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Tumor-Targeting Antibody–Anticalin Fusion Proteins for *in Vivo* Pretargeting Applications

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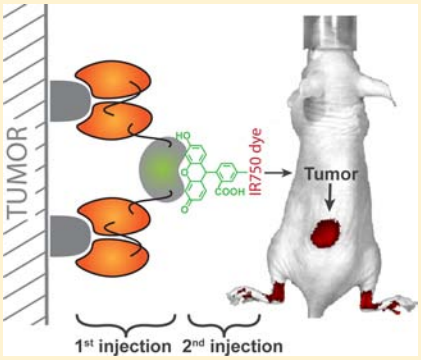
Supporting Information

ABSTRACT: Pretargeting approaches rely on the injection of bispecific antibodies capable of recognizing both an accessible disease marker and a small ligand, which is typically administered at a later stage and which serves as delivery vehicle for a payload for imaging or therapy applications. In the oncology field, pretargeting strategies have exhibited extremely promising biodistribution results and *in vivo* selectivity, but have often relied on the cumbersome preparation of multispecific antibodies by chemical conjugation techniques. Here, we describe the design, production, and characterization of a novel class of bispecific multivalent antibody products, which contain both antibody fragments and an anticalin moiety for the simultaneous recognition of tumor-associated antigens and a small organic molecule. Anticalins are derivatives of the naturally occurring binding proteins lipocalins, which have been engineered to recognize a target molecule with high affinity. In particular, we produced and compared *in vitro* and *in vivo* different fusion proteins, which contained the anticalin FluA that selectively recognizes various different fluorescein derivatives and the F8 antibody specific to the alternatively spliced EDA domain of fibronectin (a marker of tumor angiogenesis). The selective accumulation of the most promising fusion-protein scFv(F8)-FluA-scFv(F8) on solid tumors and simultaneous binding of fluorescein derivatives could be visualized *in vivo* using a fluorescein-near-infrared fluorescent dye conjugate, confirming the potential of antibody-anticalin fusion proteins for pretargeting applications.



INTRODUCTION

Monoclonal antibodies specific to accessible disease markers are increasingly being used as delivery vehicles for imaging[1] and therapeutic applications.[2] However, in spite of their exquisite specificity for target recognition and high binding affinity, intact antibodies exhibit long circulatory half-lives and slow extravasation and diffusion into tissues, resulting in a selective accumulation at sites of disease only at late time points and often with modest target-to-background ratios. Antibody fragments and other smaller binding protein scaffolds exhibit faster blood clearance, but usually result in a less efficient disease targeting since extravasation remains slow.[3−5] It has been shown that many small molecules possess the ability to rapidly diffuse into tissues. As a consequence, it would be attractive to use small organic ligands for pharmacodelivery applications.[6−8] However, unlike antibodies, it is typically difficult to identify small molecules that bind with high affinity to accessible target proteins of biomedical interest.

The pretargeting approach typically features the use of multispecific antibody products, which can simultaneously engage in the binding interaction with a marker of disease and with a small molecule, serving as a contrast agent or toxic payload. The antibody product is administered first and allowed to localize at site of disease. Due to its small molecular weight, the ligand, which is injected at a later time point, is rapidly excreted from the body, but is in part selectively retained at the site of disease due to its interaction with the multispecific antibody. This pretargeting strategy has been successfully used for the imaging and radionuclide-based therapy of tumors.[9−11]

Similarly, antibody-(strept)avidin complexes have been used in pretargeting applications, featuring the use of biotin-chelator conjugates for the selective accumulation of radionuclides at sites of cancer[12,13] or at sites of inflammation.[14]

In this article, we describe the engineering and characterization of a novel class of antibody-based fusion proteins for pretargeting applications, which rely on the use of anticalins as small monomeric binding proteins to small organic ligands.[15] Anticalins are ligand-binding proteins derived from the natural lipocalin scaffold, which can be engineered by combinatorial mutagenesis in order to alter its original binding properties and subsequent selection by *in vitro* display.[15,16] For example, PRS-050, an anticalin compound targeting vascular endothelial growth factor (VEGF), has completed phase I clinical trials in 2011 and has proven to be safe and well-tolerated for applications in patients (*ClinicalTrials.gov* Identifier NCT01141257).[15]

Due to their single polypeptide chain and small and robust structure, anticalins are ideally suited for making bispecific-

