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Tetravalent Antibody–scTRAIL Fusion Proteins with Improved Properties

Oliver Seifert, Aline Plappert, Sina Fellermeier, Martin Siegemund, Klaus Pfizenmaier, and Roland E. Kontermann

Abstract

We applied the immunoglobulin E (IgE) heavy-chain domain 2 (EHD2) as the covalently linked homodimerization module to generate antibody–scTRAIL fusion proteins. By fusing a humanized single-chain fragment variable (scFv) directed against EGFR to the N-terminus of the EHD2 and a single-chain derivative of TRAIL (scTRAIL) to the C-terminus of the EHD2, we produced a dimeric, tetravalent fusion protein. The fusion protein retained its binding activity for EGFR and TRAIL receptors. *In vitro*, the targeted antibody–scTRAIL fusion protein exhibited an approximately 8- to 18-fold increased cytotoxic activity compared with the untargeted EHD2–scTRAIL fusion protein. This resulted in increased antitumor activity in a subcutaneous Colo205 xenograft tumor murine model. In summary, the scFv–EHD2–scTRAIL fusion protein combines target cell selectivity with an increased TRAIL activity leading to improved antitumor activities. *Mol Cancer Ther*; 13(1); 101–11. ©2013 AACR.

Introduction

TRAIL is a potent inducer of the extrinsic apoptosis pathway through activation of the proapoptotic death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), which are overexpressed on tumor cells (1–4). Clinical trials of soluble TRAIL (dulanermin) demonstrated that the drug was safe and well tolerated, although in a phase II study the combination of dulanermin with chemotherapy (paclitaxel and carboplatin) and antibody therapy (bevacizumab) did not improve outcomes of patients with previously untreated advanced or recurrent non–small cell lung cancer (5–7).

The cytotoxic activity of TRAIL can be improved by targeted delivery, for example, by fusion with single-chain Fv fragments directed against tumor-associated antigens (8, 9). Applying a single-chain derivative (scTRAIL) of homotrimeric TRAIL, we showed that fusion of an anti-HER2 single-chain fragment variable (scFv) results in increased killing of HER2-positive tumor cells *in vitro* as well as in mouse xenograft tumor models (10). Recently, we

further demonstrated that conversion of the monovalent scFv–scTRAIL fusion protein into a bivalent diabody–scTRAIL fusion protein—exhibiting two antigen-binding sites and two scTRAIL moieties—further increased cytotoxic activity against tumor cells without increasing cytotoxicity toward normal cells (11). This is most likely due to the fact that the dimeric scTRAIL fusion proteins are capable of mimicking the activity of membrane-displayed, that is, multivalent, TRAIL, leading to increased receptor signal complex formation and activation (12, 13). The dimerization of scTRAIL in the diabody–scTRAIL fusion protein is mediated by the antibody moiety, thus combining targeting and dimerization in the same module. As an alternative, we have recently demonstrated that the heavy-chain domain 2 of immunoglobulin M (IgM; MHD2) can be applied to generate covalently linked homodimeric fusion proteins, thus using the MHD2 as a functionally independent dimerization module (14). This allows fusion of various targeting modules to the MHD2, as shown for scFv and bispecific scDb, thereby introducing greater flexibility.

Similar to MHD2, the IgE heavy-chain domain 2 (C_H2; EHD2) also acts as a "hinge" domain covalently connecting the two IgE heavy chains. The EHD2 is composed of 106 residues; thus, it is similar in size to the MHD2 (111 aa), also containing a single N-glycosylation site (Asn275, EU index). However, in contrast to MHD2, which forms only a single interdomain disulfide bond, the EHD2 domains are connected by two disulfide bonds formed by residues Cys247 and Cys337 between the two domains (15). For the MHD2, we showed by a mutagenesis study that not only the disulfide bond but also the N-glycan contribute to the thermal stability. It has already been shown that the IgE C_H2 domain is unaffected by heating (56°C for 30 minutes), whereas the C_H3 and C_H4 domains underwent irreversible conformational changes under

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these conditions (16), supporting the notion of an increased thermal stability of EHD2.

Here, we investigated the EHD2 as dimerization module to generate dimeric tetravalent fusion proteins using an anti-EGFR scFv for tumor cell targeting and scTRAIL as the apoptosis-inducing effector moiety. We could show that the fusion proteins formed dimers retaining the activity of the respective fusion partners, that is, binding to EGFR-expressing target cells and binding to TRAIL receptors and induction of cell death. Compared with scTRAIL and scFv-scTRAIL, the dimeric EHD2-scTRAIL showed enhanced activity *in vitro*. Importantly, a further increase in cytotoxicity toward EGFR-expressing cells was established for the scFv-EHD2-scTRAIL fusion proteins, demonstrating the beneficial effects of targeted delivery. This translated into a potent antitumor activity *in vivo* in a mouse xenograft tumor model.

Materials and Methods

Materials

A humanized anti-EGFR scFv (hu225) was generated from the antibody C225 (17) by complementarity determining region (CDR) grafting. DNA encoding the EHD2 was codon-optimized for expression in human cells and synthesized by Geneart adding appropriate cloning sites. Cell lines were cultured in RPMI-1640 medium (Invitrogen) supplemented with 5% fetal calf serum (FCS; HyClone; NCI-H460, HEK293) or 10% FCS (Colo205, HCT116, HepG2), respectively. No authentication was done by the authors. EGFR-Fc and HER2-Fc were purified from stably transfected HEK293 cells (14). Bortezomib was purchased from Sigma-Aldrich, and clinical grade bortezomib and cetuximab were kindly provided by Dr. T. Mürdter (Institute of Clinical Pharmacology, Margarete Fischer-Bosch Foundation, Stuttgart, Germany). Human soluble TRAIL protein was purchased from Peprotech.

Protein production and purification

DNA encoding the various EHD2 fusion proteins was cloned into the expression plasmid pSecTagA (Invitrogen). Fusion proteins were produced from stably transfected HEK293 cells and purified from the cell culture supernatant by immobilized metal affinity chromatography (IMAC) (scFv-EHD2) or by FLAG affinity chromatography (Sigma-Aldrich; EHD2-scTRAIL, scFv-EHD2-scTRAIL) as described previously (11, 18). TRAIL receptor-Fc fusion proteins were generated by fusing the extracellular domain of the receptors to the human Fc region, produced in stably transfected HEK293 cells, and purified from cell culture supernatant by protein A affinity chromatography as described previously (19). The protein concentration was measured with a spectrophotometer (NanoDrop) using the calculated molar extinction coefficient. Aliquots were stored at -80°C .

Deglycosylation

Proteins (10 μg) were denatured at 95°C for 5 minutes. After cooling, 2 U N-glycosidase F (Roche) was added to

the protein and incubated overnight at 37°C . The deglycosylated protein was compared with the untreated protein in SDS-PAGE under reducing conditions.

ELISA

Receptor-Fc fusion proteins (200 ng/well) were coated overnight at 4°C , and the remaining binding sites were blocked with 2% (w/v) dry milk/PBS. Proteins were titrated in duplicates and incubated for 1 hour at room temperature. Bound proteins were detected either with horseradish peroxidase (HRP)-conjugated mouse anti-His-tag antibody for the scFv-EHD2 fusion protein or with HRP-conjugated mouse anti-FLAG antibody (all scTRAIL fusion proteins) using 3,3',5,5'-tetramethylbenzidine (TMB) as substrate (0.1 mg/mL TMB, 100 mmol/L sodium acetate buffer, pH 6.0, 0.006% H_2O_2). The reaction was stopped with 50 μL of 1 mol/L H_2SO_4 . Absorbance was measured at 450 nm in an ELISA-reader.

Flow cytometry

Binding to cells was determined by flow cytometry. Cells were incubated with 20 nmol/L fusion proteins for 1 to 2 hours at 4°C . Cells were then washed with PBS, 2% FBS, and 0.02% NaN_3 (PBA), and bound antibodies were detected with anti-TRAIL antibody (R&D Systems) and phycoerythrin (PE)-conjugated anti-mouse IgG antibody (Sigma-Aldrich) for scTRAIL-conjugates or PE-conjugated anti-His-tag antibody (Miltenyi Biotec) for scFv-EHD2.

Size-exclusion chromatography

High-performance liquid chromatography (HPLC) size-exclusion chromatography of fusion proteins was performed with a BioSuite 250 column (Waters Corporation) or a BioSep SECS2000 (Phenomenex) at a flow rate of 0.5 mL/min. The following standard proteins were used: thyroglobulin, β -amylase, BSA, carbonic anhydrase, cytochrome c, and aprotinin.

Melting point

Melting points of the proteins were determined by measuring thermal denaturation with the ZetaSizer Nano ZS (Malvern). Approximately 200 μg of purified protein was diluted in PBS to a total volume of 1.0 mL and sterile-filtered into a quartz cuvette. Dynamic laser light scattering intensity was measured simultaneously as the temperature was increased in 1°C intervals from 35°C to 92°C with 2-minute equilibration for each temperature step. The melting point was defined as the temperature at which the light scattering intensity increased.

