

Potent antitumoral activity of TRAIL through generation of tumor-targeted single-chain fusion proteins

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In an attempt to improve TRAIL's (tumor necrosis factor-related apoptosis-inducing ligand) tumor selective activity a variant was designed, in which the three TRAIL protomers are expressed as a single polypeptide chain (scTRAIL). By genetic fusion with a single-chain antibody fragment (scFv) recognizing the extracellular domain of ErbB2, we further equipped scTRAIL with tumor-targeting properties. We studied tumor targeting and apoptosis induction of scFv-scTRAIL in comparison with non-targeted scTRAIL. Importantly, the tumor antigen-targeted scTRAIL fusion protein showed higher apoptotic activity *in vitro*, with a predominant action by TRAIL-R2 signaling. Pharmacokinetic studies revealed increased plasma half-life of the targeted scTRAIL fusion protein compared with scTRAIL. *In vivo* studies in a mouse tumor model with xenotransplanted Colo205 cells confirmed greater response to the ErbB2-specific scTRAIL fusion protein compared with non-targeted scTRAIL both under local and systemic application regimen. Together, *in vitro* and *in vivo* data give proof of concept of higher therapeutic activity of tumor-targeted scFv-scTRAIL molecules. Further, we envisage that through targeting of scTRAIL, potential side effects should be minimized. We propose that scFv-mediated tumor targeting of single-chain TRAIL represents a promising strategy to improve TRAIL's antitumoral action and to minimize potential unwanted actions on normal tissues.

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Subject Category: Cancer

Although for many malignancies traditional cytotoxic chemotherapy is still the treatment of choice, targeted therapies gain increasing importance because they are expected to exert higher tumor-specific activity and less dose-limiting side effects. The anti-CD20 antibody Rituximab and the Bcr-Abl-selective kinase inhibitor Gleevec, respectively, are two vivid examples for the success of targeted therapy (for review, see Gerber¹). Targeting the apoptotic machinery of malignant cells has become an attractive concept, beginning with tumor necrosis factor (TNF) and its now established clinical use for locoregional treatment of limb metastases of soft tissue sarcoma and melanoma² and followed by the current exploitation of other proapoptotic members of the TNF ligand family – CD95L and TNF-related apoptosis-inducing ligand (TRAIL) – being presently at various stages of preclinical and clinical development (for review, see Call *et al.*,³ Gerspach *et al.*⁴ and Newsom-Davis *et al.*⁵). TRAIL, similar to TNF a type II transmembrane protein, is expressed on various immune effector cells and appears critically involved in antitumor responses in the body.⁶ It is probably the best candidate of a death ligand for systemic application

because preclinical studies revealed that, in contrast to TNF and CD95 agonists, the application of trimeric, soluble TRAIL is well tolerated. Concomitantly, it demonstrated potent antitumor activity in tumor models based on tumor cell lines and primary tumor samples.^{7–10} Although first clinical trials in various advanced cancers are promising, as stable disease has been reported in many cases, TRAIL monotherapy most likely will not result in a sufficient overall therapeutic activity.¹¹ Concurrently, this faces the potential problem that under combined treatment options normal cells might also be sensitized to TRAIL-induced apoptosis (reviewed by Falschlehner *et al.*¹²). Furthermore, we have recently shown that TRAIL-R1 is activated by the soluble as well as the membrane-bound form of the ligand, whereas TRAIL-R2 is preferentially activated by membrane TRAIL or oligomerized soluble trimeric TRAIL.^{13,14} As agonistic TRAIL-R2-specific antibodies likewise display potent antitumor activity (reviewed by Johnstone *et al.*¹⁵), it is conceivable that homotrimeric TRAIL could be improved in its activity provided efficient TRAIL-R2 signaling is ensured through genetic modifications or other means. On the other hand, the spontaneous

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Abbreviations: i.v., intravenous(ly); TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR1/2, TRAIL receptor 1/2; scFv, single-chain Fv fragment; scTRAIL, single-chain TRAIL; s.c., subcutaneous(ly)

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aggregation of some recombinant TRAIL preparations, resembling secondarily crosslinked TRAIL, has been associated with unwanted actions on normal tissues and thus curtail their use as tumor-selective apoptosis inducers (reviewed by Koschny *et al.*¹⁶). Thus, TRAIL variants mimicking the full bioactivity of membrane TRAIL could present powerful cancer therapeutics provided that a tumor-restricted action can be achieved. A further apparent limitation of the currently clinically investigated recombinant TRAIL reagents is their rather short *in vivo* half-life. Therefore, an improvement of pharmacokinetic properties might also lead to increased therapeutic action. A potential solution to increase specific activity and bioavailability is the combination of TRAIL function with tumor targeting. Thus, we generated new TRAIL fusion proteins containing an antibody fragment (single-chain Fv fragment (scFv)) for targeting TRAIL to the tumor to enrich the therapeutic at the tumor site and to enhance its specific bioactivity. ErbB2, similar to EGFR/ErbB1, ErbB3 and ErbB4 belonging to the ErbB receptor tyrosine kinase family, represents a clinically relevant target antigen. It is highly expressed on a variety of human solid tumors (reviewed by Holbro and Hynes¹⁷) and there is already one humanized monoclonal antibody (mAb) directed against the extracellular domain of ErbB2 in clinical use (Trastuzumab) and approved for treatment of breast carcinoma. Several other mAbs are in different stages of clinical evaluation (reviewed by Baselga and Swain¹⁸). Although the clinical data provide clear evidence that ErbB2 is a relevant target, they also show that the therapeutic effect of the current antibody reagents is rather limited.¹⁹ This justifies the search for additional ErbB2-targeted strategies.

