7-ETA″_Cy5.5, and images were taken after 6, 24, 48, 72, and 96 hours using the NightOWL II LB891 imaging system. As shown in Fig. 3A, 6 hours after injection both fusion proteins localized to the lower abdomen, which could be identified as mainly kidney and partially liver accumulation. Ec4-ETA″ efficiently localized to tumors 24 hours after injection, and accumulation peaked between 48 and 72 hours before it declined to background values after 96 hours. In contrast, only very low background fluorescence was detectable in the tumors on injection of the nontargeted fusion protein off7-ETA″.

Furthermore, we performed *ex vivo* analysis of biodistribution in isolated organs and tumors 48 hours after injection of the fluorescently labeled probe using a fiber optic device. As shown in Fig. 3B, similar to whole animal imaging, high fluorescence activity in the tumor was detected only in mice injected with Ec4-ETA″ but not off7-ETA″. Probably as a consequence of metabolic degradation, both fusion proteins also showed localization in parts of the liver and at low levels in the intestine. These signals, however, were covered by overlaying tissue and thus undetectable by *in vivo* imaging.

Serum half-life of Ec4-ETA″ in mice

To determine the serum half-life of Ec4-ETA″, mice were injected with a single dose of 30 μg and blood was collected at intervals between 3 and 60 minutes. The concentration of Ec4-ETA″ in mouse serum was measured by sandwich ELISA. Each data point used to determine half-life represents the average of samples of 2 to 4 mice. Data were fit to a single exponential decay function with plateau (Supplementary Fig. S2) and the serum half-life of Ec4-ETA″ was determined as 11.2 minutes.

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Figure 3. Tumor localization and organ distribution of Cy5.5-labeled Ec4-ETA″ and the off7-ETA″ control fusion toxin detected by *in vivo* fluorescence imaging. Mice bearing subcutaneous HT29 tumor xenografts were intravenously injected with 30 μg of Ec4-ETA″ or off7-ETA″ conjugated with the fluorescent dye Cy5.5 ($n = 3$ for each group). A, *in vivo* images were acquired 6, 24, 48, 72, and 96 hours after injection using the NightOWL II LB891 imaging system with an exposure time of 60 seconds. For colocalization of the fluorescence image on the animal body, gray scale and pseudocolor images were merged. ND, not determined. B, *ex vivo* analysis of fluorescence intensities from isolated tumors and organs 48 hours after injection of Cy5.5-labeled Ec4-ETA″ or off7-ETA″ using a fiber optic device. Organs of 1 representative mouse of each group are shown. Data are plotted as the average of 2 mice; bars, SD.

Antitumor effect of Ec4-ETA″

To investigate how the favorable tumor localization of Ec4-ETA″ translates into therapeutic efficacy, its antitumor effect was evaluated in athymic mice bearing established subcutaneous tumor xenografts. As a first tumor model, we used the cell line HT29. In 1 group, mice received 3 doses of 30 μg Ec4-ETA″ (Ec4-ETA″ 30/3) on days 1, 3, and 5. In a second group, 6 doses of 20 μg Ec4-ETA″ (Ec4-ETA″ 20/6) were administered on days 1, 3, 5, 9, 13, and 15. Control mice received PBS or 3 doses of 30 μg off7-ETA″ (off7-ETA″ 30/3) on days 1, 3, and 5.

As shown in Fig. 4A, tumors of control mice treated with PBS grew rapidly until the end of the observation period. In contrast, in all mice treated with Ec4-ETA″ tumor growth was strongly inhibited. Tumors of mice treated with the Ec4-ETA″ 30/3 schedule almost completely disappeared after the last injection, but started to regrow when treatment was discontinued. Nonetheless, in this group, 2 of 11 mice (18%) showed complete regression, defined as nondetectable tumor or no tumor regrowth for more than 45 days. The antitumor effect of the Ec4-ETA″ 20/6 schedule

