Biodistribution studies with tumor-targeting bispecific antibodies reveal selective accumulation at the tumor site

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Keywords: antibody, biodistribution, bispecific, syngeneic, tumor

Bispecific antibodies are proteins that bind two different antigens and may retarget immune cells with a binding moiety specific for a leukocyte marker. A binding event in blood could in principle prevent antibody extravasation and accumulation at the site of disease. In this study, we produced and characterized two tetravalent bispecific antibodies that bind with high affinity to the alternatively-spliced EDB domain of fibronectin, a tumor-associated antigen. The bispecific antibodies simultaneously engaged the cognate antigens (murine T cell co-receptor CD3 and hen egg lysozyme) and selectively accumulated on murine tumors in vivo. The results, which were in agreement with predictions based on pharmacokinetic modeling and antibody binding characteristics, confirmed that bispecific antibodies can reach abluminal targets without being blocked by peripheral blood leukocytes.

Bispecific and multispecific antibodies were proposed over 20 years ago as pharmaceutical agents capable of redirecting the lytic activity of immune cells toward malignant target cells.1,2 These proteins are frequently designed to simultaneously engage in a binding interaction with a surface antigen on a tumor cell and with a surface protein on an immune cell. Indeed, it has been shown in vitro that bispecific antibodies mediate the formation of an artificial immunological synapse by bringing cytotoxic T lymphocytes (CTLs) and tumor cells into close proximity, which results in the selective killing of the target cell.3,4

The potential of bispecific antibodies as cancer therapeutics relates to their ability to mediate repeated and selective rounds of target cell lysis in an MHC- and T cell receptor independent manner. Some bispecific antibodies can be effective in vitro at picomolar concentrations and have been shown to eradicate tumors implanted in mice.5-9 Furthermore, there is emerging evidence that certain bispecific antibodies may provide a substantial benefit to patients with cancer, in particular, those with hematological malignancies.10,11

Strategies used in the 1980s for the construction of dual specificity products involved creation of murine IgG antibodies or F(ab')2 molecules by fusion of two different hybridoma lines (quadroma) (Fig. 1A), but, because of random pairing of heavy and light chains, the yield of antibodies with the desired bispecificity was extremely low.12 In addition, these constructs had from low antitumor activity and caused side effects due to the presence of the Fc region. The development of diabodies and bispecific T cell engagers (BiTEs; scFv fragments arranged in a tandem array; Fig. 1A) greatly improved the process for the preparation of homogenous bispecific antibodies, but these antibody formats may display reduced uptake at the site of disease because of the monovalence of each scFv binding moiety.13-16 Multivalent engagement with both tumor cell and immune cell can be achieved using the tetravalent bispecific “TandAb” format, which consists of a sequential fusion of VH-VL-VH-VL domains of two antibodies, arranged in a single polypeptide (Fig. 1B).17,18

Leukocyte antigens frequently targeted by bispecific antibodies for re-directing an immune response against tumor cells include CD3 (a component of the T cell receptor found on T lymphocytes), and the Fc receptors CD64 (constitutively expressed on macrophages and monocytes) and CD16 (found on natural killer cells, neutrophils and macrophages). A multispecific antibody, catumaxomab (Removab), which simultaneously targets the carcinoma marker EpCam, CD3 and Fc receptors), is approved in the European Union for the locoregional treatment of malignant ascites.19 The bispecific T cell engaging antibody blinatumomab (AMG103, formerly MT103) in BiTE format, which simultaneously recognizes the CD3xCD19 antigens, induced tumor regressions in the majority of relapsed non-Hodgkin B cell lymphoma and acute lymphoblastic leukemia (ALL) patients treated with this drug.10,11

The pharmaceutical development of bispecific antibodies is made more difficult by the fact that two binding moieties have to be optimized to achieve biological activity in vivo. The biochemical binding properties of the two antibodies can have a profound effect both on target cell lysis and on pharmacokinetic parameters. For example, objective responses with blinatumomab were only achieved when patients were treated by continuous intravenous
Figure 1. For figure legend, see page 777.
infusion for a period of 4–8 weeks, while the administration of the same product by bolus injection did not confer a substantial benefit to patients and led to adverse side effects. In spite of many years of research and considerable industrial investments in the field, not much is known about the real ability of bispecific antibodies to localize at the site of disease in vivo. Cochliovius et al. reported a biodistribution of 125I-labeled TandAb in Rag2 deficient mice bearing subcutaneous (s.c.) Raji xenografts. It is to be noted that Rag2 knockout mice have very few T cells that lack expression of extracellular CD3. In 2009, Kontermann and colleagues reported a quantitative biodistribution study in which bispecific antibodies fused to different half-life prolonging moieties were administered to nude mice. Although it could be shown that PEGylation increases serum half-life and tumor accumulation of bispecific antibodies, the biodistribution experiments were performed in nude mice, which lack functional T cells, xenografted with human cancer cells. To our knowledge, and as stated by Stork et al., no biodistribution study has been published so far using bispecific antibodies in fully syngeneic immunocompetent models of cancer. The use of such models is crucial to assess whether the binding of a bispecific antibody to circulating immune cells inhibits extravasation and prevents a selective accumulation at the site of disease, which is a prerequisite for therapy.