Recently, a single-chain TNF molecule, consisting of three TNF monomers fused by short peptide linkers, was described to exert enhanced stability and antitumoral activity.²⁰ We have designed an analogous single polypeptide chain TRAIL variant (scTRAIL), which was used to construct a scFv–scTRAIL fusion protein for tumor targeting. We have compared the bioactivities of this fusion protein with non-targeted scTRAIL in *in vitro* and *in vivo* tumor models. We show here that the tumor antigen-targeted scTRAIL fusion protein showed higher apoptotic activity to scTRAIL *in vitro*, with a predominant action by TRAIL-R2 signaling on Colo205 colon carcinoma cells. *In vivo* studies in a mouse xenograft tumor model confirmed significantly higher response to the tumor-targeted ErbB2-specific scTRAIL fusion protein.

Results

Construction and production of TRAIL fusion proteins.

To generate a single-chain TRAIL molecule, we followed the design principle described for scTNF²⁰ and covalently fused three TRAIL monomers, each consisting of the extracellular domain of TRAIL (aa 95–281) through two peptide linkers comprising four repeats of the sequence GGS (Figure 1a). To facilitate purification and analysis an N-terminal FLAG tag was added. Furthermore, by additional N-terminal fusion of a single-chain antibody fragment (scFv) recognizing the FRP5 epitope within the extracellular domain of ErbB2,²¹ we generated an ErbB2-specific scTRAIL fusion protein (Figure 1a).

Both scTRAIL and scFv–scTRAIL were purified by affinity chromatography on monoclonal M2 anti-FLAG agarose from the supernatant of stably transfected HEK293 cells with yields of about 1 mg protein per liter cell culture supernatant. Immunoblot analysis and SDS-PAGE (Figure 1b) of the purified protein showed single protein bands with a molecular mass of ~70 kDa for scTRAIL and ~100 kDa for scFv–scTRAIL matching the expected calculated molecular masses of 71 and 98 kDa, respectively. Gel filtration analysis indicated a monomeric organization of both scTRAIL variants, corresponding, with respect to the TRAIL part, to a noncovalently assembled trimer of a conventional recombinant TRAIL molecule (Figure 1c). The molecular masses deduced from SEC were slightly lower compared with that derived from SDS-PAGE (Figure 1b), which did not result from degradation as verified by immunoblot analysis of collected fractions (Figure 1c). In the scTRAIL preparation, the minor fraction eluting in SEC at apparent higher molecular weight, thus potentially comprising aggregated complexes, did not display disproportionately high bioactivity, as revealed from comparison of cytotoxic activity of fraction 2 and 15, taking relative protein amounts into consideration (data not shown).

Target antigen-specific binding of scFv–scTRAIL. To analyze specific binding of scFv–scTRAIL to ErbB2-positive cells, flow cytometry analysis was performed. ErbB2 expression analysis confirmed a median *versus* low expression level for Colo205 colon carcinoma and HT1080 fibrosarcoma cells, respectively (Figure 2a), compared with SKBR3 cells that are well-known to highly express the ErbB2 protein (data not shown).²² Incubation of Colo205 and HT1080 cells with the fusion protein revealed a specific, binding to ErbB2-positive cells (Figure 2a and b) compared with incubation with the non-targeted scTRAIL, which resulted only in a weak fluorescence signal, either by detection with anti-TRAIL (Figure 2a) or with anti-FLAG antibodies (data not shown). As binding of homotrimeric FLAG-tagged TRAIL showed similar weak signals (data not shown), this could reflect a low level of TRAIL receptor (TRAILR) expression on the investigated target cells and/or insufficient sensitivity of FACS analyses. ErbB2-specific binding was confirmed by using TRAILR1- and R2-Fc fusion proteins for detection of scFv–scTRAIL binding to target cells (Figure 2b) and competition with anti-ErbB2 antibodies (FRP5). This led to a near complete inhibition of scFv–scTRAIL binding to the ErbB2-positive cell line (Figure 2b).

Target antigen-restricted induction of cell death by scFv–scTRAIL.

To address the bioactivity of scFv–scTRAIL *in vitro*, we first analyzed induction of cell death on ErbB2-negative SKW6.4 cells, which are known to be TRAIL-R1 and TRAIL-R2 positive^{23,24} (Figure 3a). Although scTRAIL and scFv–scTRAIL did not show cytotoxic activity up to 8 nM, respectively, in the presence of the crosslinking anti-FLAG antibody M2 comparable bioactivities of both fusion proteins were observed (Figure 3a). In contrast, incubation of ErbB2-positive Colo205 and HT1080 cells with scTRAIL or scFv–scTRAIL revealed an approximately three- to fivefold higher bioactivity of the ErbB2-specific scFv–scTRAIL compared with the non-targeted scTRAIL