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binding constructs by fusion with antibody fragments. We focused on the use of "FluA", an anticalin which binds to a broad range of fluorescein derivatives with low dissociation constants. FluA was generated by selection from a large combinatorial library of mutants of the bilin-binding protein, a lipocalin from *Pieris brassicae*.[17] Fluorescein binds into a deep cavity inside the eight-stranded antiparallel β-barrel of FluA, and its fluorescence is quenched by the interaction with residue Trp 129.[18,19] Drawing information from a crystal structure of the FluA-fluorescein complex,[18] the affinity of the anticalin toward fluorescein was further improved by rational protein design. Suboptimal interactions between FluA residues and fluorescein were eliminated, yielding the fluorescein binding protein FluA(R95K, A45I, S114R) with an affinity in the low nanomolar range.[19]

We designed and produced fusion proteins consisting of the F8 antibody (specific to the alternatively spliced EDA domain of fibronectin, a marker of tumor angiogenesis[20,21]) and FluA(R95K, A45I, S114R) [hereafter termed "FluA"], arranged in three different molecular formats. The F8 antibody binds with comparable affinity to the human and murine EDA of fibronectin,[22] and localizes with high selectivity on solid tumors in mice.[23−25]

We found that antibody-anticalin fusion proteins can be expressed and purified to homogeneity in various domain arrangements, retaining full binding activities of both the F8 and FluA moieties. We also confirmed that radioiodinated preparations of F8-FluA can selectively localize on solid tumors, following intravenous administration, and that the fusion protein can be targeted *in vivo* by fluorescein derivatives.

EXPERIMENTAL PROCEDURES

Cell Lines and Mouse Tumor Model. CHO-S cells (Invitrogen, Switzerland) were cultured in suspension in PowerCHO-2CD (Lonza, Switzerland), containing 8 mM Ultraglutamine, HT supplement (Lonza, Switzerland), at 37 °C and 180 rpm. Adherent CHO-S cells were were cultured in RPMI 1640 (Gibco, Switzerland) containing 10% FCS (Invitrogen) and 2 mM Ultraglutamine (Lonza, Switzerland). The murine teratocarcinoma F9 cell line (CRL-1720, ATCC) was cultured as described before.[26] Female Balb/c nude mice were obtained from Charles River (Germany). *In vivo* experiments were performed under project licenses granted by the Veterinäramt des Kantons Zürich, Switzerland (169/2008 and 42/2012). Twenty-five million tumor cells were injected subcutaneously and tumors grown for 4−6 days prior to the experiment.

Cloning and Protein Expression. The protein sequence of FluA (R95K, A45I, S114R)[19] was reverse translated (www.bioinformatics.org) with codon usage optimized for expression in Chinese hamster cells. The synthetic gene was obtained from DNA2.0 and cloned into pcDNA3.1(+) (Invitrogen) in arrangement with the a secretion peptide and the F8 antibody in the scFv[27] or diabody format[22] as previously described. The heavy (V_H) and light (V_L) chain variable domains of the antibody and the FluA domain are linked by a flexible amino acid sequence V_H-SGGSG-V_L-GGGSGGGGSG-FluA for FluA-Db(F8)-FluA, V_H-GGASGAGGSG-FluA-GAGSGGAGSG-V_L V_H(F8)-FluA-V_L(F8), and V_H-GGGGSGGGGSGGGG-V_L-FluA-V_H-GGGGSGGGGSGGGG-V_L for scFv(F8)-FluA-scFv-(F8).

The fusion proteins were expressed using transient gene expression in CHO-S cells[26] and purified by protein A chromatography. F8 binds to protein A since its V_H belongs to the V_H3 family, which is recognized by protein A.[28] The purified proteins were analyzed by SDS-PAGE, size exclusion chromatography (Superdex200 10/300GL, GE Healthcare), and Biacore on an EDA antigen-coated CM5 sensor chip. Monoclonal cell cultures of stably transfected CHO cells were selected by FACS cell sorting method as described by Zuberbuhler et al.[29]

The stability of the fusion proteins at different storage temperatures over time was analyzed by SDS-PAGE and size exclusion chromatography (Supporting Information Figure 1).

Isothermal Titration Calorimetry and Fluorescence Titration. Isothermal titration calorimetry (ITC) measurements were performed using a VP-ITC instrument (Microcal). The fusion proteins were dialyzed against phosphate buffered saline (PBS) pH 7.4, set to a concentration of 2 μM, and titrated with a solution of fluorescein dissolved in the dialysis buffer at a concentration of 17 μM at 30 °C. Typically, titrations were performed until a ratio of 1:1 of ligand to binding sites on protein was reached. The concentration of binding molecule was determined by absorption measurement at 495 nm. The resulting titration curves were processed and fitted with the *Origin 7* software (Microcal) to obtain K_d values.