was even more pronounced, resulting in 2 of 5 mice (40%) with complete regressions. Lower doses of Ec4-ETA″ (5 μg) did not result in significant tumor responses (data not shown). Treatment with the control fusion protein off7-ETA″ 30/3 had no effect on tumor growth compared with mice treated with PBS. To better discriminate between the response rates in the various groups, Kaplan–Meier curves were plotted with an endpoint defined as a tumor size more than 100 mm³. As shown in Fig. 4B, all control mice treated with PBS or off7-ETA″ developed tumors more than 100 mm³ already 9 days after the start of treatment. At this time point, all mice in the Ec4-ETA″-treated group had tumors clearly less than this size. On day 31, 18% of mice treated with Ec4-ETA″ 30/3 and 60% of mice treated with Ec4-ETA″ 20/6 still showed tumors less than 100 mm³, indicating that treatment with the Ec4-ETA″ 20/6 schedule was more effective in tumor control than with Ec4-ETA″ 30/3. At the end of the experiment (day 31), the average size of tumors was significantly reduced from $1,005 \pm 275$ mm³ (PBS group) to 188 ± 125 mm³ in the Ec4-ETA″ 30/3 treated group ($P < 0.05$) and to 89 ± 87 mm³ in the Ec4-ETA″ 20/6

Figure 4. Antitumor effect of Ec4-ETA″ in athymic mice. A, mice bearing subcutaneously growing HT29 tumor xenografts of 50 to 100 mm³ in size received tail-vein injections of 3 × 30 μg Ec4-ETA″, 3 × 30 μg off7-ETA″ control, 6 × 20 μg Ec4-ETA″, or PBS as vehicle control. Tumor growth was monitored by caliper measurement during the course of 31 days. Data represent the mean tumor volume ± SD of 5 to 11 mice per group. B, Kaplan–Meier survival curves with an endpoint defined as tumor volume of 100 mm³. The curves show the percentage of treated mice in each group in which tumors did not exceed 100 mm³ in size at the various time points after the start of treatment. C, mice bearing subcutaneously growing SW2 tumor xenografts of 40 to 80 mm³ in size received tail-vein injections of 6 × 20 μg Ec4-ETA″, 6 × 20 μg off7-ETA″ control, or PBS as vehicle control. Tumor growth was monitored by caliper measurement during the course of 31 days. Data represent the mean tumor volume ± SD of 5 to 6 mice per group. D, pictures of representative tumors isolated from mice of the different treatment groups.
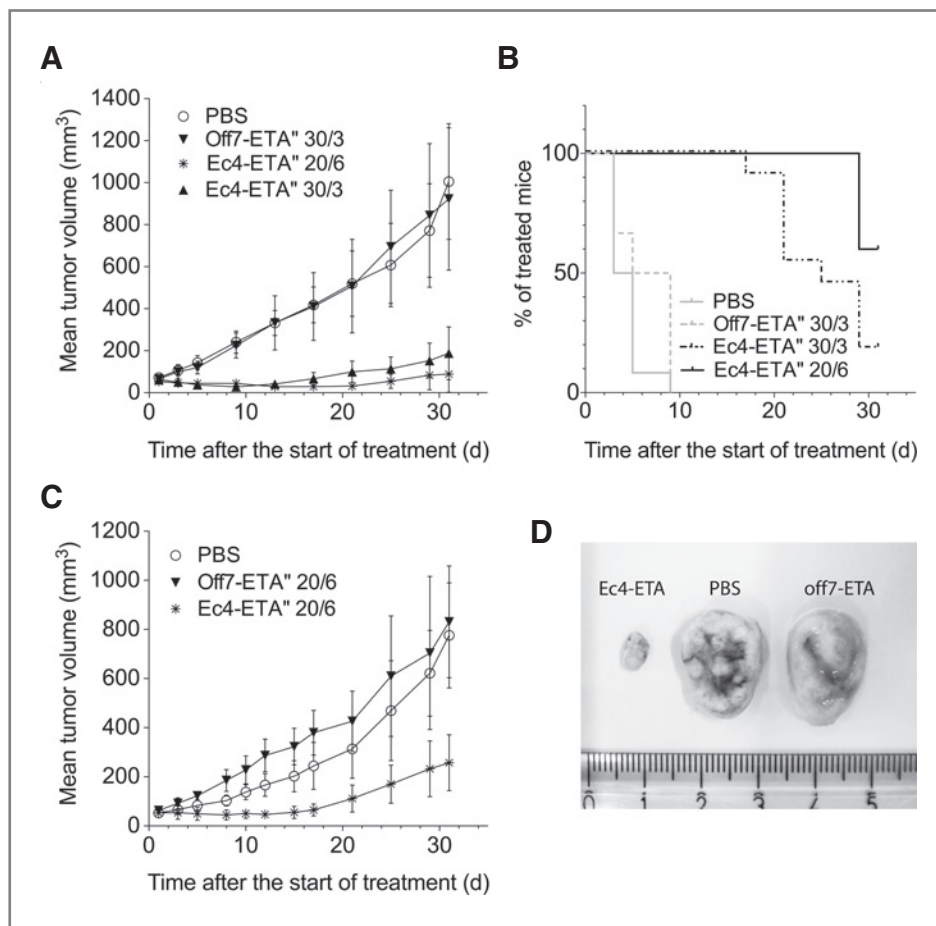
treated group ($P < 0.05$). This reflects a reduction in tumor volume of 81% and 91%, respectively. The favorable effect of the Ec4-ETA″ 20/6 schedule, which delivered a higher overall dose but without increased toxicity, is also reflected by the 40% complete regressions (as shown earlier). The high standard deviations observed for Ec4-ETA″ are due to the fact that some mice showed complete regressions. No difference in tumor size was found in mice treated with off7-ETA″ 30/3 compared with PBS.