Cytotoxicity assay

Colo205 (5×10^4 cells/well), NCI-H460 (2×10^4 cells/well), HepG2 (2×10^4 cells/well), or HCT116 (1×10^4 cells/well) cells were grown in 100 μL culture medium in 96-well plates for 24 hours, followed by treatment with serial dilutions of the EHD2-constructs, diabody-scTRAIL (Db-scTRAIL), or scTRAIL in duplicates. Cytotoxic assays were performed in the absence or presence of 250 ng/mL

bortezomib. Before adding the serial dilution of the EHD2 fusion proteins or scTRAIL, the cells were preincubated with bortezomib for 30 minutes to sensitize them to TRAIL-induced apoptosis. After 16 hours of incubation, cell death was determined with crystal violet staining. The targeting effect was demonstrated by preincubating cells with cetuximab (200-fold molar excess) for 30 minutes.

Pharmacokinetic studies

Female SWISS (Janvier, CD1, 8-week old, 3 animals per construct) received an intravenous injection of 25 µg of the recombinant protein in a total volume of 100 µL. Blood samples (50 µL) were taken at time intervals of 2 minutes and 30 minutes, then at 1, 2, 4, and 6 hours, and at 1 and 3 days and incubated on ice for 10 minutes. Clotted blood was centrifuged at $16,100 \times g$ for 20 minutes, 4°C and serum samples were stored at -20°C. Serum concentration of TRAIL fusion proteins were analyzed with BD OptEIA Human TRAIL ELISA Set (BD) according to the manufacturer's instructions. The scFv-EHD2 construct was detected via ELISA as described earlier. For comparison, the first value (2 minutes) was set to 100%. The $T_{1/2\beta}$ and area under the curve (AUC) were calculated with MS Excel.

In vivo antitumor activity

Female NMRI nu/nu mice (Janvier, 8-weeks old) were injected subcutaneously at the left and right dorsal sides each with 3×10^6 Colo205 cells in 100 µL PBS. Treatment with proteins was done when tumors reached a volume of approximately 100 mm³. In a first experiment, mice received four intravenous injections of 0.35 nmol of EHD2-scTRAIL and scFv-EHD2-scTRAIL, or 0.70 nmol of scTRAIL every fourth day (days 7, 11, 15, and 19). Mice

received an additional 5 µg bortezomib in 100 µL PBS intraperitoneally on alternate days (days 7, 9, 11, 13, 15, 17, 19, and 21). In a second experiment, mice received four intravenous injections of 1 nmol of scFv-EHD2 or scFv-EHD2-scTRAIL every second day (days 9, 11, 13, and 15). Mice received an additional 5 µg bortezomib in 100 µL PBS intraperitoneally on alternate days. Tumor growth was monitored and calculated as described previously (10). Statistical analysis was performed with ANOVA and Tukey *post hoc* tests.

Alanine aminotransferase assay

Female SWISS (Janvier, CD1, 8-weeks old, 3 mice per construct) received an intravenous injection of 1 nmol scFv-EHD2-scTRAIL together with 5 µg bortezomib intraperitoneally or only PBS intravenous (negative control), respectively, in a total volume of 150 µL. Blood samples (100 µL) were taken after 4 and 24 hours, and incubated on ice for 10 minutes. Clotted blood was centrifuged at $16,100 \times g$ for 20 minutes at 4°C, and serum samples were stored at -20°C. The activity of Alanine aminotransferase (ALT) was measured using an enzymatic assay (BIOO Scientific).

Results

EHD2

The EHD2 (see Fig. 1 for an overview) was produced in HEK293 cells and purified by IMAC. In SDS-PAGE under reducing conditions, the purified EHD2 revealed two bands with apparent molecular masses of 14 and 16 kD (Fig. 2). Under nonreducing conditions, three bands with apparent molecular masses of 26, 38, and 45 kD were observed, confirming the disulfide linkage of the two domains and indicating heterogeneity, probably caused by various degrees of N-glycosylation. Dimer formation

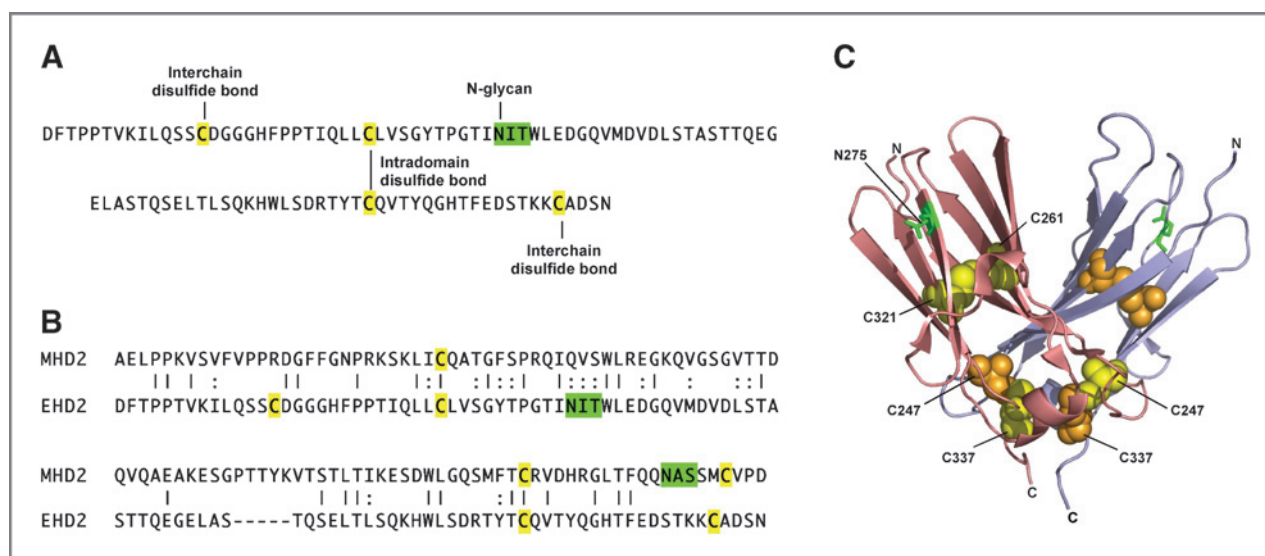


Figure 1. A, sequence of the human IgE heavy-chain domain 2 (EHD2). Inter- and intrachain disulfide bonds as well as the N-glycosylation site are marked. B, alignment of EHD2 with the corresponding human IgM heavy-chain domain 2 (MHD2). C, structure of the EHD2 (from pdb entry 1O0V; ref. 15). The two domains are colored in red and blue. Cysteine residues in the two domains are shown as spheres in yellow and orange, respectively. The potential N-glycosylation site is shown in green. Numbering of residues according to the EU index (43).