Fluorescence measurements were carried out on a microplate reader (Polarion, Tecan). In a black 384 well plate (Greiner), the fusion proteins at varying concentrations between 0 and 12 μM in PBS were prepared. Upon addition of 0.5 μM fluorescein and equilibration in the dark, the ligand fluorescence was excited at 494 nm and emission was detected at 521 nm. The data were fitted by nonlinear least-squares regression using *Kaleida Graph v 4.1.2* (Synergy Software).

Biodistribution Studies. The *in vivo* targeting performance was assessed by quantitative biodistribution studies as described before.[26] Fusion proteins (20 μg) radioiodinated with ^{125}I were injected into the tail vein of tumor-bearing Balb/c nude mice (5 mice per group). Mice were sacrificed 24 h after injection.

Immunoreactivity of the labeled protein was confirmed by analyzing the retention of radiodinated proteins on EDA coupled to CNBr-activated sepharose (GE Healthcare) as described previously[26] (data not shown).

Immunoreactivity of Fusion Proteins and Dye Conjugates. EDA-sepharose (1 mL) was filled into columns and equilibrated with PBS. Fusion protein (0.1 mg) labeled with Alexa Fluor 546-NHS ester (Invitrogen), 1 nmol fluorescein, 1 nmol Alexa Fluor 546-NHS quenched with 10 equiv Tris-HCl, 1 nmol Fluo-Alexa Fluor 546 (synthesis described in Supporting Information) or 0.1 mg unlabeled fusion protein followed by 1 nmol Fluo-Alexa Fluor 546 were applied to the resin. After washing with 500 μL PBS, the columns were photographed and imaged under UV light.

Imaging Studies. The synthesis of Fluo-IR750 and Boc-IR750 is described in the Supporting Information. For imaging studies, mice received 5 nmol of the pretargeting protein scFv(F8)-FluA-scFv(F8) in PBS intravenously on day 4 after tumor cell injection. After 24 h, 5 nmol Boc-IR750 or Fluo-IR750 was administered intravenously in 5% DMSO/PBS. Mice were anesthetized with 2% isoflurane and positioned inside an IVIS Spectrum (Xenogen, Caliper Life Sciences) on their ventral side. The imaging parameters were as follows: λ_{ex} = 745 nm, λ_{em} = 800 nm, exposure time = 1 s, f/stop = 2, small binning. Measurements were repeated at 0 h, 1 h, 2 h, 4 h, 8 h and 10 h upon dye administration.

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RESULTS

Figure 1 depicts a schematic representation of how a disease-targeting antibody (e.g., F8), fused to FluA, could be used for tumor-targeting applications, using fluorescein as a small organic ligand for the delivery of contrast agents (e.g., fluorophores or radionuclides) or therapeutic payloads (e.g., a cytotoxic drug or a photosensitizer). In order to practically implement F8-FluA pretargeting strategies, we explored three different molecular formats, in which the V_H and V_L domains of the F8 antibody were sequentially fused with a FluA moiety, resulting in fusion proteins with two antigen binding sites and one or two FluA molecules (Figure 2). More specifically, we used (i) the F8 antibody in diabody format, fusing FluA at the C-terminal end of the polypeptide (owing to the short amino acid linker between V_H and V_L, the diabody structure gives rise to a stable noncovalent homodimer) (FluA-Db(F8)-FluA; Figure 2a); (ii) the FluA moiety as a linker between the V_H and V_L domains of the F8 antibody, resulting in the monomeric structure V_H(F8)-FluA-V_L(F8); Figure 2b, (iii) a sequential fusion of scFv(F8), FluA, and scFv(F8), resulting in a single polypeptide with two F8 binding sites and one FluA moiety scFv(F8)-FluA-scFv(F8) (Figure 2c).