We confirmed the antitumor activity of Ec4-ETA″ in a SW2 small cell lung carcinoma xenograft model, which shows a slower growth rate and is known to form a very solid tumor mass. Mice bearing subcutaneous SW2 tumors were treated with 6 doses of 20 μg Ec4-ETA or off7-ETA″ on days 1, 3, 5, 8, 10, and 12. As shown in Fig. 4C, treatment with Ec4-ETA″ decreased the tumor size compared with controls (PBS and off7-ETA). On day 31, mice treated with Ec4-ETA″ had an average tumor size of 257 ± 114 mm³ whereas mice treated with off7-ETA″ and PBS already reached tumor sizes of 856 ± 198 mm³ and 776 ± 214 mm³, respectively. There was thus a significant reduction in tumor volume of treated compared with PBS control mice ($P < 0.05$), although the effect was slightly less pronounced than on faster growing HT29 tumors.

Toxicity of Ec4-ETA″

To determine treatment-related unspecific toxicity on Ec4-ETA″ and off7-ETA″ administration, mice were monitored for weight loss, dehydration, and signs of distress (apathy, hyperalgesia, and ungroomed appearance) throughout the course of the study. Representative for all therapy experiments, Fig. 5 shows for HT29 tumor-bearing mice that treatments were well tolerated and after reversible marginal weight loss after the third injection of Ec4-ETA″ 30/3 no further signs of toxicity were observed. Furthermore, to assess liver toxicity as a frequent dose-limiting side effect of ETA fusion toxin therapy in patients, blood from 4 PBS-treated mice and 4 mice receiving 3 doses of 30 μg Ec4-ETA″ was collected and analyzed for activity of the liver transaminases AST and ALT. As shown in Table 1, liver toxicity could be excluded for this treatment schedule, as there was no significant elevation of ALT and AST activity in the plasma of Ec4-ETA″-treated (ALT: 54 ± 22 U/L, and AST: 141 ± 47 U/L) compared with PBS-treated mice (ALT: 57 ± 51 U/L, and AST: 99 ± 53 U/L).

Discussion

Chemotherapy still has remained the mainstay of cancer therapy. The great majority of approved treatments has no
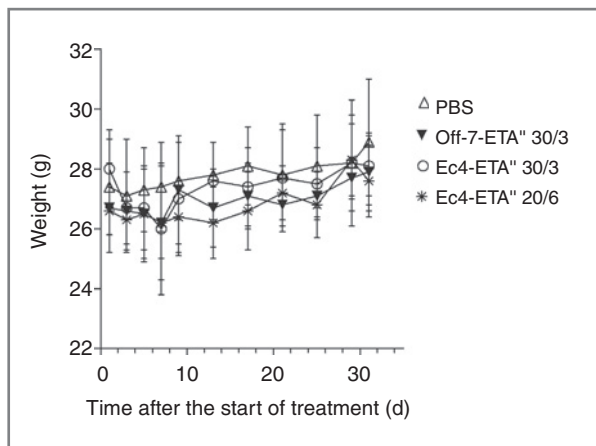
Figure 5. Average weight of mice on treatment with different dose schedules of Ec4-ETA″, or off-7-ETA″ or with PBS control. Animals were weighed 3 times per week during the whole experiment to monitor treatment-related toxicity.

inherent specificity for tumor cells but attack all dividing cells. More recently many investigations have been carried out in which a toxic principle was coupled to a recognition function. Immunotoxins based on *Pseudomonas aeruginosa* exotoxin A (ETA, also termed PE), particularly its N-terminally truncated variant carrying a C-terminal KDEL peptide (ETA″), have been generated against various cell surface receptors and extensively tested in preclinical and early clinical studies (5, 34). Most of them use a single-chain fragment (scFv) or a disulfide-stabilized dsFv fragment of an antibody as targeting moiety. We previously also reported on the potent antitumor effect of an EpCAM-specific scFv-ETA″ immunotoxin (11), which is currently under phase II clinical investigation (9).