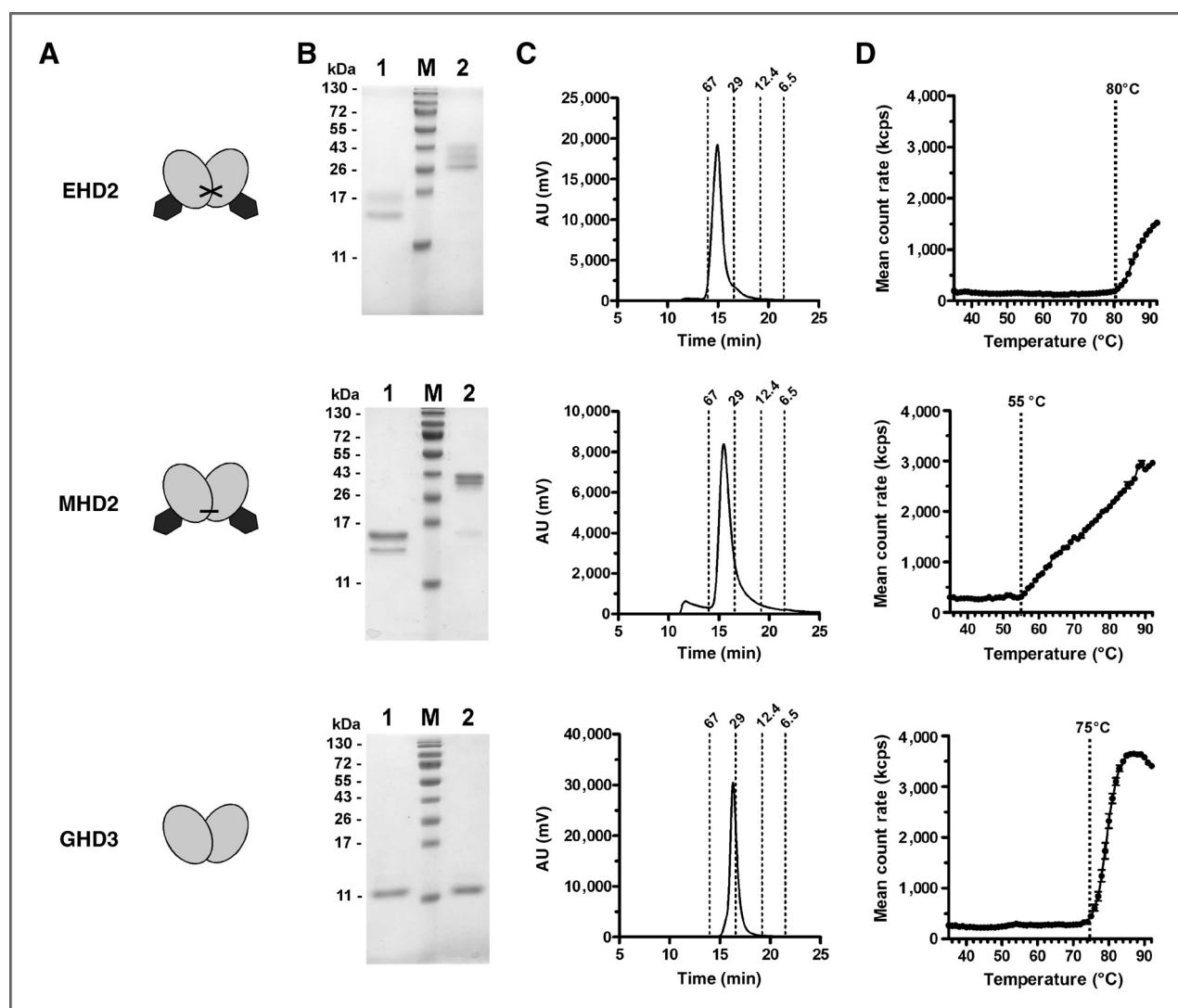


Figure 2. Comparison of EHD2 with the IgM heavy-chain domain 2 (MHD2) and the IgG1 heavy-chain domain 3 (GHD3). A, schematic representation of the domains. N-glycans are shown as black hexagons, disulfide bonds as lines. B, SDS-PAGE analyses of the individual domains expressed in HEK293 cells and purified by IMAC from the cell culture supernatant. Proteins were analyzed under reducing (1) and nonreducing (2) conditions. C, SEC analysis of the domains. D, determination of melting points by dynamic light scattering. The measured melting points are indicated by dotted lines.

was further confirmed by SEC, which showed a single peak with an apparent molecular mass of approximately 49 kD. Purified EHD2 displayed a melting point of 80°C in dynamic light scattering analysis. The biochemical characteristics of EHD2 were compared with those of purified MHD2 (14) and of domain 3 of the human IgG1 heavy chain (GHD3); the latter is routinely used to generate dimeric minibodies (Fig. 2; ref. 20). Similarly as the EHD2, the MHD2 migrated in two bands in SDS-PAGE under reducing conditions (13 and 15 kD) and in three bands under nonreducing conditions (between 38 and 42 kD), whereas GHD3 showed a single band of approximately 12 kD. SDS-PAGE further showed disulfide linkage of the MHD2. Dimeric assembly of MHD2 and GHD3 was confirmed by SEC. Here, MHD2 migrated with an apparent molecular mass of 41 kD and GHD3 migrated with an

apparent molecular mass of 31 kD. MHD2 showed a rather low thermal stability with a melting point of approximately 55°C, whereas GHD3 exhibited a melting point of approximately 75°C.

Generation of EHD2 fusion proteins

Having confirmed the ability of EHD2 to form covalently linked dimers, we generated various fusion proteins, fusing an anti-EGFR scFv to the N-terminus (scFv-EHD2), scTRAIL to the C-terminus (EHD2-scTRAIL), or the scFv to the N-terminus and scTRAIL to the C-terminus of EHD2 (scFv-EHD2-scTRAIL; Fig. 3). All fusion proteins were produced in stably transfected HEK293 cells and purified by affinity chromatography with yields of 7.9 mg/L supernatant for the hexahistidyl-tagged scFv-EHD2, and 2.8 and 4.6 mg/L supernatant for

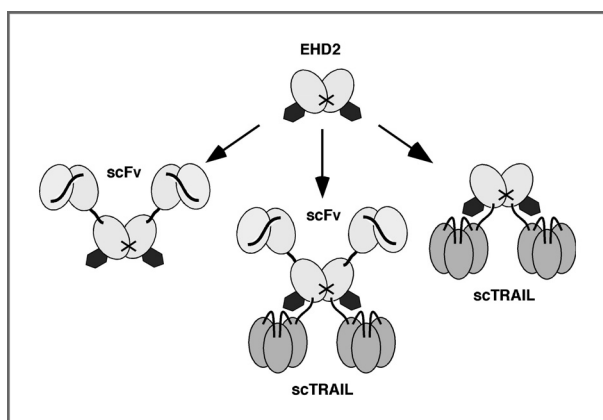


Figure 3. Schematic illustration of the various EHD2 fusion proteins (scFv-EHD2, scFv-EHD2-scTRAIL, and EHD2-scTRAIL).

the FLAG-tagged EHD2-scTRAIL and scFv-EHD2-scTRAIL fusion proteins, respectively. SDS-PAGE analysis confirmed purity and integrity of the fusion proteins as well as formation of disulfide-linked dimers, although only a fraction of the EHD2-scTRAIL and the scFv-EHD2-scTRAIL molecules showed covalent linkage (Fig. 4A). Nevertheless, correct assembly into dimeric molecules was demonstrated by SEC, indicating the presence of dimeric molecules even in the absence of interchain disulfide bonds (Fig. 4B). *N*-glycosylation of the EHD2 was confirmed by deglycosylation of the scFv-EHD2 fusion protein with PNGase F, revealing only a single band in SDS-PAGE under nonreducing conditions, corresponding in size to the faster migrating band observed for the untreated fusion protein (data not shown). For the scTRAIL molecule, a melting point of 46°C was determined by dynamic light scattering, and this is identical to that of the homotrimeric sTRAIL (Supplementary Fig. S1). The EHD2-scTRAIL exhibits a slightly increased melting point of approximately 50°C. The melting point of scFv-EHD2 is 63°C, identical to that of the scFv hu225. The bifunctional scFv-EHD2-scTRAIL fusion protein showed two melting points of 50°C and 66°C, respectively, indicating that the thermal stability is determined by the individual building blocks.

Functionality of the fusion proteins was shown by ELISA (Fig. 4C). Here, scFv-EHD2 and scFv-EHD2-scTRAIL bound to immobilized EGFR-Fc fusion protein, whereas no binding was seen for EHD2-scTRAIL. None of the fusion proteins was capable of binding to the HER2-Fc included as the negative control (not shown). A titration of scFv and scFv-EHD2 for binding to immobilized EGFR in ELISA revealed an approximately 3-fold increased binding of scFv-EHD2 indicating avidity effects of the divalent scFv-EHD2 fusion protein, with an EC_{50} value of 0.84 nmol/L for the scFv and 0.27 nmol/L for scFv-EHD2 (Fig. 4D). In addition, EHD2-scTRAIL and scFv-EHD2-scTRAIL showed binding to recombinant human TRAIL receptor-Fc fusion proteins (TRAILR1-4) in ELISA. Furthermore, scFv-EHD2 and scFv-EHD2-scTRAIL showed

binding to cell lines (Colo205, NCI-H460, and HCT116) expressing different amounts of EGFR, whereas only marginal binding was observed for HepG2 cells lacking detectable expression of EGFR (Fig. 4E and F). Only weak binding of EHD2-scTRAIL was observed for Colo205, NCI-H460, and HepG2, indicating a rather low expression of TRAIL receptors in these cell lines, whereas an increased binding was seen with HCT116. This was confirmed by flow cytometric analysis of the cell lines with monoclonal antibodies directed against TRAIL receptors 1 to 4 (data not shown). These results indicate that both EGFR and TRAIL receptors contribute to the binding of the scFv-EHD2-scTRAIL fusion protein.

***In vitro* induction of cell death by EHD2-scTRAIL fusion proteins**

The cytotoxic activity of the fusion proteins were determined on NCI-H460 and Colo205 cells incubated with the fusion proteins for 16 hours in the absence or presence of the proteasome inhibitor bortezomib, which is known to sensitize tumor cells for TRAIL-induced apoptosis (21). In the absence of bortezomib, scTRAIL did not reach 50% cell death over the analyzed concentration range (1 pmol/L–10 nmol/L). In contrast, EHD2-scTRAIL caused cell death with an EC_{50} of 220 pmol/L (NCI-H460) and 570 pmol/L (Colo205), respectively (Fig. 5, Supplementary Table S1). Compared with EHD2-scTRAIL, cytotoxic activity was further increased for the scFv-EHD2-scTRAIL fusion protein (8-fold for NCI-H460, 18-fold for Colo205), supporting the important role of targeted delivery of TRAIL molecules for efficient apoptosis induction. In the presence of the apoptosis sensitizer bortezomib (250 µg/mL), a left shift of the dose response curve was noted for all scTRAIL fusion proteins. Again, EHD2-scTRAIL was more potent than scTRAIL and strongest bioactivity was observed for scFv-EHD2-scTRAIL (Supplementary Table S1). The NCI-H460 cell line was more sensitive than Colo205; higher amounts of expressed TRAIL receptors 1 and 2 as revealed by flow cytometric analysis (data not shown) may contribute to the observed higher sensitivity. The scFv-EHD2 fusion protein showed no cytotoxic activity over the analyzed concentration range (Supplementary Table S1). To investigate the contribution of scFv-mediated targeting to cell death induction, experiments were repeated in the presence of excess amount of cetuximab, recognizing the same epitope as hu225 (in the presence or absence of bortezomib). Cytotoxic activity of scFv-EHD2-scTRAIL in the presence of cetuximab was reduced to that observed for EHD2-scTRAIL, whereas cetuximab had no effects on the cell death induced by EHD2-scTRAIL (Fig. 5; Supplementary Table S1). In addition, we tested cell-death induction of scTRAIL and the EHD2 fusion proteins on HCT116 and HepG2 in the absence and presence of bortezomib (Supplementary Fig. S2). On these cell lines, the dimeric EHD2-scTRAIL fusion protein was more active than scTRAIL, too. For HCT116, the scFv-EHD2-scTRAIL fusion proteins displayed even stronger activity, similar to that observed for NCI-H460