To study the performance of tumor-targeting bispecific antibodies in vivo in an immunocompetent context, we used the L19 antibody, which is specific to the alternatively-spliced EDB domain of fibronectin, a marker of angiogenesis. The EDB domain is virtually undetectable in normal adult organs (with the exceptions of the endometrium in the proliferative phase and the placenta), but is strongly expressed in the majority of malignancies, with a prominent perivascular pattern of staining (Fig. 1G). The sequence of EDB is identical in mouse and man. The tumor targeting properties of the L19 antibody have been extensively studied in tumor-bearing mice and in patients with cancer. Interestingly, while a large variety of payloads can be delivered to the tumor subendothelial extracellular matrix using the L19 antibody as delivery vehicle, payloads that prevent extravasation due to receptor blockade, extreme isoelectric point values or large size were found to completely abrogate selective tumor targeting in vivo. As a T cell specific antigen, we chose the CD3 epsilon (CD3ε) subunit of the T cell receptor (TCR) expressed on the majority of T lymphocytes, which is frequently used as target in pharmaceutical development programs. We used the Armenian hamster antibody 145-2c11, which specifically recognizes the murine CD3ε isoform, and which has previously been used for the study of bispecific antibodies in mice.

To generate a negative control bispecific antibody of irrelevant specificity in the mouse that would not able to engage in a binding interaction with murine T cells, we used the KSF antibody moiety, which is specific to hen egg lysozyme. Schematic representations of the TandAb structures for the mCD3εxEDB and HELxEDB antibodies, forming non-covalent homodimers with four binding sites and two antigen specificities are shown in Figure 1B. Both proteins were purified to near homogeneity using Protein A chromatography (Fig. 1C) and eluted as a single peak of expected size in size-exclusion chromatography (Fig. 1D), after an initial polishing step (Fig. S1). Figure 1E shows the schematic representation of the domain structures and the biochemical purity data (anti-His western blot) for a recombinant antigen (termed mCD3εy26) that was used to assess the binding properties of the bispecific antibodies to mCD3ε. The L19 moiety, but not the KSF moiety, is capable of recognizing neo-vascular structures in the F9 murine teratocarcinoma model of cancer, chosen for this study, as evidenced by immunofluorescence analysis of tumor sections (Fig. 1F).

Binding of the mCD3εxEDB antibody to the cognate recombinant mCD3εy26 (Fig. 2A) and EDB (Fig. 2B) antigens was assayed in vitro by surface plasmon resonance (SPR) on a BIAcore instrument. Simultaneous binding of mCD3εxEDB to immobilized mCD3εy26 and soluble EDB was further confirmed in a sandwich SPR experiment (Fig. S2). Figure 2C shows association of the parental 145-2c11 IgG antibody as positive control on a mCD3εy26-coated chip. As expected, HELxEDB did not bind to mCD3εy26 (Fig. 2D), but bound equally well as mCD3εxEDB to immobilized EDB on a sensor chip (data not shown). In addition, binding of mCD3εxEDB (Fig. 2E), but not HELxEDB (Fig. 2F), to the CD3-positive cells of murine CTLL2 T cell lymphoma could be shown by FACS. Simultaneous binding of the bispecific mCD3εxEDB TandAb to CD3 on the cell surface and to soluble fluorescently-labeled EDB domain could also be detected by FACS using CTLL2 cells (Fig. 2G).