Commonly used antibody Fv-based formats, however, are difficult to produce in high amounts when compared with other proteins and are often aggregation-prone (24, 26, 35). ScFv and dsFv fragments used for tumor-targeted fusion proteins must either be expressed in the periplasm or refolded after expression in inclusion bodies. Here, we investigated another class of highly advanced binding molecules, DARPins, for targeted delivery of ETA″ to tumor cells *in vitro* and *in vivo*. DARPins can be selected for high affinity, which is a major requirement for efficient tumor targeting (36), their inherent robustness allows easy conjugation with various types of effector molecules, and pharmacology and tumor targeting properties can be easily modulated, for example, by site-specific PEGylation. Therefore, we are not limited to the simple fusions described here, as the molecules are robust enough to allow efficient production of more complicated constructs. As shown recently, the possibility to produce DARPins in functional form in multigram amounts by fermentation of *E. coli* and their easy purification can significantly reduce the time from preclinical development to clinical investigation (http://clinicaltrials.gov/ct2/show/NCT01042678, http://clinicaltrials.gov/ct2/show/NCT01086761).

We are expressing the DARPin-ETA″ fusions in the cytoplasm of *E. coli* and find that these fusion proteins are produced in soluble form. Even though the ETA″ part contains 2 disulfide bonds, which appear to be beneficial in the internalization process (37), we find in the purified protein that they have been formed almost quantitatively, possibly by air oxidation. Thus, we can use the convenient production of DARPins in the *E. coli* cytoplasm also with the DARPin-ETA″ fusion proteins.

A major challenge for the application of immunotoxins is the choice of targets that provide sufficient tumor specificity and, at the same time, promote intracellular delivery of the payload. Several targets have been explored, which serve this purpose, for example, EGFR, LewisY, and various hematopoietic markers (2). Most tumor-targeting with fusion toxins has been studied in hematopoietic malignancies, but even there, only 1 (denileukin diftitox, Ontak), a fusion of IL-2 with diphtheria toxin, finally received FDA approval (38).

EpCAM is overexpressed in many solid tumors and on the basolateral cell surfaces of some normal epithelia (15, 18) where it is, however, poorly accessible to circulating anti-EpCAM antibodies (17). Because it also occurs on normal hepatic stem cells and hepatoblasts, EpCAM-targeted immunotherapy may in principle impair liver regeneration. However, liver toxicity has never been reported in clinical trials with anti-EpCAM antibodies, probably because the regenerative capacity of the adult liver is sufficiently maintained by mitotic division of mature hepatocytes (39). In contrast, the inherent liver toxicity of ETA is based on inflammatory responses caused by liver macrophages, independent on specific hepatocyte targeting (40).

Recently, EpCAM was also identified as a marker of cancer initiating cells in colon (21), breast (22), pancreatic (23), and hepatocellular carcinomas (24). Because cancer stem cells respond poorly to standard therapy and thus are largely responsible for treatment failure, their elimination must be a prime objective for all kinds of innovative cancer therapy. Thus, from a therapeutic point of view, EpCAM is particularly interesting as a docking site for targeting ligands delivering external effector molecules. In fact, EpCAM efficiently mediates internalization of bound ligands by receptor-mediated endocytosis and thus perfectly matches the need of anticancer agents acting on intracellular targets such as protein toxins, chemotherapeutic agents, and antisense compounds (11–13).

Recently, we have described for the first time the production and biochemical characterization of EpCAM-specific DARPins, and a first generation binder was used to successfully deliver therapeutic siRNA into tumor cells in the form of mono- and multivalent binders fused to highly charged protamine (14). Subsequent affinity maturation efforts then resulted in DARPin Ec4, which displayed affinity to EpCAM in the low nanomolar range and excellent biophysical properties (P. Martin-Killias et al., in preparation).