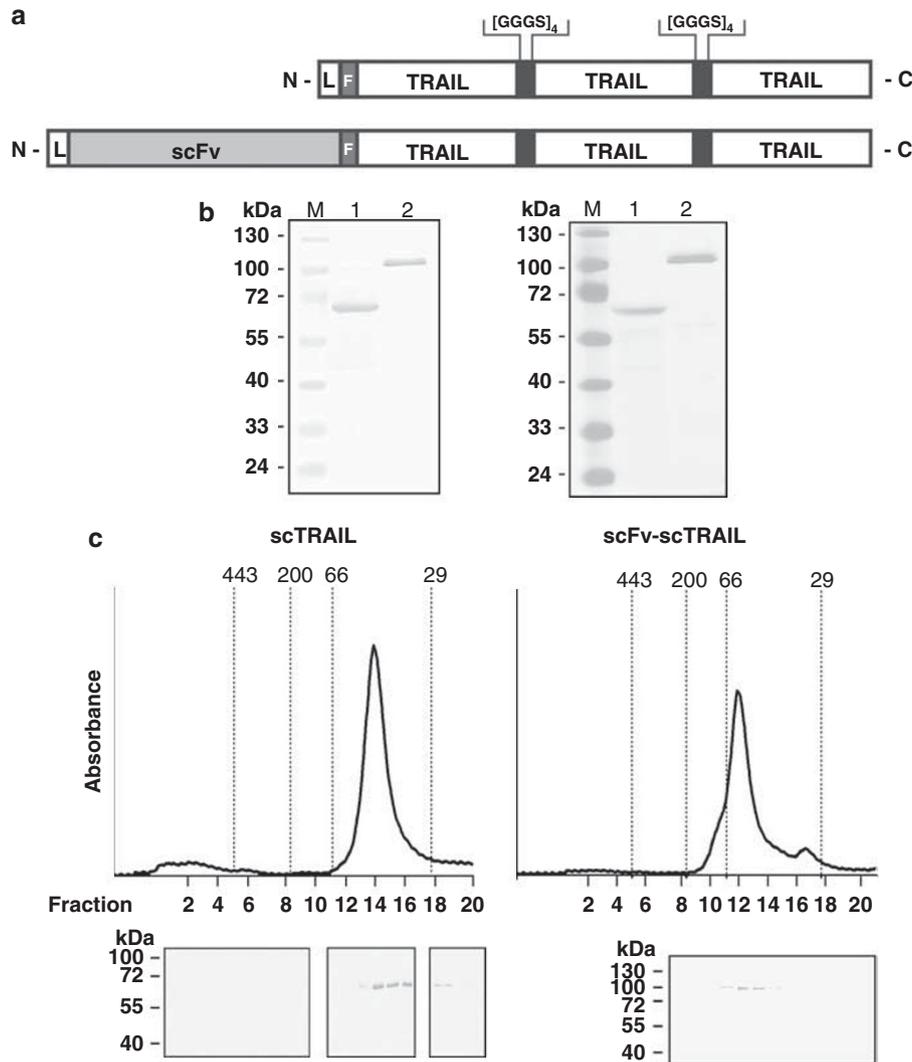


Figure 1 Biochemical analysis of scTRAIL and scFv-scTRAIL. (a) Scheme of the scTRAIL fusion proteins used in this study. L, leader peptide; scFv, human ErbB2-specific single-chain antibody fragment; F, FLAG tag; TRAIL, human TRAIL (aa 95–281). (b) Purified scTRAIL (lane 1) and scFv-scTRAIL (lane 2) were analyzed by SDS-PAGE (10%, reducing conditions) followed by immunoblot analysis (left, 250 ng protein/lane) or silver staining (right, 1 μ g protein/lane). For immunoblotting, the monoclonal anti-FLAG M2 antibody and an alkaline phosphatase-conjugated secondary antibody was used. (c) The TRAIL variants were separated by gel filtration on a BioSuite250 column. Dotted lines indicate the retention time of the molecular weight standards apoferritin (443 kDa), β -amylase (200 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). Collected fractions were analyzed by immunoblotting as in b. For scTRAIL fractions 1–9 (every second fraction; first part), fractions 11–16 (second part) and fractions 19–26 (every second fraction; third part) were immunoblotted. For scFv-scTRAIL fractions 8–24 were immunoblotted

(Figure 3b; Colo205: $EC_{50_{scFv-scTRAIL}}$: 30 ± 10 pM, $EC_{50_{scTRAIL}}$: 130 ± 70 pM; HT1080: $EC_{50_{scFv-scTRAIL}}$: 50 ± 5 pM, $EC_{50_{scTRAIL}}$: 140 ± 10 pM). Furthermore, bioactivity of the targeted scFv-scTRAIL compared well with that of a commercially available high activity TRAIL, the so-called ‘Killer-TRAIL’, with about twofold higher (Colo205) or at least comparable (HT1080) activity of the scFv-scTRAIL reagent (Figure 3c). Furthermore, on target-negative SKW6.4 cells, the secondarily crosslinked scTRAIL or scFv-scTRAIL displayed comparable cytotoxic activity to ‘Killer-TRAIL’ (data not shown). From blocking studies using anti-ErbB2 antibodies (FRP5, Figure 3d) or recombinant ErbB2 peptides comprising the epitope of the erbB2-specific scFv²¹ (Figure 3e), it became evident that the increased bioactivity

depended on ErbB2 target binding. Preincubation with FRP5 or ErbB2 peptides before addition of the ErbB2-specific scFv-scTRAIL fusion protein restored cell viability of Colo205 and HT1080 cells to a significant extent (Figure 3d and e). Furthermore, as for both tested cell lines TRAIL-induced cell death is primarily mediated by TRAIL-R2,^{13,25} the higher bioactivity of the scFv-scTRAIL on ErbB2-positive cells pointed to a membrane TRAIL-mimetic activity upon specific cell surface immobilization of the fusion protein (see below). Furthermore, inhibition of TRAIL bioactivity of the scFv-scTRAIL by the use of a TRAIL-neutralizing antibody led to complete restoration of cell viability of Colo205 and HT1080 cells (Figure 3f). Thus, the ErbB2-specific scFv domain *per se* did not directly contribute to apoptosis induction.