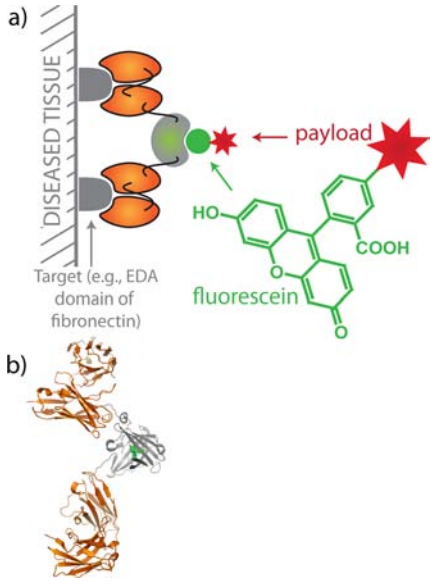

Figure 1. (a) Schematic representation of the pretargeting system with the FluA-F8 fusion protein targeting the tumor associated antigen EDA, and a fluorescein−payload conjugate homing to the fluorescein binding site of FluA. (b) Possible structural arrangement of a two scFv antibody fragments (PDB 1DZB, orange) and the anticalin FluA-fluorescein complex (PDB 1N0S, protein in gray, fluorescein in green). Please note the difference in size between the protein moieties and the fluorescein molecule.

The three fusion proteins were cloned and expressed in mammalian cells. From the culture supernatants, the F8-FluA products were purified to homogeneity using Protein A chromatography. The purified proteins had the expected dimension both in SDS-PAGE analysis and in gel-filtration (Figure 2). Inspection of the Biacore binding profiles to immobilized EDA domain of fibronectin revealed a high functional affinity for FluA-Db(F8)-FluA and scFv(F8)-FluA-scFv(F8), but a slightly lower kinetic stability for the V_H(F8)-FluA-V_L(F8) format. For this reason, we continued our investigations with the bivalent formats. It has to be noted that the functional affinity of the F8 antibody toward its cognate antigen, present at high concentrations in the subendothelial extracellular matrix, is likely to be higher than in solution, due to avidity effects.

The affinity of the FluA moiety of the fusion proteins toward fluorescein was studied by fluorescence quenching and isothermal titration calorimetry (ITC). Quenching revealed