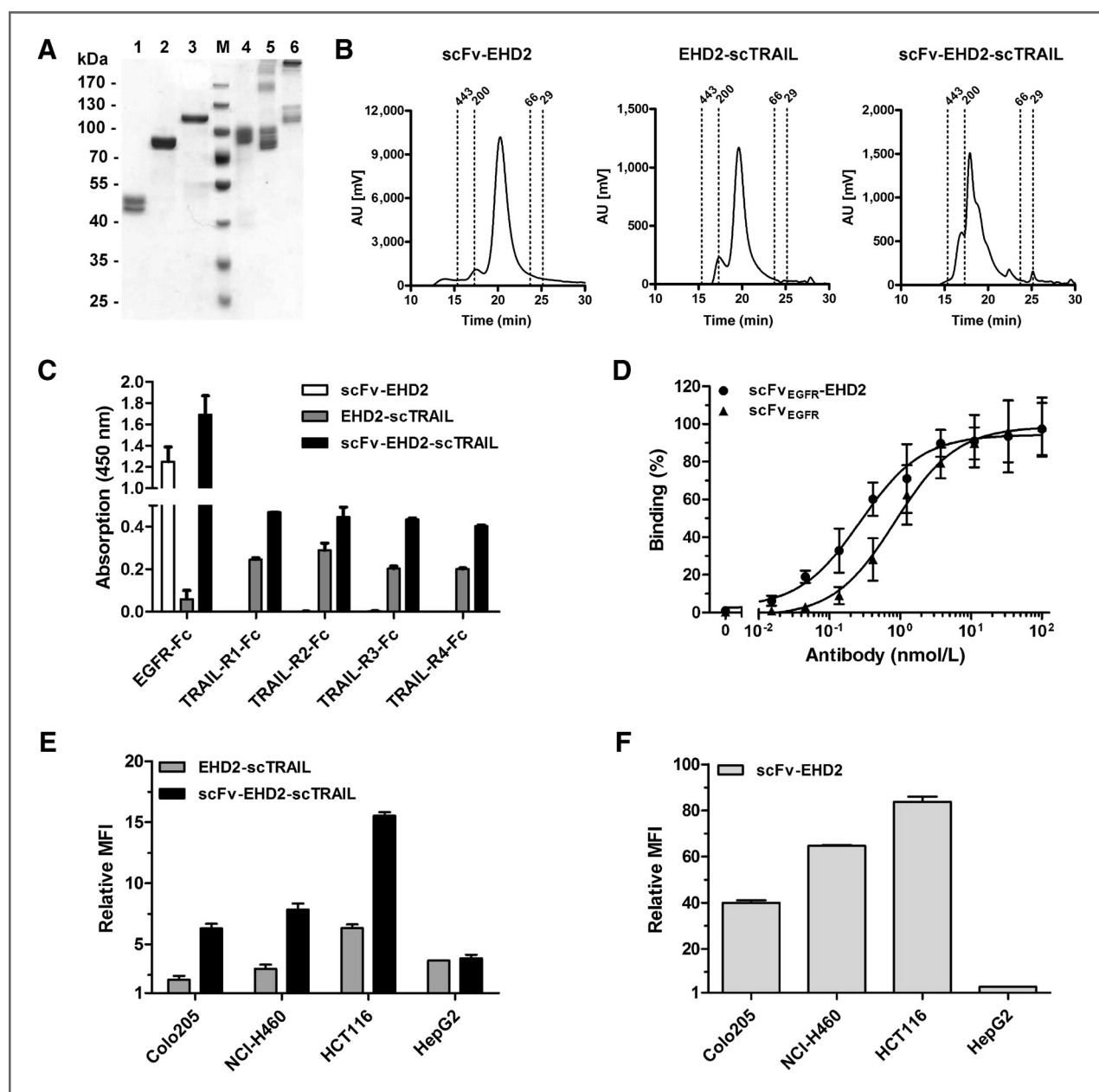


Figure 4. A, SDS-PAGE analysis of the purified fusion proteins under reducing (lanes 1–3) or nonreducing (lanes 4–6) conditions (scFv-EHD2: lanes 1, 4; EHD2-scTRAIL: lanes 2, 5; scFv-EHD2-scTRAIL: lanes 3, 6). B, SEC analysis of the purified fusion proteins. C, ELISA for binding of the fusion proteins to EGFR and human TRAIL receptors 1 to 4 using receptor Fc fusion proteins. D, titration of binding of anti-EGFR scFv and scFv-EHD2 to EGFR-Fc analyzed by ELISA. E, binding of EHD2-scTRAIL and scFv-EHD2-scTRAIL to Colo205, NCI-H460, HCT116, and HepG2 analyzed by flow cytometry using anti-TRAIL antibody and anti-mouse antibody for detection. F, binding of scFv-EHD2 to Colo205, NCI-H460, HCT116, and HepG2 analyzed by flow cytometry using an anti-His-tag antibody for detection.

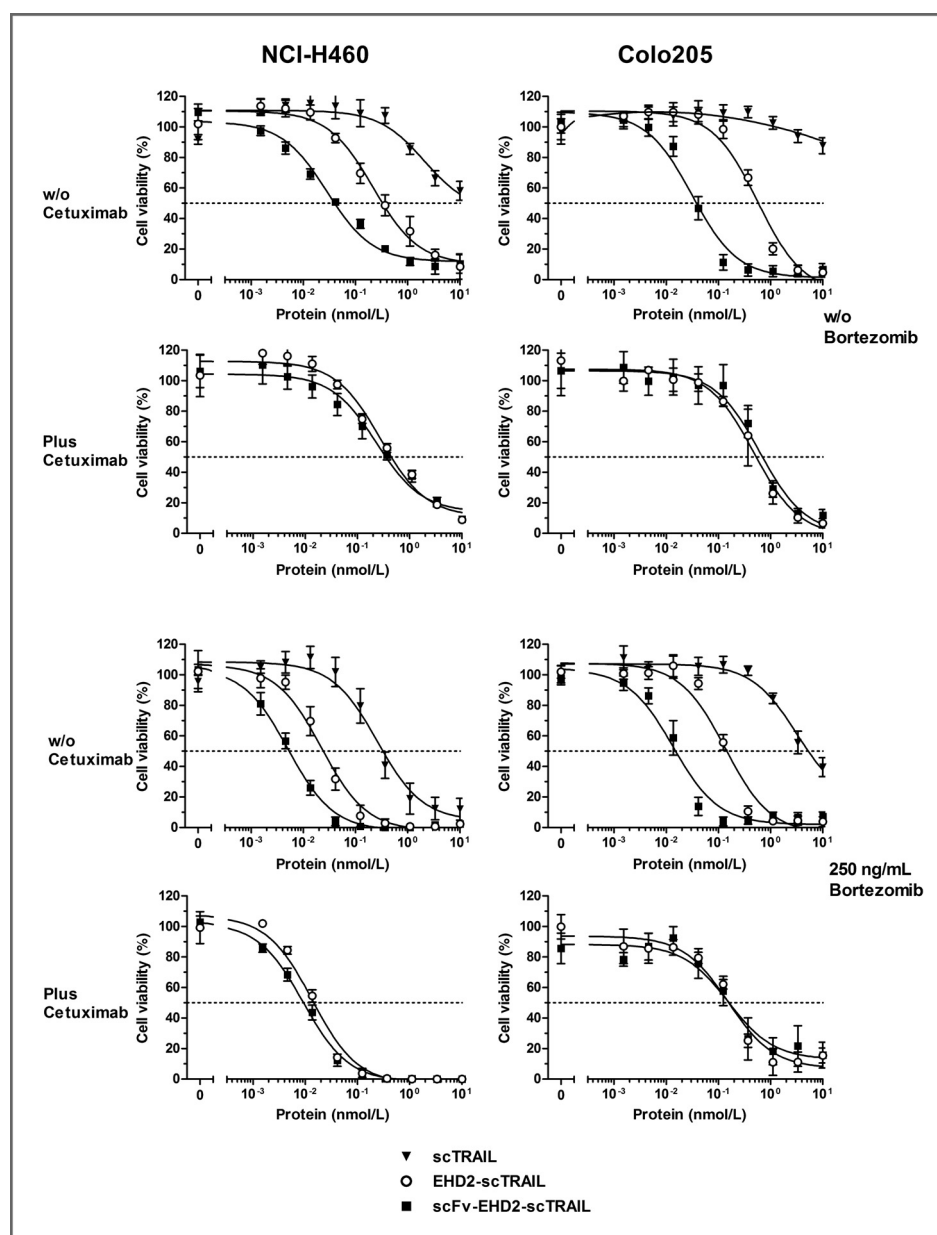
and Colo205, with EC₅₀ values in the low pmol/L range. As a control, on EGFR-negative HepG2 cells, no increased cell death of scFv-EHD2-scTRAIL fusion proteins as compared with the nontargeted dimeric TRAIL was revealed.