Figure 3 shows biodistribution results in immunocompetent 129Sv mice bearing F9 murine teratocarcinomas, 24 h after injection of 125I radiolabelled mCD3εxEDB TandAb (Fig. 3A) and HELxEDB TandAb (Fig. 3B). Both bispecific antibodies exhibited preferential accumulation at the tumor site, with tumor:organ ratios comparable to the one of the parental L19 antibody in diabody format.
Having demonstrated the ability of mCD3xEDB to accumulate at the tumor site and to simultaneously engage in a binding interaction with its cognate target antigens in vitro, we tested the therapeutic potential of this TandAb in vivo. We did not anticipate that the targeted redirection of a T cell response to the sub-endothelial extracellular matrix would mediate a therapeutic effect, but we could not exclude that small amounts of EDB(+) fibronectin on the surface of tumor cells would be sufficient to elicit an anti-cancer response.48 The results of a therapy experiment in 129Sv mice bearing murine F9 teratocarcinomas (n = 6 per treatment group) are shown in Figure 4. Mice were injected i.v. in the tail vein with 40 μg mCD3xEDB, HELxEDB or saline solution every 48h for 6 injections in total. Therapy was stopped at days 15 and 16 because treatment with mCD3xEDB...
did not result in a statistically significant anti-tumor effect compared with control treatments. Our study shows that TandAb mCD3εxEDB could preferentially accumulate at the tumor site, without being trapped in blood by circulating T cells. These findings are compatible with a pharmacokinetic prediction, based on a two-compartment model and on the knowledge of kinetic binding parameters, concentrations and affinity constants. The velocity of complex formation between two molecular ligands A (target) and B (antibody) in solution can be described by the following differential equation:\(^{36}\)

\[
\frac{d[AB]}{dt} = -\frac{d[A]}{dt} = k_{on}[A][B] - k_{off}[AB]
\]

where \([AB]\) is the concentration of the complex and \([A]\) and \([B]\) the concentrations of the free ligands, \(k_{on}\) the kinetic association constant and \(k_{off}\) the kinetic dissociation constant. Neglecting blood clearance and assuming that ligand B is present in large molar excess to A and that \(k_{off}\) is negligibly low in comparison to \(k_{on}\), Equation 1 can be written as:

\[
-\frac{dx}{dt} = k_{on}x[B]
\]

where \(x\) is the fraction of unbound ligand A. The solution to this differential equation has the form of an exponential rate of change:

\(x(t) = x(0)e^{-k_{on}t[B]}\)

where \(x(t)\) is the fraction of unbound target antigen A at the time \(t\) and \(x(0)\) the initial amount of unbound antigen A. From Equation 3 we can derive the equation describing the time \(T_{1/2}\) required for semi-saturation of the target antigen by the antibody:

\[T_{1/2} = \frac{\ln 2}{k_{on}[B]}\]

The BIAcore binding curves for the interaction between mCD3εxEDB and its cognate mCD3γ26 antigen allowed the determination of kinetic binding parameters \(k_{on}\) (1.1 × 10^4 M\(^{-1}\).s\(^{-1}\)) and \(k_{off}\) (2.0 × 10\(^{-3}\) s\(^{-1}\)). From these values, an apparent mean \(K_a\) of 200 ± 78 nM could be derived \([K_a = (k_{off}/k_{on})]\). The high \(K_a\) value indicates that CD3 antigen molecules on the surface of circulating T lymphocytes would not be saturated by antibody administered at submicromolar concentrations in biodistribution experiments. Furthermore, knowledge of blood TandAb concentration and the \(k_{on}\) value (1.1 × 10^4 M\(^{-1}\).s\(^{-1}\)) predicts an association kinetic between bispecific antibody and circulating T cell that is slow, compared with the antibody extravasation and localization to EDB(+) fibronectin in the tumor neo-vascularure.

In summary, we produced and characterized novel bispecific antibodies that recognize the alternatively-spliced EDB domain of fibronectin in vitro and in vivo. The use of an antibody moiety specific to the murine mCD3 antigen allowed the execution of biodistribution studies in syngeneic immunocompetent models of cancer, revealing that the bispecific antibodies were capable of selective localization at site of disease. A pharmacokinetic model, based on the knowledge of antibody and antigen concentrations, as well as of kinetic binding parameters, indicates that the use of bispecific antibodies at concentrations below the dissociation constant \(K_a\) for the mCD3 binding interaction (200 ± 78 nM) is permissible to a good antibody accumulation at the tumor site in vivo.