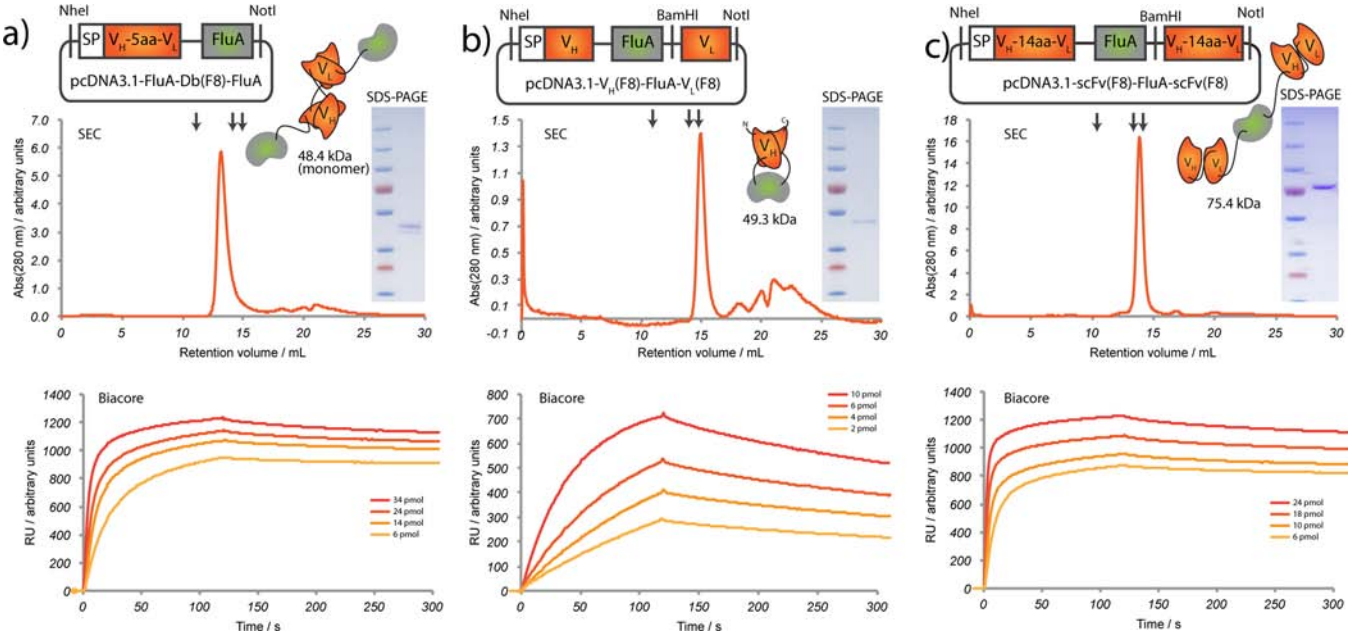

Figure 2. Characterization of the fusion proteins (a) FluA-Db(F8)-FluA, (b) VH(F8)-FluA-VL(F8), and (c) scFv(F8)- FluA-scFv(F8). The cloning scheme of the fusion proteins is shown as well as a schematic representation of the folded proteins. The fusion proteins were analyzed by size-exclusion chromatography (SEC; arrows indicate standard proteins at 11 mL, Ferritin 440 kDa; 14.1 mL, BSA 67 kDa; 15.4 mL, β-lactoglobulin 35 kDa), SDS PAGE (molecular mass marker with bands from top: 185, 115, 80, 65 (red), 50, 30, 25 (red), 15 and 10 kDa) and Biacore.

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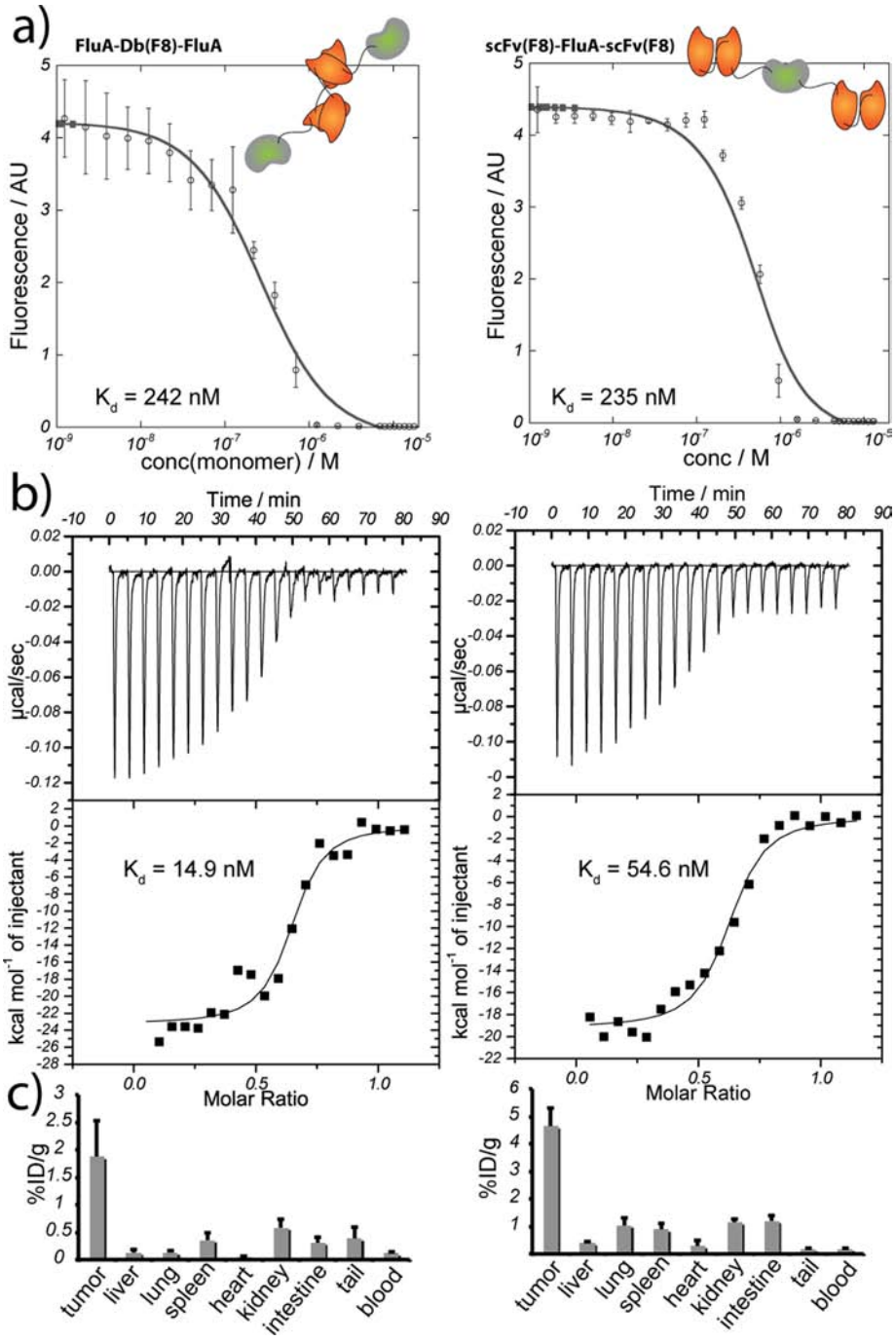