In further experiments, we compared cell death induced by a monomeric scFv-scTRAIL fusion protein with that of the dimeric scFv-EHD2-scTRAIL fusion protein. Using NCI-H460 and Colo205, the scFv-EHD2-

scTRAIL fusion protein exhibited an approximately 3.0- to 4.6-fold increased cytotoxic activity compared with scFv-scTRAIL in the presence of bortezomib (data not shown).

Finally, we compared the induction of cell death of dimeric molecules scFv-EHD2-scTRAIL and Db-scTRAIL using Colo205 and NCI-H460 in the absence or presence of bortezomib. In flow cytometry experiments, both

Figure 5. A, *in vitro* cytotoxicity of EHD2-scTRAIL and scFv-EHD2-scTRAIL in comparison with scTRAIL. Cytotoxicity was analyzed with two cell lines (NCI-H460 and Colo205) in the absence or presence of bortezomib (250 ng/mL) and/or cetuximab (200-fold molar excess). Cells were incubated for 16 hours with the fusion proteins, and viable cells were determined by crystal violet staining.



molecules bound equally to these cell lines (data not shown). For both cell lines, scFv-EHD2-scTRAIL appeared slightly more potent than Db-scTRAIL in cell-death induction; however, EC_{50} values were not significantly different ($P > 0.05$; Supplementary Fig. S3).

Pharmacokinetics, *in vivo* tolerance, and therapeutic activity of EHD2-scTRAIL fusion proteins

Pharmacokinetic properties of the fusion proteins were determined in CD1 mice receiving a single intravenous injection of 25 μ g protein (Fig. 6A). All three EHD2 fusion proteins exhibited a prolonged circulation time compared with scTRAIL. Terminal half-lives were increased from 3.0 hours for scTRAIL to between 7.2 and 9.4 hours for the

EHD2 fusion proteins resulting also in a 3- to 4-fold increased AUC_{0-24h} (Supplementary Table S2). We further determined the pharmacokinetic properties of the dimeric Db-scTRAIL fusion protein, which exhibited a terminal half-life of approximately 3.5 hours and an AUC_{0-24h} similar to that of scFv-EHD2 and EHD2-scTRAIL (Supplementary Table S2). Differences of the terminal half-life and AUC between scTRAIL and the EHD2 fusion proteins were all statistically significant ($P < 0.05$), whereas the differences in the AUC of the fusion proteins were statistically nonsignificant from each other ($P > 0.05$). Next, we performed an ALT assay to investigate possible liver toxicity of the scFv-EHD2-scTRAIL fusion protein in combination with bortezomib (Fig. 6B). Blood samples

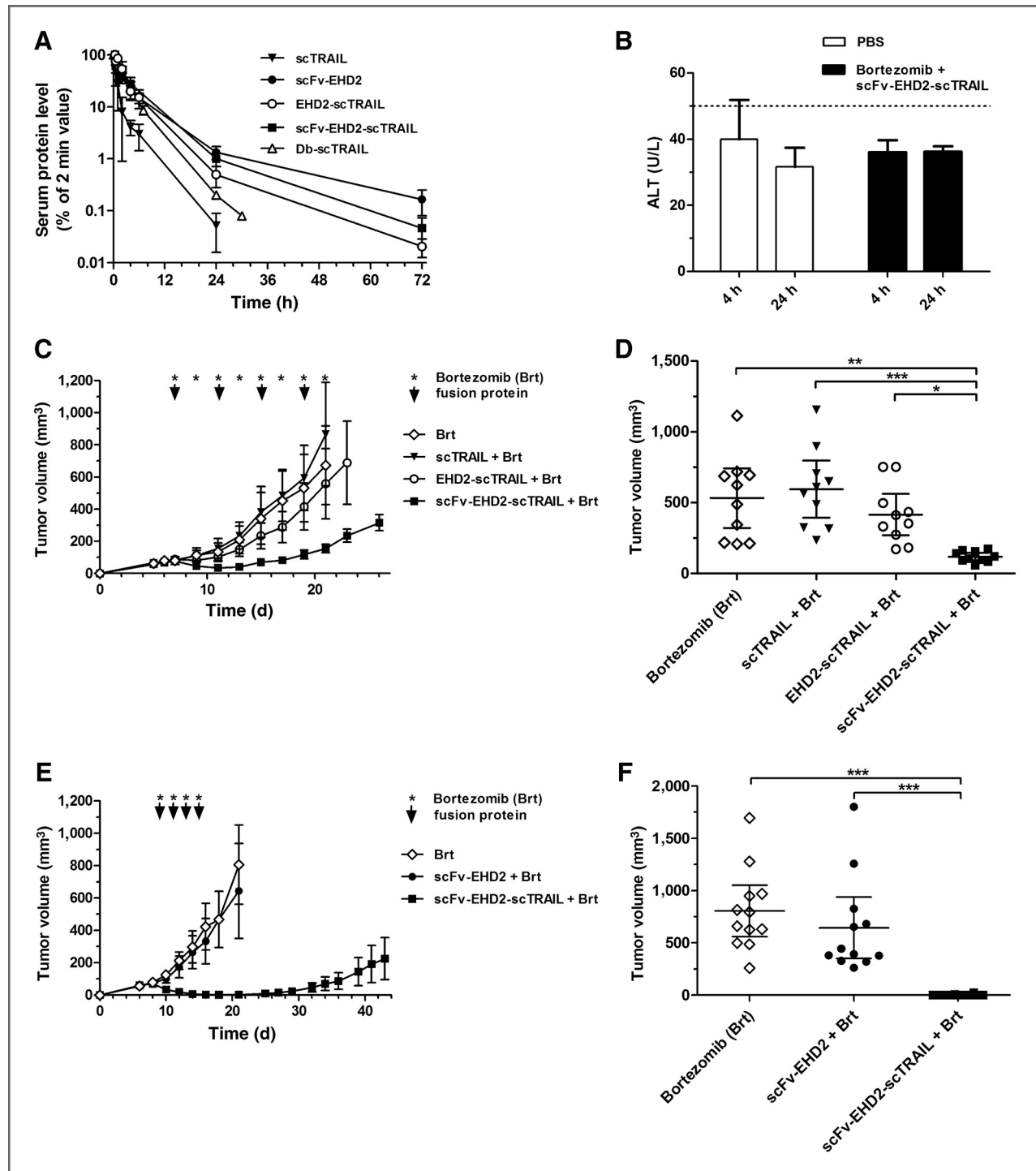


Figure 6. A, pharmacokinetics of EHD2 fusion proteins in mice. ScTRAIL, Db-scTRAIL, and the EHD2 fusion proteins (25 μ g per animal) were injected intravenously into CD1 mice ($n = 3$) and serum concentrations were determined by ELISA. B, ALT assay performed 4 or 24 hours after injection of PBS or scFv-EHD2-scTRAIL together with bortezomib. C, *in vivo* antitumor activity of EHD2-scTRAIL and scFv-EHD2-scTRAIL in comparison with scTRAIL. NMRI nude mice bearing subcutaneous Colo205 xenograft tumors received four intravenous injections of the proteins (0.35 nmol of the fusion proteins and 0.7 nmol of scTRAIL) every 4 days as well as eight intraperitoneal injections of bortezomib (Brt; 5 μ g per injection) on alternate days. D, tumor volumes at day 19. E, *in vivo* antitumor activity of scFv-EHD2-scTRAIL in comparison with scFv-EHD2. NMRI nude mice bearing subcutaneous Colo205 xenograft tumors received four intravenous injections of the proteins (1 nmol fusion proteins) as well as four intraperitoneal injections of Brt 5 μ g per injection on alternate days. F, tumor volumes at day 21.

analyzed at 4 or 24 hours after injection of the fusion protein (1 nmol) and bortezomib (5 µg) into CD1 mice did not reveal any increased serum ALT activity.