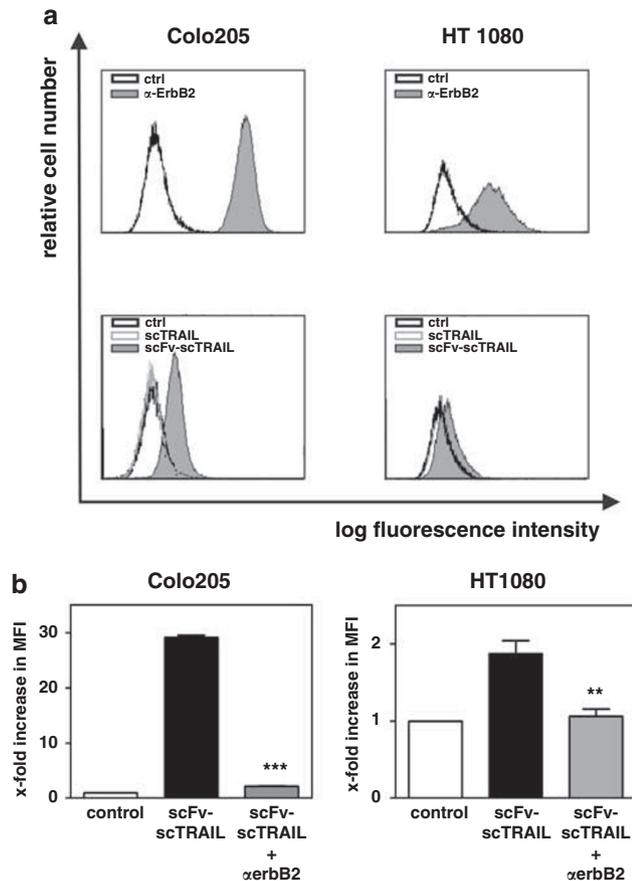


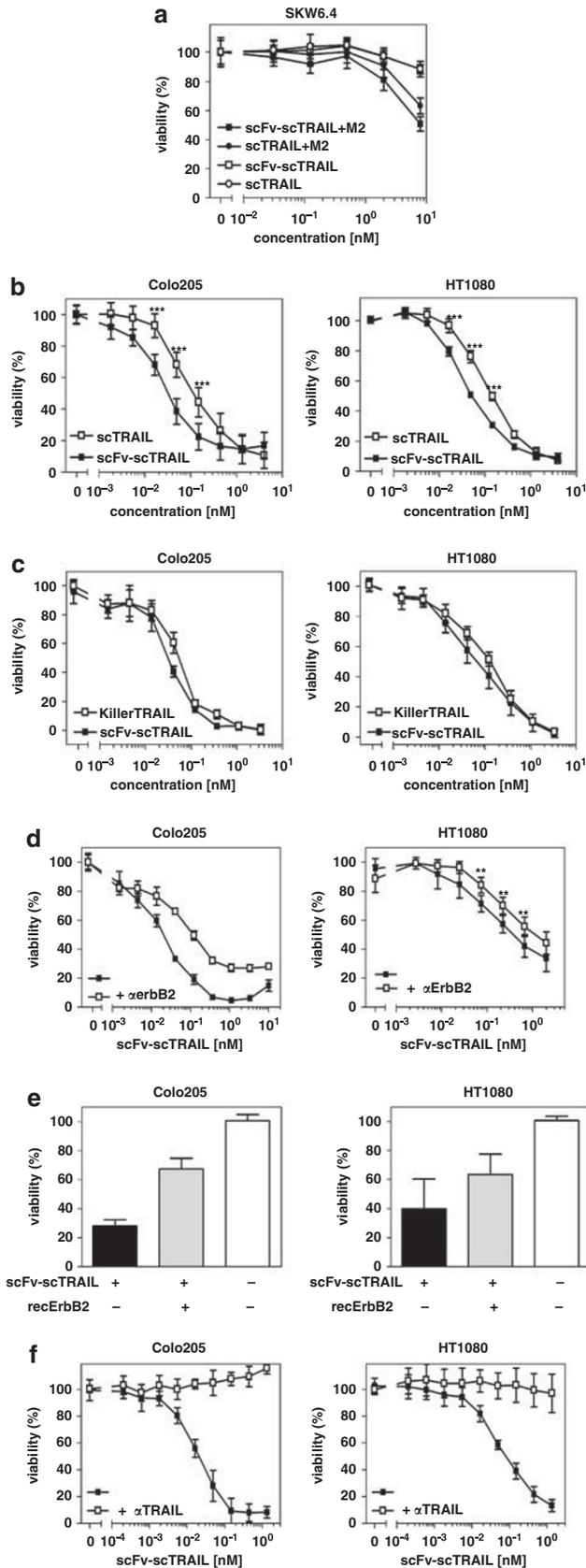
Figure 2 Target antigen-specific binding of scFv-scTRAIL. (a) ErbB2 expression of Colo205 and HT1080 cells: 5×10^5 cells were incubated with anti-ErbB2 (gray filled) or isotype control antibody (black lines) followed by FITC-conjugated anti-mouse antibody and fluorescence intensity was measured by FACS analysis. Binding of scFv-scTRAIL to ErbB2-positive Colo205 and HT1080 cells compared with non-targeted scTRAIL: 5×10^5 cells were incubated with 50 nM scTRAIL (gray lines) or 50 nM scFv-scTRAIL (gray filled) and, after repeated washes, bound proteins were detected using anti-human TRAIL mAb and FITC-labeled anti-mouse IgG. As control, cells treated with anti-human TRAIL mAb and FITC-labeled anti-mouse IgG were used (black lines). (b) Binding competition of scFv-scTRAIL to ErbB2-positive Colo205 cells by ErbB2-specific antibodies: 5×10^5 Colo205 or HT1080 cells were preincubated with FRP5 or mouse IgG1 (10 μ g/ml, respectively) before addition of scFv-scTRAIL (50 nM). Detection was carried out by TRAILR-Fc fusion proteins and FITC-labeled anti-human IgG, Fc-specific antibodies. Shown are X-fold increased mean fluorescence intensities (MFI) compared with the control (FITC-labeled anti-human Fc) from three independent experiments (mean \pm S.D.). Statistical analysis was carried out with unpaired *t*-test, two-tailed test, ***P* = 0.0021, ****P* < 0.0001