Figure 3. Characterization of the binding affinity of the FluA moiety of FluA-Db(F8)-FluA (left) and scFv(F8)-FluA-scFv(F8) (right) toward fluorescein by (a) quenching of ligand fluorescence upon binding and (b) isothermal titration calorimetry. (c) Quantitative biodistribution shows selective accumulation of the fusion proteins in the tumor.

high nanomolar dissociation constants (Figure 3a), which is in line with the results from ITC (Figure 3b). A radioiodinated preparation of FluA-Db(F8)-FluA and scFv(F8)-FluA-scFv-(F8) was used for a quantitative biodistribution analysis in athymic mice bearing subcutaneously grafted murine F9 teratocarcinoma (Figure 3c). Based on the results of the biodistribution 24 h after i.v. injection of the radiolabeled fusion, we continued our investigations with scFv(F8)-FluA-scFv(F8), which gave the highest tumor accumulation values. The stability of this fusion protein under storage conditions and at 37 °C was studied by SDS PAGE and gel filtration (Supporting Information Figure 1).

In order to investigate the ability of scFv(F8)-FluA-scFv(F8) to simultaneously engage in a binding interaction with the cognate EDA antigen and with fluorescein derivatives, we immobilized the antigen on a sepharose resin and applied the fusion protein on it. The subsequent addition of a fluorescein-Alexa Fluor 546 fluorophore conjugate allowed a clear detection of the bispecific fusion protein on the column, in full analogy to experiments performed with the scFv(F8)-FluA-scFv(F8) directly labeled with the dye. The Alexa Fluor 546 fluorophore was used in these experiments, as its purple color facilitates the visualization of binding interactions. By contrast, neither fluorescein alone, Alexa Fluor 546 dye alone, nor the

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Fluo-Alexa Fluor546 conjugate were able to bind to the resin in the absence of the scFv(F8)-FluA-scFv(F8) adaptor protein (Figure 4).

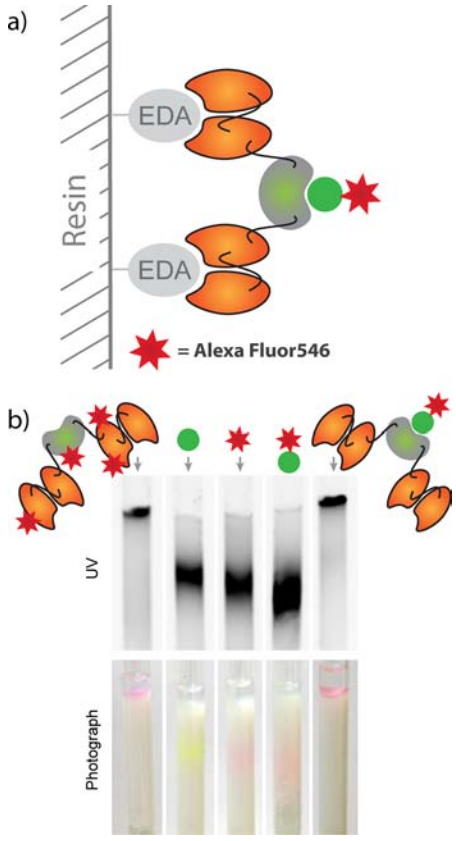

Figure 4. Interaction of the FluA-F8 fusion protein in a pretargeting system. (a) Scheme of the fusion protein binding to EDA immobilized on sepharose, and a fluorescein−dye conjugate binding into the FluA binding pocket. (b) Alexa Fluor 546-labeled scFv(F8)-FluA-scFv(F8), fluorescein, Alexa Fluor 546, Fluo-Alexa Fluor 546, or scFv(F8)-FluA-scFv(F8) and subsequently Fluo-Alexa Fluor 546 was applied to columns packed with EDA sepharose. The columns were imaged under UV light and by photography.