### Materials and Methods

**Construction of bispecific antibodies.** TandAbs were genetically assembled by successive overlap PCR in the order
VH2c11-Linker10aa-VL19-Linker12aa-VH19-Linker10aa-VL2c11 and cloned into the pcDNA3.1 expression vector downstream of a mammalian excretion signal sequence. In the first step, cDNA sequences of VH19, VL19, VH2c11, and VL2c11 were amplified with the following primer pairs (italic indicates overhangs, underline indicates BamHI restriction cutting site) respectively (1) 5'-GGT GGA TCC GCC GCT GGT GAG GTG CAG CTG TTG GAG TCT GGG G-3' and (2) 5'-CAG GGA ACC CTG TGC ACC GTC TCG AGT TCC GCC AAG ACC CCC AAG CTG GTC ACC GGA GTG CAG TCT CCA GG-3' and (4) 5'-GCC AAG GGA CCA AGG TGG AAA TCA AAC GG G AAC AAA AAC TCA TCT CAG AAG GGC-3'. In the second step, VH2c11-Linker10aa-VL19 and VH19-Linker10aa-VL2c11 fragments were assembled using the primer pairs (9) 5'-CCC AAG CTT GCC GCC GTG GCC GCC GCT GCA ACC CAG TCT CCA TC-3' and (8) 5'-CTG GCA CCA AGC TGG AAA TCA AAC GG G AAC AAA AAC TCA TCT CAG AAG GGC-3'. Both fragments were subsequently digested and ligated into one at the BamHI restriction site before insertion into the vector.

**Cell lines and mice.** For the production of the bispecific antibodies, Chinese hamster ovary cells (CHO-S; Invitrogen) in suspension were used. The tumor cell line used for the therapy study was the murine teratocarcinoma cell line F9 [CRL-1720, American Type Culture Collection (ATCC)]. For flow cytometry experiments, the murine cytotoxic T cell line CTLL2 [TIB-214, American Type Culture Collection (ATCC)] was used. CHO-S cells in suspension were cultured in shaker incubators (37°C) in PowerCHO-2CD medium containing 8 mM Ultraglutamine and HT supplement. F9 cells were cultured on 0.1% gelatin-coated tissue flasks in Dulbecco's modified Eagle Medium (Gibco) supplemented with 10% Fetal Calf Serum (FCS) incubated at 37°C and 5% CO₂. CTLL2 cells were cultured at 37°C and 5% CO₂ in RPMI-1640 medium (Gibco) supplemented with 10% FCS (Invitrogen, Germany), Antibiotic-Antimycotic (Gibco), Ultraglutamine, 1 mM Na-Pyruvate, 50 μM β-mercaptoethanol, human IL2 (20 units/ml) (Sino Biological). Female 129/SvEv mice were obtained from Charles River.

**Protein expression and purification.** Bispecific antibodies were expressed using transient gene expression. For 200 mL of production, 200.10⁶ CHO-S cells were resuspended in 100 mL ProCHO4 (Lonza). Plasmid DNA (250 μg) was mixed with 150 mM NaCl to reach a final volume of 5 mL. Five milliliters of 250 mg/ml 25-kDa linear polyethylene imine (PEI; 1 mg/mL solution in water at pH 7.0; Polysciences) in 150 mM NaCl were added to the DNA NaCl solution and allowed to stand at room temperature for 10 min. The solution containing the PEI-DNA complexes was then added to the cells and gently mixed. The transfected cultures were incubated in a shaker incubator at 37°C. At 4 h post-transfection, the transfected culture was diluted with 100 mL Power-CHO-2CD medium and then incubated at 31°C in a shaking incubator for 6 d. The fusion proteins were purified from the cell culture medium by protein A affinity chromatography and then dialyzed against phosphate buffered saline (PBS,}

![Figure 4](image-url). Therapy with mCD3xEDB TandAb in mice. Mean tumor volume ± SD (mm³) is shown over time in 129Sv mice (n = 6 per treatment group) implanted s.c. with F9 tumor cells. Mice were injected i.v. in the tail vein with 40 μg TandAb mCD3xEDB, HELxEDB or saline solution every 48 h for 6 injections in total.
6 mg/ml NaCl, 3.12 mg/ml NaH₂PO₄, 5.34 mg/ml Na₂HPO₄, 300 mM NaOH, pH 7.4).

**Construction and bacterial expression of mCD3εγ26 and EDB antigens.** The mCD3εγ26 construct is an N-terminally His-tagged heterodimer consisting of the murine CD3 epsilon (mCD3ε) and gamma (mCD3γ) chains linked by a 26 amino acid linker (GSA DDA KKD AAK KDD AKK DDA KKD GS) as first reported by Kim et al. The mCD3ε and mCD3γ cDNA was purchased from Sino Biological Inc. The mCD3εγ26 nucleotide sequence was assembled by overlap PCR using the following primers: (1) 5’-CCC CCA TAT GGA CGA TGC CGA GAA CAT TGA ATA C-3’ (2) 5’TGT CTT TGC ATC GTC CTT TTT GGC TGC GTC CTT GTC AGC TGA CCC GTA CGT GTT TTT ATT TGA GGC TG-3; (3) 5’-GCA GCC AAA AAG GAC GAT GCA AAG AAA GAC GAT GCC AAG AAG GAC GGC AGC CAG ACA AAT AAA GCA AAG AAT TGG G-3’ (4) 5’-CTG CAA GTG TAT TAC AGA ATG TAG GCC GCC GC-3’. The mCD3εγ26 was purified from the supernatant soluble lysate fraction by immobilized nickel affinity chromatography (Sephadex 75). The purity of the resulting 30 kD antigen was confirmed by Coomassie staining, anti-His western blot and size exclusion chromatography (Sephadex 75).