We measured efficient tumor localization of Ec4-ETA″, but not of the control fusion off7-ETA″, by *in vivo* fluorescence imaging upon systemic administration in a HT29 colon carcinoma xenograft model. These data

Table 1. Liver transaminase activity in the plasma from mice treated with Ec4-ETA″ or PBS

| | Treatment[a] | | | | | | | |
| | Ec4-ETA″ | | | | PBS | | | |
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 1	Mouse 2	Mouse 3	Mouse 4
ALT U/L	34	84	57	41	134	40	26	29
AST U/L	141	200	138	85	177	62	86	70

[a]Mice received 3 × 30 μg of Ec4-ETA″ or PBS intravenously. Activity of the transaminases was measured 24 hours after the final injection.

showed that localization in the tumor was specific and dependent on EpCAM binding with peak tumor accumulation 48 to 72 hours after injection.

Fluorescence imaging records the distribution of molecules at any particular time point, whereas the previously used residualizing label $^{99m}Tc(CO)_3$ (36) gives an integral localization since the beginning of injection, because any label will accumulate at the site of cellular internalization. This probably accounts for the difference in kidney accumulation, which is seen to decay rapidly when measured by fluorescence imaging but not by $^{99m}Tc(CO)_3$ radioactivity. Although immunotoxins (scFv fragments or DARPins fused to a protein toxin such as ETA) would be expected to not fall in an ideal molecular weight range for maximum accumulation (36), we see very encouraging enrichments and therapeutic effects already with the constructs described here, and there may be even better effects with different molecular formats.

In this study, Ec4 was fused with ETA″ to assess for the first time the potential of a rationally engineered DARPin for tumor targeting and therapy. The fusion protein could indeed be well produced in soluble form in *E. coli* at approximately 80-fold higher yield from shake flasks than our previously described scFv-ETA″ fusion toxin (4D5MOCB-ETA; ref 11), could be easily purified and was stable, and resistant to aggregation. This will facilitate subsequent scaling up of the production process required for clinical trials.

We found that the high-affinity binding of Ec4 to intact cells was fully preserved on fusion with ETA″ and that Ec4-ETA″ showed extremely high and specific *in vitro* cytotoxicity against EpCAM-positive tumor cells of various histotypes with IC_{50} values of 0.005 pmol/L. This is remarkably low compared with other immunotoxins (41–43) and likely mirrors its high stability, affinity, and efficient internalization by receptor-mediated endocytosis. These data, combined with efficient tumor targeting, indeed translated into potent antitumor effects at well-tolerated doses with some mice showing complete regression of their tumors. The higher overall dose administered as 6 × 20 μg could prove more effective than the lower dose schedule (3 × 30 μg). Probably due to the short half-life of the DARPin-ETA″ fusion toxin in the circulation, which may limit tumor accumulation and also toxicity to normal tissues, the dose required to achieve tumor responses was higher than for a previously described scFv-ETA″ immunotoxin (11). The repeatable response measured after repeated injections further suggests that tumors retained a stable EpCAM expression profile and that antigen loss did not occur during treatment.

A phase II study with this previously reported EpCAM-specific immunotoxin (11) consisting of ETA″ fused to a scFv antibody is ongoing and will be completed soon (9). On the basis of the findings of this study, it is tempting to speculate that DARPins such as Ec4 can replace the antibody fragment as cell binding ligands also in forthcoming generations of tumor-targeted fusion toxins and other drug delivery systems.

In summary, we describe for the first time the generation and preclinical evaluation of an EpCAM-specific fusion toxin consisting of a high-affinity DARPin (Ec4) and ETA″ as a catalytic biotoxin. We provide evidence for its potent activity against various tumor cell types *in vitro*, and its favorable tumor localization and antitumor activity *in vivo*. The advantages of DARPins enabling high yield expression, resistance against aggregation, and stability also in the form of fusion toxins in conjunction with a tumor-associated target such as EpCAM, opens new avenues for the generation of rationally designed protein therapeutics with outstanding efficacy.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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Clinical Cancer Research

Correction: A Novel Fusion Toxin Derived from an EpCAM-Specific Designed Ankyrin Repeat Protein Has Potent Antitumor Activity

In this article (Clin Cancer Res 2011;17:100–10), which was published in the January 1, 2011 issue of *Clinical Cancer Research* (1), the name of the first author appeared incorrectly on the online journal. The correct name of the first author is Patricia Martin-Killias. This error has been corrected.

Reference

1. Martin-Killias P, Stefan N, Rothschild S, Plückthun A, Zangemeister-Wittke U. A novel fusion toxin derived from an EpCAM-specific designed ankyrin repeat protein has potent antitumor activity. Clin Cancer Res 2011;17:100–10.

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