We investigated the tumor targeting performance of Fluo-IR750 and of the corresponding dye devoid of fluorescein in a pretargeting experiment in tumor-bearing mice (Figure 5). Here, we used the near-infrared dye IR750, as light penetration through tissues is maximal at approximately 800 nm.[30] The Boc-IR750 conjugate was used as negative control dye, devoid of FluA binding activity. Mice bearing subcutaneously grafted F9 tumors first received 5 nmol of scFv(F8)-FluA-scFv(F8) intravenously, and 24 h later 5 nmol of either cognate Fluo-IR750 conjugate or 5 nmol of the nontargeted Boc-IR750 dye. Imaging immediately after intravenous injection of the dye showed a distribution of fluorescence throughout the body. Eight hours later, both dye conjugates had been cleared almost completely from tissues, but a portion of the fluorescein-IR750 conjugate was retained at the tumor site in mice which had received an injection of the scFv(F8)-FluA-scFv(F8) fusion protein. Similarly, administration of Fluo-IR750 to mice that had not been injected with the fusion protein did not result in tumor visualization (Figure 5 and Supporting Information Figure 2).

DISCUSSION

We have developed a novel class of bispecific trivalent antibody-based fusion proteins, which incorporate two antibody fragments and an anticalin moiety, for recognition of small organic molecules. We used the anticalin mutant FluA, as it recognizes fluorescein derivatives with dissociation constants in the nanomolar range[19] (Figure 3). One particular format scFv(F8)-FluA-scFv(F8), in which the FluA domain is used as a linker between two scFv fragments, embedded in a single polypeptide, exhibited full retention of binding affinities of both antibody and anticalin moieties and favorable expression, purity, and stability profiles. The scFv(F8)-FluA-scFv(F8) was shown to preferentially localize to solid tumors after intravenous administration to mice bearing subcutaneously grafted F9 carcinomas. Furthermore, the fusion protein could be targeted *in vitro* and *in vivo* by fluorescein-dye conjugates. However, retention of the dye conjugate at the tumor site was gradually lost over time, as a kinetically stable 1:1 complex would require dissociation constants in the picomolar range. It should be possible to improve this interaction by increasing the binding affinity of FluA to fluorescein (ideally by exploiting irreversible binding strategies[31−33]) or by the use of bivalent ligands.[34]

A number of pretargeting strategies have been investigated both preclinically and clinically, primarily for the imaging and therapy of cancer. Most strategies have either relied on antibody-(strept)avidin combinations or bispecific antibodies. The (strept)avidin approach makes use of radioactive biotin and either fusion proteins between streptavidin and a tumor targeting antibody[13,35] or a biotinylated antibody chased with avidin that clears circulating antibody and due to its tetravalent nature generates binding sites for radioactive biotin on bound antibody.[36] Moreover, intraoperative injection of avidin in the tumor bed after quadrantectomy for the homing of radioactive biotin has been used in a clinical setup.[37,38] Bispecific antibody-based strategies have produced spectacular biodistribution results in tumor-bearing animals,[39] and the results of clinical trials are promising.[11,40] The tumor accumulation in pretargeting settings can reach values as high as 278 ± 130%ID/g and tumor-to-blood ratios >30 at 1 h post injection in mouse models of cancer.[41] However, such high values may only be reached in very homogeneous tumors, which display an extremely high and uniform level of tumor-associated antigen expression. In most human tumors, a more heterogeneous microscopic structure of the neoplastic tissue is typically observed. Biodistribution studies involving antibodies specific to oncofetal fibronectin may be a more relevant comparison of the pretargeting data presented in this study. Moosmayer et al. used a bispecific antibody recognizing the EDB domain of fibronectin in pretargeting biodistribution studies in mice bearing human glioblastoma xenografts. The authors reported a tumor accumulation of 16.9 ± 3.6%ID/g at 4 h post injection of a radiolabeled hapten, 41 h after administration of the bispecific antibody.[42]

Compared to conventional bispecific antibodies, the scFv-(F8)-FluA-scFv(F8) format is modular, easy to produce and well-behaved from a biochemical point of view. No chemical coupling step is needed for the preparation of this trivalent bispecific fusion protein, which can be produced recombinantly in mammalian cells. In contrast to the situation encountered with recombinant bispecific antibodies, which may display problems associated with the wrong domain pairing,[43,44] the