TRAIL-R2-mediated apoptosis induction of scFv-scTRAIL. In absence of the ErbB2 antigen, crosslinking of scFv-scTRAIL by its internal FLAG tag with the FLAG-specific mAb M2 was necessary to induce cell death in SKW (Figure 3a) and in Jurkat cells (Figure 4a). Thus, similar to homotrimeric TRAIL, secondary crosslinking conferred TRAIL-R2-stimulating capacity, confirming the nonaggregated state of the single-chain fusion protein with a TRAIL domain resembling a trimeric soluble TRAIL. The ErbB2-positive Colo205 and HT1080 cells express both, TRAIL-R1 and -R2. To define through which of the two TRAIL death receptors the

scFv-scTRAIL molecule signals apoptosis, neutralization experiments with antagonistic, TRAIL-R2-specific monovalent antibody fragments (Fab fragments) were performed. Both ErbB2-positive cell lines showed dominant TRAIL-R2-mediated cell death at low concentrations of scFv-scTRAIL (Figure 4b), demonstrating that cell surface immobilized scFv-scTRAIL exerted membrane TRAIL-like activity, similar to what has been previously described for conventional scFv-TRAIL fusion proteins.^{13,26,27} The concentration of anti-TRAIL-R2 Fab fragments used in these experiments was sufficient to almost completely block cell death of Jurkat cells induced by secondary crosslinked, homotrimeric FLAG-tagged TRAIL (Figure 4b). In addition, cell surface antigen-bound scFv-scTRAIL also activated the murine TRAILR, which is, similarly to human TRAIL-R2, not or hardly activated by soluble TRAIL. This was shown by mixed cultures using equal numbers of Colo205 cells and L929 mouse fibroblasts (Figure 4c). The latter do not express human ErbB2 and thus, in contrast to Colo205 cells, are not able to immobilize the scFv-scTRAIL by the ErbB2-targeting domain. Thus, if scFv-scTRAIL is able to induce cell death in L929 cells by mimicking membrane TRAIL after binding to the ErbB2 receptor on Colo205 cells, more than 50% of the cells are expected to die. Indeed, incubation with scFv-scTRAIL induced cell death in over 70% cells of the co-culture, whereas equal molar amounts of scTRAIL induced cell death in only about 30% of the mixed cells (Figure 4c). In monocultures, the same concentration of scFv-scTRAIL and scTRAIL induced cell death in about 90% of Colo205 cells, respectively, but not in L929 cells, confirming that, in the soluble form, both proteins are non-toxic to the latter (data not shown). Furthermore, due to the lack of human ErbB2, the L929 cells might also be considered as antigen-negative bystander cells, potentially occurring in a tumor tissue. Thus, scFv-scTRAIL bound to antigen is expected to elicit an antitumoral effect also on neighboring antigen-negative cells within the tumor environment.

Serum half-life of scFv-scTRAIL. To evaluate the pharmacokinetic characteristics of scFv-scTRAIL in comparison with scTRAIL, serum samples were collected after a single dose intravenous (i.v.) injection of 400 pmol into Balb/c mice. The relative serum concentrations plotted over time fitted well to a two-phase exponential decay regression curve in both cases. Within 20 min post-injection, both TRAIL variants displayed similar kinetics with a serum half-life of \sim 35 min ($t_{1/2}$), respectively. At later time points, a slight but significant difference in bioavailability was detected with half-lives ($t_{1/2}$) of 160 and 124 min and AUCs of 11 191 and 8125 for scFv-scTRAIL and scTRAIL, respectively (Figure 5a). Thus, the ErbB2-specific fusion protein was more efficiently retained in the bloodstream, which might positively affect therapeutic activity.

Tumoricidal effects on human colon carcinomas in nude mice. To investigate whether the improved target antigen-restricted tumoricidal activity of scFv-scTRAIL observed *in vitro* is effective *in vivo*, in a first experiment a local treatment strategy was applied. NMRI nu/nu mice were injected subcutaneously (s.c.) with Colo205 cells. Treatment commenced 8 days later when vascularized tumor could be



clearly discerned. Regimen was 4 daily s.c. injections of 1 nmol of scTRAIL and scFv-scTRAIL, respectively, in an area close to the established tumors. Tumor growth was monitored for 22 days. With this treatment protocol, we observed significant tumor regression in the scFv-scTRAIL-treated group compared not only with the control group (days 1–19; e.g., day 11, $**P=0.003$) but also with the non-targeted scTRAIL-treated group (days 1–11; e.g., day 11, $*P=0.04$, Figure 5b). These data are in accordance with one pilot experiment also showing significantly reduced tumor growth up to 11 days after scFv-scTRAIL treatment started ($**P<0.003$, data not shown). These data prompted studies with a systemic treatment protocol to assess the feasibility of such fusion proteins for potential clinical use. Again treatment was initiated when a solid, vascularized tumor had been established. Mice received 8 daily i.v. injections of 1 nmol of the TRAIL fusion proteins or control injections. All treatments were well tolerated by the animals, and we observed no weight loss or other obvious signs of systemic toxicity. Tumor size in control mice increased progressively over the study period (Figure 5c). Similar to the peritumoral treatment, systemic application of the two TRAIL reagents resulted in clear antitumoral activity during treatment. We observed significantly delayed tumor growth on scTRAIL treatment compared with control groups (days 1–19), and an even stronger effect for the tumor-targeted scFv-scTRAIL fusion protein (days 1–19). Remarkably, during scFv-scTRAIL treatment a clear reduction in tumor size was recorded, which resulted in a significantly enhanced anti-tumoral effect compared with non-targeted scTRAIL (days 1–19). Although tumors of both treatment groups began to re-grow shortly after discontinuation of treatment, these differences remained significant during the complete observation period (e.g., day 19 (Figure 5c)).