The scTRAIL fusion proteins were then tested for their antitumor activity in nude mice bearing subcutaneous Colo205 tumors, which is an established *in vivo* model to study antitumor activities of TRAIL (22–24). In a first experiment, mice received four consecutive intravenous injections of scTRAIL, EHD2-scTRAIL, or scFv-EHD2-scTRAIL, respectively, over a period of 12 days. Treatment was started when tumors had a size of approximately 100 mm³. Doses of 0.7 nmol scTRAIL and 0.35 nmol EHD2-scTRAIL and scFv-EHD2-scTRAIL were used; thus, mice received equimolar doses in respect to scTRAIL. In addition, all mice, including a control group, received bortezomib (intraperitoneally) on alternate days over a period of 14 days (Fig. 6C). In a previous study, we showed that bortezomib at this dose does not induce any antitumor effects in this xenograft tumor model (11). With the doses of reagents applied, a statistically significant reduction of tumor growth was observed for scFv-EHD2-scTRAIL, whereas EHD2-scTRAIL resulted only in a minor response and scTRAIL had no effect compared with the bortezomib control (Fig. 6D).

In a second experiment, we compared the activity of scFv-EHD2-scTRAIL and scFv-EHD2 to investigate the potential contribution of EGFR receptor (EGFR) blocking on the therapeutic activity of the fusion protein. In this experiment, a dose of 1 nmol protein (2 nmol with regard to scTRAIL) was applied. Animals received, in total, four intravenous injections of the proteins together with bortezomib (intraperitoneally) on alternate days starting at day 8 after tumor cell inoculation (Fig. 6E). Bortezomib alone was included as control group. A strong reduction of tumor growth with macroscopically complete remissions during treatment was observed for scFv-EHD2-scTRAIL, whereas scFv-EHD2 did not interfere with tumor growth. Approximately 10 days after the last treatment with scFv-EHD2-scTRAIL, slow tumor regrowth became detectable. Nevertheless, a comparison of tumor volumes at day 21 revealed a statistically significant inhibition of tumor growth for scFv-EHD2-scTRAIL (Fig. 6F).

Discussion

Using an scFv directed against EGFR and a single-chain derivative of human TRAIL (scTRAIL) as the effector moiety, we established, in this study, that the EHD2 is a suitable dimerization building block for the generation of tetravalent and bifunctional molecules (scFv-EHD2-scTRAIL) with improved antitumoral activity *in vitro* and *in vivo*. Compared with the previously described MHD2 (14), the EHD2 displays superior stability, evident from a strongly increased thermal stability (melting point 80°C vs. 56°C for EHD2 and MHD2, respectively). In addition, this melting point is higher than that of the CH3 domain derived from human IgG1 (GHD3), which is not cova-

lently linked by disulfide bonds, emphasizing the superior properties of the EHD2 for the generation of dimeric fusion proteins. Similar to MHD2 (14), the use of EHD2 as a dimerization module has the advantage that molecules can be fused to both ends of this domain, allowing great flexibility and modularity in generating bivalent and bifunctional fusion proteins.

Our results support previous findings (11) which showed that the valency of scTRAIL fusion proteins has a tremendous impact on the induction of apoptosis in cancer cell lines and the antitumor activity *in vivo*. Thus, we found that dimeric EHD2-scTRAIL and scFv-EHD2-scTRAIL fusion proteins are more potent in inducing cancer cell death as compared with monomeric scTRAIL and scFv-scTRAIL. Although not mechanistically addressed here, we reason that similar mechanisms apply for the enhanced cell-death induction by the EHD2-dimerized bifunctional TRAIL fusion proteins described here and those reported previously, in which dimerization was achieved through generation of diabody-scTRAIL fusion proteins (11). Accordingly, enhanced apoptosis-inducing activity might be attributed to two distinct structural features of the fusion protein: (i) a covalently linked dimer of a scTRAIL molecule, which on its own already displays higher bioactivity compared with scTRAIL, and (ii) an increased scTRAIL-TRAIL receptor interaction stabilized by binding to the tumor-associated antigen, leading to sustained receptor activation. In particular, for optimum activation, TRAILR2 apparently requires natural membrane TRAIL or membrane-targeted TRAIL in the form of fusion proteins (12). Importantly, targeting of the scTRAIL to EGFR-expressing tumor cells enhanced apoptotic activity approximately 8- to 18-fold, depending on the cancer cell lines studied, compared with the untargeted bivalent EHD2-scTRAIL fusion protein, demonstrating the beneficial effects of scFv-mediated binding of the proapoptotic molecule to target cells. In itself, the scFv-EHD2 fusion protein did not exhibit cytotoxic activity against these tumor cells, indicating that the improved activity is not caused by the inhibition of EGFR-mediated signaling in the tested cell lines. This finding is in accordance with our previous results on EGFR-targeting diabody-scTRAIL molecules (11). Both, Colo205 and NCI-H460 are described to be nonresponsive to anti-EGFR antibodies such as cetuximab due to mutations in downstream signaling pathways (11, 25). In contrast, other anti-EGFR scFv-TRAIL fusion proteins showed rapid inactivation of the EGFR signaling pathways in various other tumor cell lines (e.g., A431; ref. 26). In our study, we applied a humanized version of clinically approved antibody cetuximab for the generation of the antibody-scTRAIL fusion proteins. Thus, tumor cells sensitive for inhibition by cetuximab are potentially particularly sensitive to treatment with the bifunctional, EGFR-blocking, and TRAILR-activating scFv-EHD2-scTRAIL fusion protein.

The activity of the scTRAIL fusion proteins was strongly increased in the presence of bortezomib known to

sensitize cells for TRAIL-mediated apoptosis induction. Bortezomib is a proteasome inhibitor, and is clinically approved as Velcade for the treatment of multiple myeloma and mantle cell lymphoma (27, 28). Bortezomib can directly or indirectly affect signaling and apoptosis induction by TRAIL at multiple levels, including upregulation of TRAIL receptors 1 and 2 and modulation of the expression or activity of proapoptotic as well as antiapoptotic molecules (29). In addition, other TRAIL receptor sensitizers might be useful in combination with the EHD2-scTRAIL fusion proteins, including for example Smac mimetics and chemotherapeutic drugs (30–34).

Compared with scTRAIL, all EHD2 fusion proteins exhibited an approximately 2.5- to 3-fold prolonged serum half-life. This is probably due to an increased hydrodynamic radius diminishing clearance by renal filtration (35). The longer half-life can have a direct impact on the therapeutic activity by maintaining an effective concentration over a prolonged period of time, reflected by an increased AUC. However, the terminal half-lives of the fusion proteins are in the range of 6 to 10 hours, which is much shorter than that of full IgG molecules. Approaches to further increase the half-life of the EHD2 fusion proteins, for example, by PEGylation, fusion to albumin or Fc fragments, or alternatively to albumin or Ig-binding domains, might further increase the therapeutic activity (36–38). However, these strategies also affect the hydrodynamic radius of the molecules and, thus, may influence tissue penetration and receptor binding.

In summary, our *in vitro* and *in vivo* experiments demonstrated a potent antitumor activity of the scFv-EHD2-scTRAIL fusion protein and further established the advantages of combining a tumor-targeting moiety with dimerization of scTRAIL (effector moiety) through a separate dimerization moiety. This modular composition allows a combination of the EHD2 with different targeting and effector molecules, including also dual targeting strategies (39). In a previous study, we obtained dimeric scTRAIL molecules through dimeric assembly of the

targeting moiety driven by the expression as a bivalent diabody (Db; ref. 11). This approach is, thus, limited to the use of V_H and V_L domains of antibodies for targeting. In contrast, the EHD2 is applicable to any kind of targeting ligand, including other antibody formats (e.g., Fab, single-domain antibodies, nanobodies; ref. 40), novel scaffold proteins (41), as well as natural ligands (growth factors, hormones, cytokines) and peptides (42). Therefore, the EHD2 represents a versatile building block for the generation of targeted multivalent and multifunctional protein therapeutics.

Disclosure of Potential Conflicts of Interest

O. Seifert, A. Plappert, and R.E. Kontermann have ownership interest (including patents) covering the EHD2 technology. K. Pfizenmaier and R. E. Kontermann have ownership interest (including patents) covering the scTRAIL technology; both K. Pfizenmaier and R.E. Kontermann are consultants and have received commercial research funding from SME and BioNTech, respectively. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A. Plappert, R.E. Kontermann
Development of methodology: O. Seifert, A. Plappert, R.E. Kontermann
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Seifert, S. Fellermeier, M. Siegemund
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Seifert, A. Plappert, M. Siegemund, R.E. Kontermann
Writing, review, and/or revision of the manuscript: O. Seifert, A. Plappert, K. Pfizenmaier, R.E. Kontermann
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Plappert
Study supervision: R.E. Kontermann