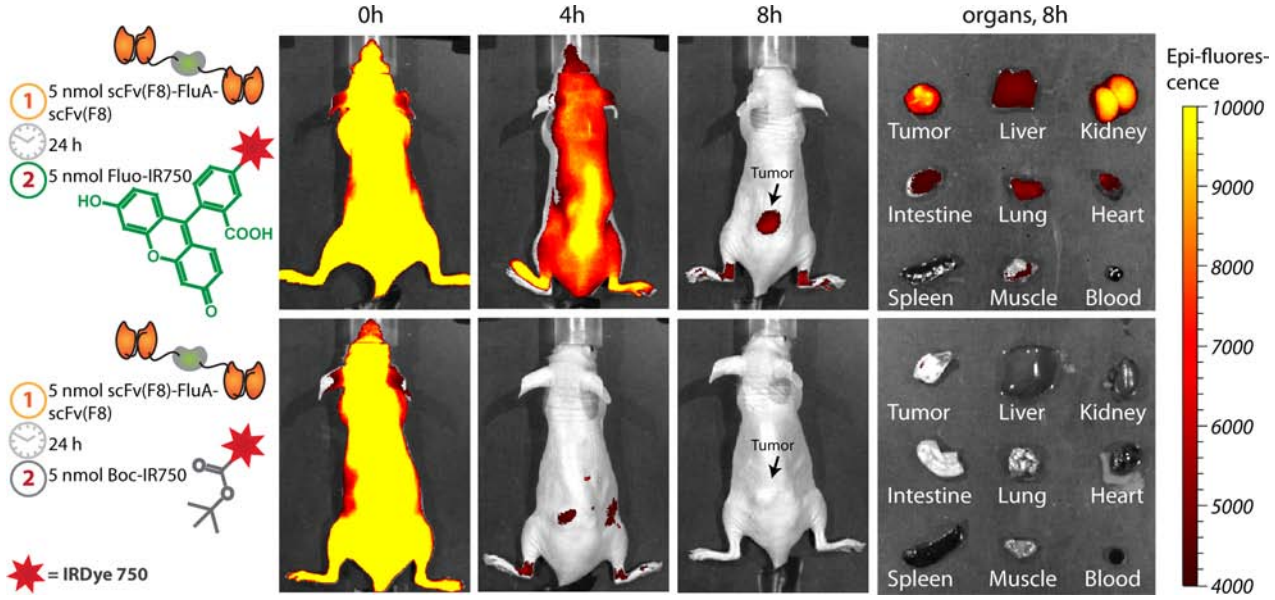

Figure 5. *In vivo* imaging of cancer with a pretargeted infrared dye (IRDye 750). Twenty-four hours after intravenous administration of the pretargeting protein scFv(F8)-FluA-scFv(F8) in Balb/c nude mice bearing a subcutaneous F9 murine teratocarcinoma, either the targeting Fluo-IR750 conjugate or the nontargeting Boc-IR750 was injected. Mice were imaged at 0, 4, and 8 h after injection of the dye, and the organs were imaged 8 h post injection (number of mice: n = 4 per group; representative results of one mouse per group are shown here).

simultaneous use of scFv and FluA domains ensures a full retention of binding activities.

Fluorescein is an FDA-approved drug and widely used contrast agent for diagnostic fluorescein angiography or angioscopy of the retina and iris vasculature. The low molecular weight of this fluorophore and its easily modifiable structure facilitate the production of low-molecular-weight derivatives, which retain a high binding affinity toward scFv(F8)-FluA-scFv(F8) fusion proteins. The fluorescence properties of fluorescein are lost upon binding, but targeting properties can be detected using near-infrared fluorophores (as described in this application) or with radionuclides. For therapy applications, it would be conceivable to use scFv(F8)-FluA-scFv(F8) in combination with fluorescein-drug conjugates, which incorporate chemical linkers compatible with a slow release of cytotoxic agent at the site of disease, such as disulfide bonds[45,46] or thiazolidines.[47]

While pretargeting approaches promise to offer unprecedented targeting selectivity shortly after intravenous administration, certain challenges need to be considered and some problems are still unresolved. The timely separation of two drug components multiplies the number of parameters chosen for an effective administration regime such as dosing of each agent, the time interval, and the precise stoichiometric matching in relation to the individual pharmacokinetics. However, the need to develop two pharmaceutical agents is not necessarily a negative feature of pretargeting strategies. The system is extremely flexible in that the hapten binding to the artificial receptor could be kept constant, while fusion proteins with different specificities could be used for different applications. In imaging procedures, results are needed shortly after injection of the contrast agent, and it would therefore be conceivable to administer the bispecific antibody product outside of specialized imaging departments, while the hapten-based contrast agent would be administered at the hospital site, shortly before the imaging procedure.

■ ASSOCIATED CONTENT

Supporting Information

SDS-PAGE, size exclusion chromatography, gel filtration, synthesis of compounds, and tumor imaging. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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