Discussion

We here show that through genetic engineering and targeting, TRAIL's antitumoral activity can be improved. TRAIL is considered as a tumor-selective agent, as cells from normal

Figure 3 Target antigen-dependent bioactivity of scFv-scTRAIL. (a) Bioactivity on target antigen-negative cells. 2×10^4 SKW6.4 cells/well were seeded in 96-well plates. The next day, cells were treated with serial dilutions of the fusion proteins in the presence or absence of anti-FLAG M2 antibody (2 μ g/ml). (b) Bioactivity of scTRAIL versus scFv-scTRAIL. 5×10^4 Colo205 or 2×10^4 HT1080 cells/well were seeded in 96-well plates. The following day, cells were treated with 2.5 μ g/ml cycloheximide and challenged in duplicates with increasing concentrations of scTRAIL and scFv-scTRAIL, respectively. After overnight incubation, cell viability was determined by MTT assay or KV staining. Results from three to five independent experiments are shown (mean \pm S.D.). (c) Bioactivity of scFv-scTRAIL compared to 'KillerTRAIL': Same assay as in (b) but incubation with serial dilutions of scFv-scTRAIL or 'KillerTRAIL'. (d, e) Target antigen-dependent apoptosis induction of scFv-scTRAIL: Same assay as in (b) with scFv-scTRAIL titrated in the presence or absence of anti-ErbB2 (FRP5, 5 μ g/ml) (d) or with scFv-scTRAIL (0.05 nM) in the presence or absence of recErbB2 (10 μ g/ml) (e). (f) Contribution of the scFv domain in the scFv-scTRAIL to apoptosis induction. Same assay as in (b) with scFv-scTRAIL titrated in the presence or absence of neutralizing anti-TRAIL antibodies (2E5, 1 μ g/ml). (a, c–f) Results from three independent experiments are shown (mean \pm S.D.). (b, d) Statistical analysis was carried out with unpaired t-test, two-tailed test, $**0.0026 < P > 0.0098$, $***P < 0.0001$