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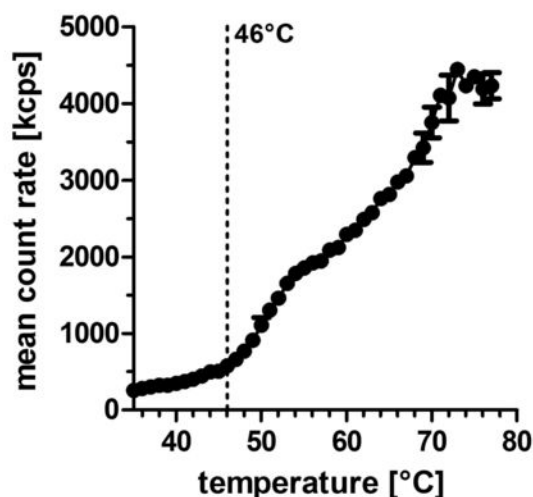
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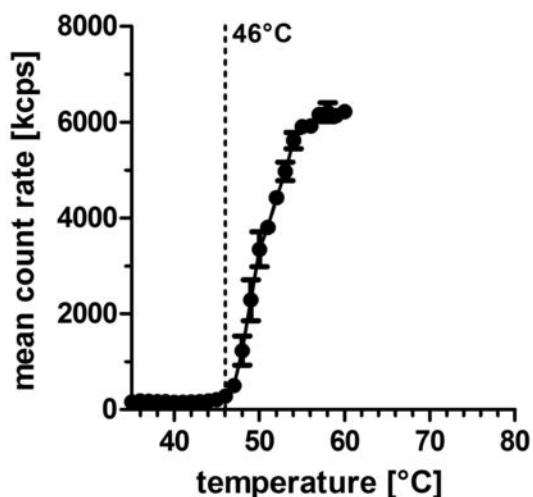
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Suppl. Figure 1

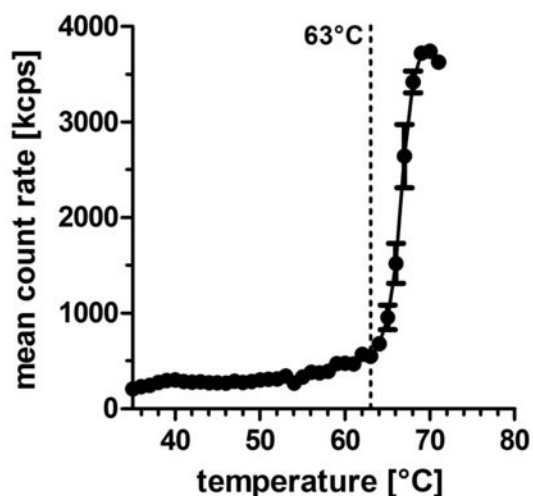
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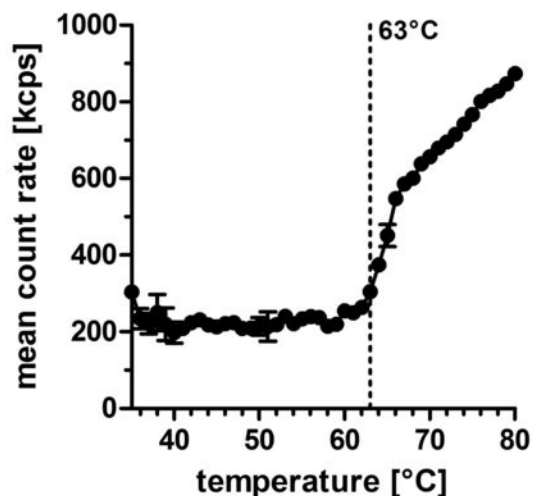
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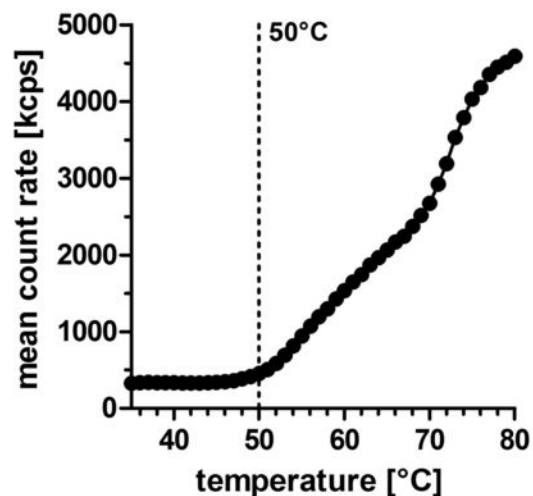
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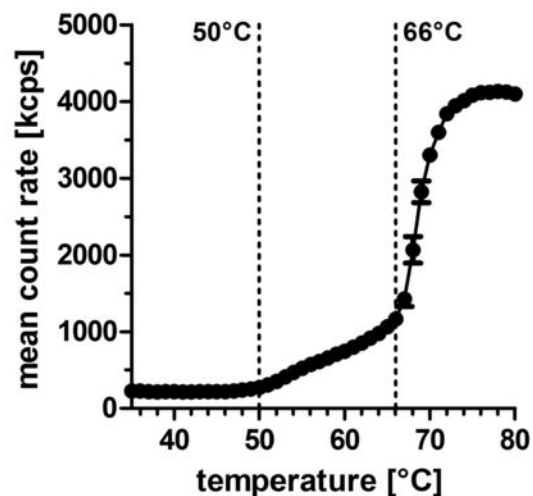
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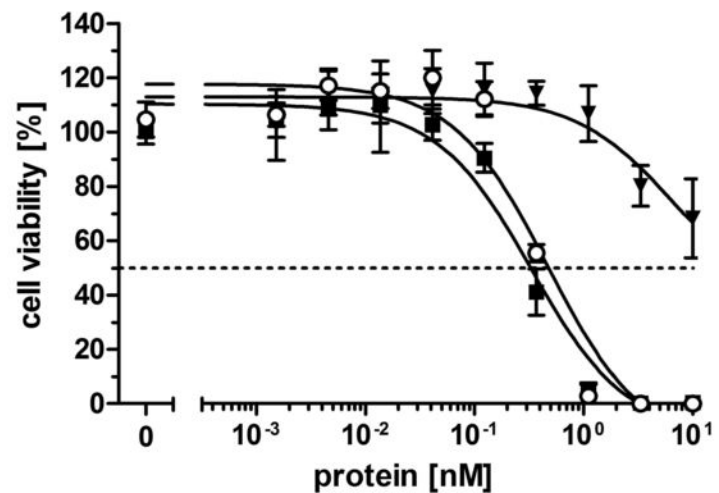
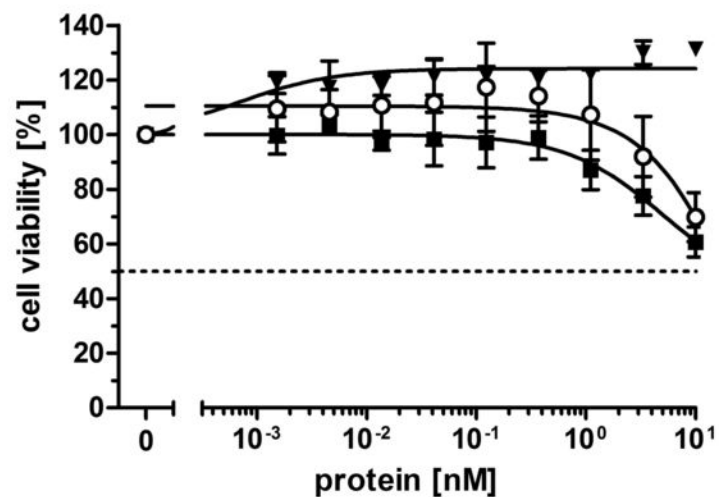
EHD2-scTRAIL



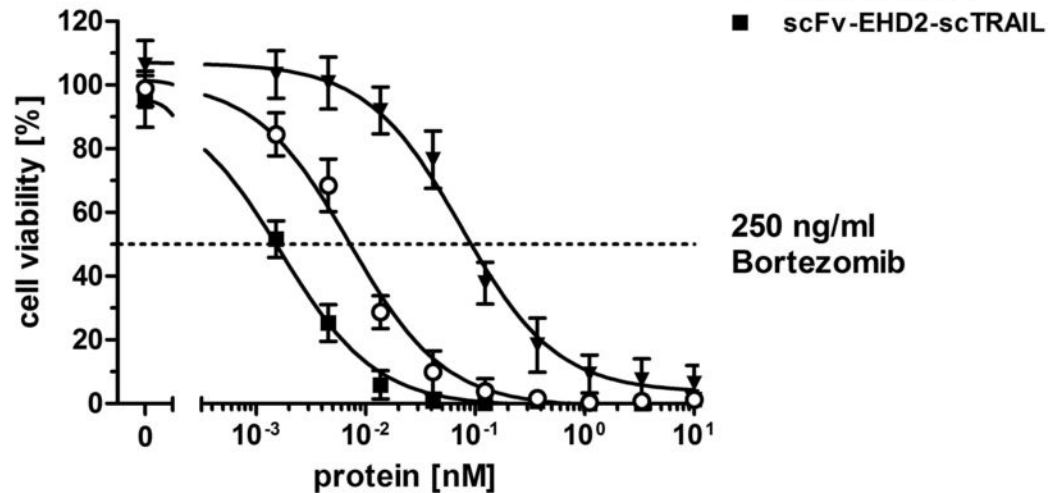
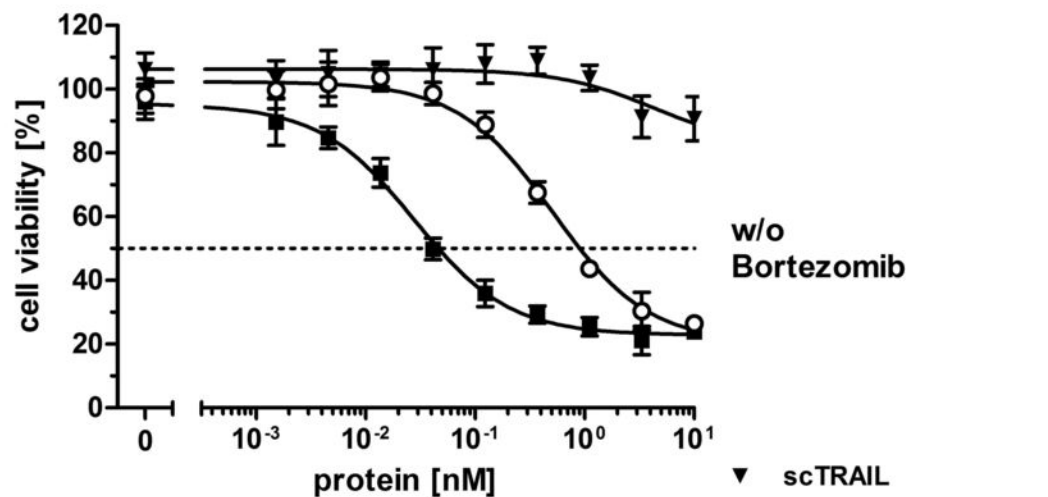
scFv-EHD2-scTRAIL