**Conjugation of EDB to FITC.** Protein was incubated 32 h with 10-fold molar excess Fluorescein isothiocyanate (FITC) after pH was adjusted to 8.4 by addition of 500 mM NaHCO₃. Excess FITC was removed by buffer exchange column (PD-10, GE Healthcare) according to manufacturer’s protocol. The 145-2c11 IgG antibody used as positive control was purchased from eBiosciences.

**Flow cytometry analysis.** 10⁵ CTLL2 cells were resuspended in a 96-well U-bottom plate in 200 μl per well FACS buffer (5% FBS, 2 mM EDTA). Cells were pelleted and incubated on ice for 1 h with bispecific antibodies in PBS. Cells were then washed 3 times with FACS buffer and incubated 30 min with 200 μl of a 1:600 dilution of protein-A-Alexa488 conjugate (Invitrogen, Life Technologies) or FITC-conjugated EDB (0.1 mg/ml). Cells were then again washed 3 times with FACS buffer and analyzed on a FACS Canto II Flow Cytometer (BD Biosciences).

**Immunofluorescent staining.** For immunofluorescence staining, 9F tumors were excised, embedded in cryo-embedding medium (Thermo Scientific) and stored at -80°C. Cryostat sections (10 mm) of tumors were fixed in ice-cold acetone, rehydrated with PBS and blocked with 10% fetal bovine serum (Invitrogen) in PBS. Rat-anti-mouse CD31 antibody (BD Biosciences) and was used for staining endothelial cells from blood vessels. Rat-Alexa488 (Invitrogen) coupled secondary antibody was used for detection. Biotinylated L19 small immunoproteins (SIP), revealed with Alexa594-conjugated streptavidin (Invitrogen) was used to stain EDB. Slides were mounted with fluorescent mounting medium (Dako) and analyzed with an Axioskop2 mot plus microscope (Zeiss).

**Surface plasmon resonance.** Bispecific antibody solutions were filtered (using 0.22 μm PVDF filters) and their binding properties analyzed using a BIAcore3000 instrument (BIAcore) and an antigen coated chip, which was prepared by covalent coupling of mCD3εγ26 (at different concentrations) to a CM5 sensor chip (GE Healthcare) according to the manufacturer’s protocol. The 145-2c11 IgG antibody used as positive control was purchased from ebiosciences.

**Biodistribution.** Tumor-bearing mice were obtained by subcutaneous injection of F9 teratocarcinoma cells (10⁵) in the flank of 11–13 weeks old female 129/SvEv mice. Five days after tumor implantation, 10 μg (0.09 nmol) ¹²⁵I radiolabelled mCD3εEDB TandAb and HELxEDB TandAbs were injected into the lateral tail vein of 129/SvEv (Charles River) mice bearing subcutaneous implanted F9 murine teratocarcinoma cells. Bioreactivity after radiiodination was confirmed by binding to EDB resin. Mice were sacrificed 24 h after injection. Organs were weighed and radioactivity was counted using a Packard Cobra gamma counter. Radioactivity content of representative organs was expressed as the percentage of the injected dose per gram of tissue (%ID/g ± SE).

**Therapy experiment.** 129Sv mice aged between 11–13 weeks were injected s.c. with 10⁷ F9 teratocarcinoma cells. Five days after tumor implantation, when tumors were clearly palpable, mice were randomized in different treatment groups (n = 6 per treatment group). Therapy was performed by injection in the tail vein. 129Sv mice were injected 6 times, every 48 h with 40 μg mCD3εEDB or HELxDNB TandAb or saline (PBS). Experiments were performed under a project license granted by the Veterinaramt des Kantons Zuerich, Switzerland (169/2008).

**Disclosure of Potential of Conflicts of Interest** No potential conflicts of interest were disclosed.

**Acknowledgments** Financial contribution from the ETH Zürich, the Swiss National Science Foundation, the Kommission für Technologie und Innovation, the Swiss Cancer League and Philochem AG is gratefully acknowledged.

**Supplemental Material** Supplemental material may be downloaded here: www.landesbioscience.com/journals/mabs/article/22271
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