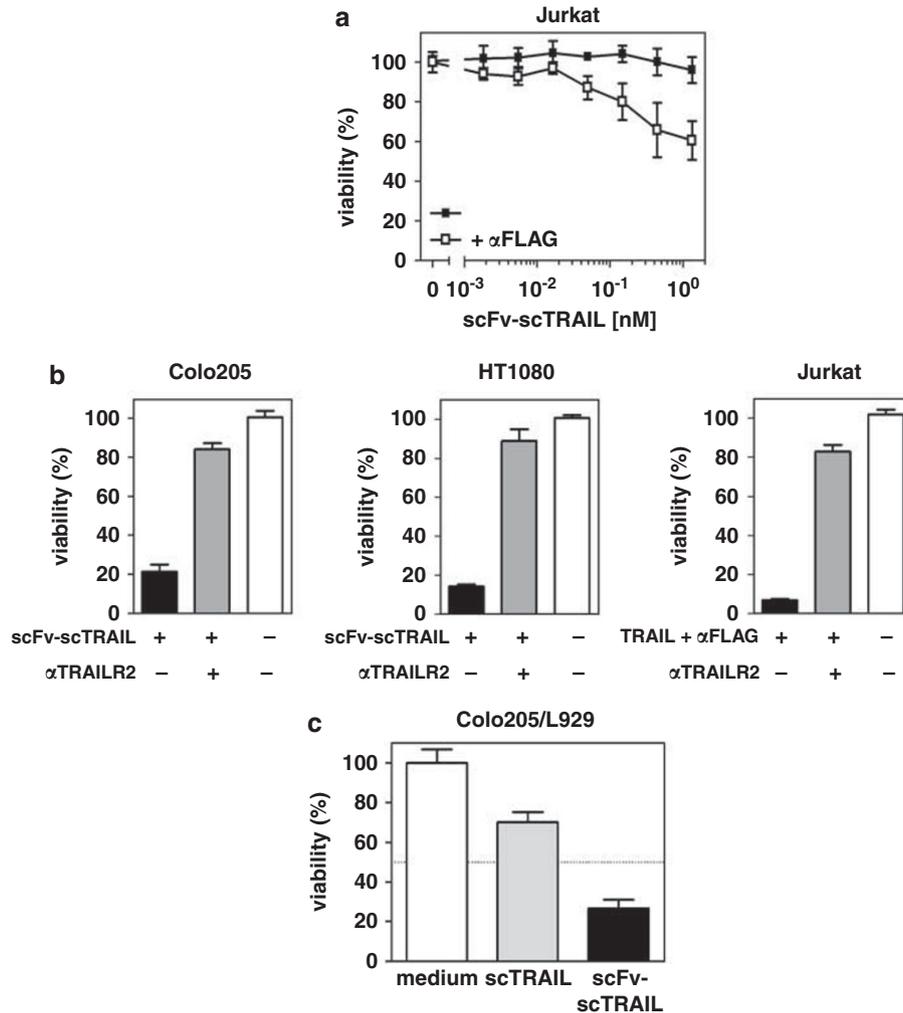


Figure 4 TRAIL-R2-mediated apoptosis induction of scFv-scTRAIL. (a) 1×10^5 Jurkat cells/well were seeded in 96-well plates. The following day, cells were challenged with the indicated concentration of scFv-scTRAIL in the absence (open symbols) or presence (filled symbols) of $1 \mu\text{g/ml}$ of the crosslinking anti-FLAG mAb M2. After additional 16 h, cell viability was determined by MTT assay. (b) Cells (5×10^4 Colo205, 2×10^4 HT1080, 1×10^5 Jurkat cells/well) were seeded in 96-well plates. The next day, cells were treated with $2.5 \mu\text{g/ml}$ cycloheximide (HT1080, Colo205) and challenged with scFv-scTRAIL (1.35 nM) (HT1080, Colo205) or 2 nM TRAIL + $1 \mu\text{g/ml}$ anti-FLAG mAb M2 (Jurkat) in the presence or absence of TRAIL-R2 Fab ($50 \mu\text{g/ml}$). After additional 16 h, cell viability was determined by MTT assay or KV staining. (c) 1×10^4 Colo205 and 1×10^4 L929 cells/well were seeded and co-cultured in 96-well plates. The next day, cells were treated with $2.5 \mu\text{g/ml}$ cycloheximide and challenged with 1 nM scTRAIL or scFv-scTRAIL. After additional 16 h, cell viability was determined by MTT assay. (a-c) Results from three independent experiments are shown (mean \pm S.D.)

tissue appear largely resistant toward apoptosis induction by trimeric TRAIL derivatives,⁸ and clinical studies so far revealed that homotrimeric TRAIL is well tolerated.^{28,29} However, in contrast to many investigated tumor cell lines, diverse primary cancer cells are inherently resistant to TRAIL-mediated apoptosis (reviewed by Newsom-Davis *et al.*⁵). Moreover, it has been demonstrated that TRAIL may under certain conditions even promote survival, proliferation, migration and invasion in TRAIL-resistant tumor cells.^{30,31} Thus, combination therapy with other drugs will be required, for example, for sensitization of apoptotic pathways or suppression of potentially protumoral TRAIL effects, to achieve major and lasting tumor remissions. In this context, recent data appear of relevance showing that the resistance of nontransformed cells toward the apoptotic action of TRAIL may vanish, at least in some tissues such as the liver, when

these are subjected to various stress conditions, such as infection and chemotherapeutics.^{12,32-34} Recently, it has also been shown that TRAIL-R2-mediated apoptosis might substantially contribute to chronic cholestatic disease.³⁵ Accordingly, an improvement of the tumor-selective action of TRAIL appears mandatory. Along this line, we and others have already shown earlier that tumor-targeting approaches through the generation of TNF ligand fusion proteins, which aim to restrict TNF ligand's full cytotoxic activity to the cancerous tissue are very promising approaches to improve therapeutic efficacy of TRAIL and other members of the TNF ligand family.⁴ Conventional fusion proteins of TRAIL and antibody-derived scFv fragments reveal homotrimeric proteins with a molecular mass of $\sim 150 \text{ kDa}$. This nearly corresponds in size to complete antibodies that are reported to penetrate tumor tissues slowly and nonuniformly.³⁶