HepG2



HCT116

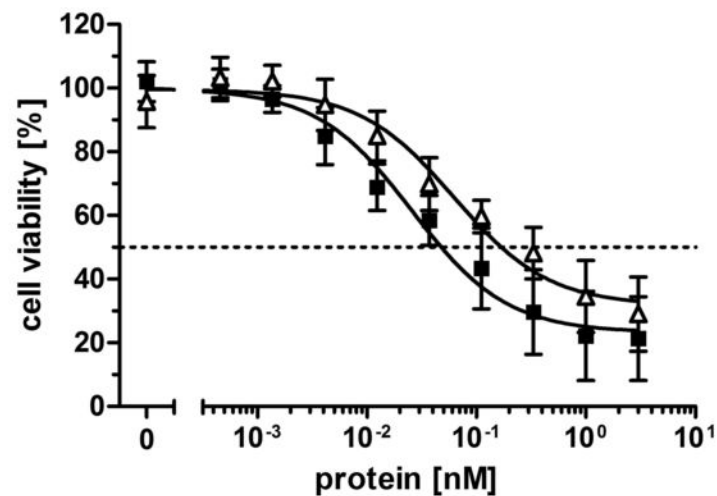


w/o
Bortezomib

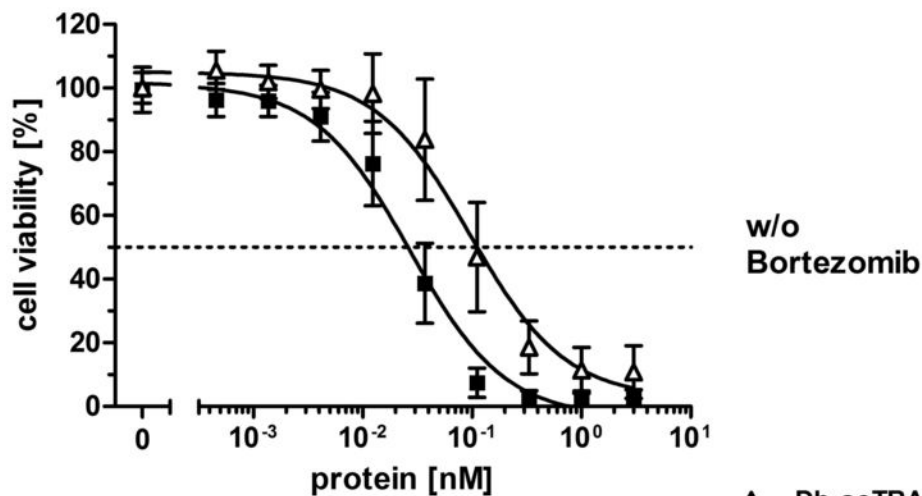
250 ng/ml
Bortezomib

▼ scTRAIL
○ EHD2-scTRAIL
■ scFv-EHD2-scTRAIL

NCI-H460

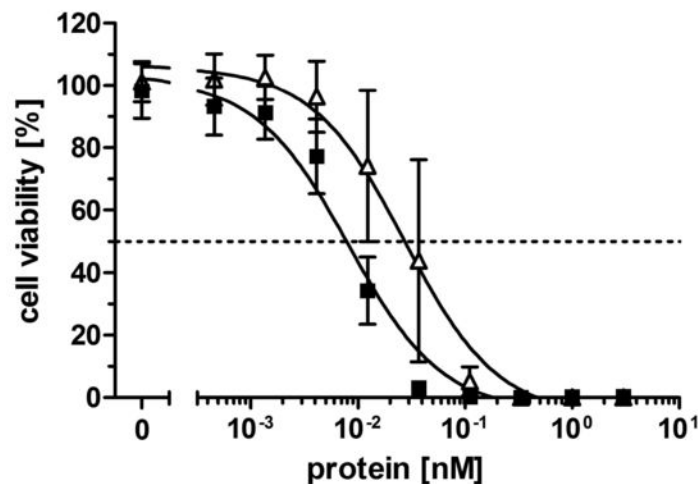
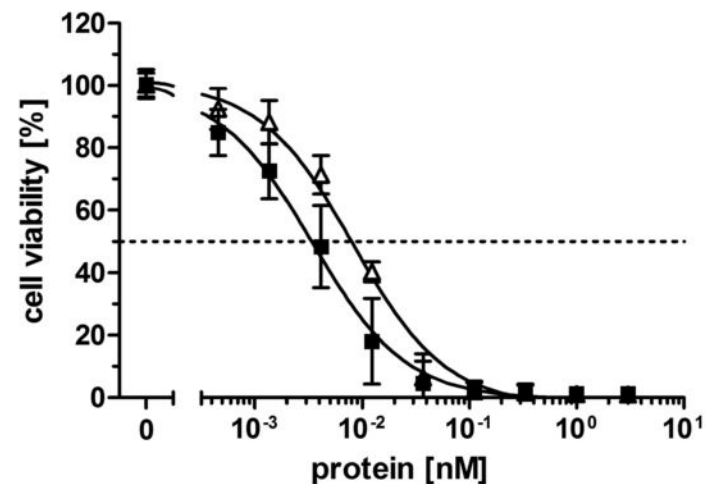


Colo205



w/o
Bortezomib

Δ Db-scTRAIL
■ scFv-EHD2-scTRAIL



250 ng/ml
Bortezomib

Supplementary Figures legends

Supplementary Figure 1. Determination of melting points of the different fusion proteins by dynamic light scattering. The measured melting points are indicated by dotted lines.

Supplementary Figure 2. In vitro cytotoxicity of EHD2-scTRAIL and scFv-EHD2-scTRAIL in comparison with scTRAIL. Cytotoxicity was analyzed with two cell lines (HepG2, HCT116) in absence or presence of bortezomib (250 ng/ml). Cells were incubated for 16 h with the fusion proteins and viable cells were determined by crystal violet staining.

Supplementary Figure 3. In vitro cytotoxicity of scFv-EHD2-scTRAIL in comparison with Db-scTRAIL. Cytotoxicity was analyzed with two cell lines (NCI-H460, Colo205) in absence or presence of bortezomib (250 ng/ml). Cells were incubated for 16 h with the fusion proteins and viable cells were determined by crystal violet staining.

Supplementary data

Supplementary Table 1: In vitro cytotoxicity

fusion protein	Bortezomib	Cetuximab	EC ₅₀ (pM)	
			NCI-H460	Colo205
scTRAIL	-	-	-	-
	+	-	270 ± 40	3,470 ± 1,590
scFv-EHD2	-	-	-	-
	-	+	-	-
	+	-	-	-
	+	+	-	-
EHD2-scTRAIL	-	-	220 ± 90	570 ± 50
	-	+	280 ± 10	530 ± 180
	+	-	22 ± 6	140 ± 20
	+	+	14 ± 2	160 ± 0.7
scFv-EHD2-scTRAIL	-	-	28 ± 2	31 ± 6
	-	+	240 ± 50	610 ± 90
	+	-	5 ± 1	14 ± 4
	+	+	9 ± 0	156 ± 8

Supplementary Table 2: Pharmacokinetic properties of EHD2 fusion proteins

construct	M_r (kDa)	S_r (nm)	$t_{1/2}\beta$ (h)	AUC_{0-24h} (%h)
scTRAIL	67.5	3.3 [#]	3.0 ± 0.3	127 ± 30
scFv-EHD2	82.8	4.4	9.4 ± 0.5	358 ± 89
EHD2-scTRAIL	164.6	4.6	6.5 ± 0.4	388 ± 135
scFv-EHD2-scTRAIL	218.6	5.1	7.2 ± 0.9	508 ± 112
Db-scTRAIL	187.0	5.0	3.5 ± 0.3	326 ± 29

The molecular masses were calculated for the dimeric molecules

[#] *data from (11)*