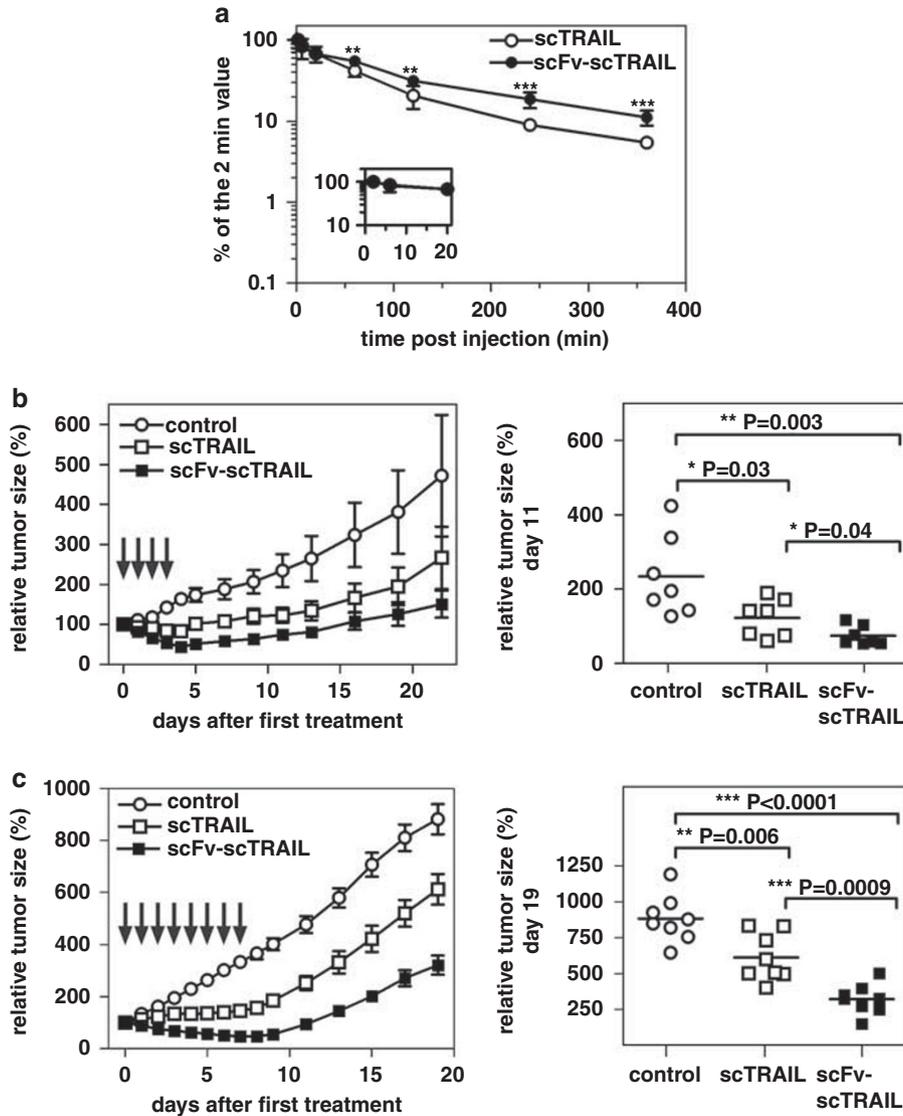


Figure 5 Pharmacokinetic properties and antitumoral activity of scTRAIL and scFv-scTRAIL. (a) Reduced elimination of scFv-scTRAIL from circulation. scTRAIL and scFv-scTRAIL were detected by ELISA in serum samples obtained at the indicated time points after i.v. injection into Balb/c mice. For each protein three mice were treated. Significant differences between both proteins were marked by asterisks. (b) Antitumoral activity in a xenograft tumor model – local treatment. NMRI nu/nu mice were injected s.c. with 3×10^6 human colon carcinoma Colo205 cells. Treatment started when tumors reached about 25 mm^2 (8 days after inoculation). Mice received four daily s.c. injections of 1 nmol of the corresponding proteins beginning day 0. $n = 7$ tumors per treatment group; symbols, mean of tumor volumes; bars, S.E.M. Individual tumor sizes at day 11 with bars indicating the mean are shown on the right. (c) Antitumoral activity in a xenograft tumor model – systemic treatment. 3×10^6 Colo205 cells were s.c. inoculated at the left and right dorsal site of NMRI nu/nu mice. Treatment started when tumors reached about 25 mm^2 (5 days after inoculation). Mice received eight daily i.v. injections of 1 nmol of the corresponding proteins beginning at day 0. $n = 8$ tumors per treatment group; symbols, mean of tumor volumes; bars, S.E.M. Individual tumor sizes at day 19 with bars indicating the mean are shown on the right

Furthermore, antibody fragments may have an intrinsic tendency to aggregate, thereby potentially leading to higher order complexes, which in turn not only results in a further increase in size but may also cause target-independent TRAIL-R2 activity, too. To circumvent or at least minimize these potential problems, we have developed a new format of a TRAIL fusion protein based on scTRAIL, a single polypeptide chain composed of three TRAIL monomers. Gel filtration and western blot analysis revealed that the ErbB2-specific scFv-scTRAIL fusion protein could be expressed in the expected monomeric state corresponding formally to

a trimeric TRAIL variant. This is of great importance, as it became evident that the formation of high order complexes and aggregates of recombinant forms of TRAIL (e.g., His-TRAIL, crosslinked FLAG-TRAIL) increases the toxicity of the ligand to normal cells (reviewed by Koschny *et al.*¹⁶). As expected, the ErbB2-specific scFv-scTRAIL specifically bound to ErbB2-positive cells. This could be confirmed by the use of TRAILR-Fc fusion proteins to detect the ErbB2 bound scFv-scTRAIL and by blocking of fusion protein binding with the ErbB2-specific antibody FRP5, thereby indicating predominant binding of the fusion protein by the antibody

moiety to ErbB2 rather than by the TRAIL domain and TRAIL-R. Compared with the non-targeted scTRAIL molecule, the tumor antigen-targeted scTRAIL fusion protein showed higher apoptotic activity *in vitro* on ErbB2-positive cells. This target antigen-dependent cytotoxicity could be confirmed by preincubation with competing ErbB2-specific antibody FRP5 or with ErbB2 peptide, restoring cell viability to a significant extent. We and others have previously described that, compared with soluble homotrimeric TRAIL, homotrimeric scFv–TRAIL fusion proteins gain a higher activity on TRAIL-R2 upon cell surface binding by the targeting domain within the fusion proteins.^{13,26,27} Of note, although there is some controversial discussion on this, it has been shown that efficient activation of TRAIL-R2 is only reached by natural membrane TRAIL, crosslinked soluble or cell surface immobilized TRAIL.^{13,26,27,37,38} Importantly, the scFv–scTRAIL fusion protein likewise converted into a functional equivalent of membrane TRAIL on target-antigen binding, and thus efficiently induced cell death already at low concentrations by TRAIL-R2, the predominant TRAIL-induced apoptosis pathway in the cell lines studied here. Accordingly, receptor binding and activation could be blocked by TRAIL-R2-neutralizing monovalent Fab fragments. Moreover, Jurkat cells, which only express TRAIL-R2, but not ErbB2 and TRAIL-R1 are resistant against soluble scFv–scTRAIL, whereas crosslinking of the fusion protein led to an increase in activity. These properties are especially important based on observations that on tumor cells TRAIL-R2 is more frequently and strongly expressed than TRAIL-R1,³⁹ that apoptotic signaling through TRAIL-R2 may be more potent than through TRAIL-R1¹⁴ and that, on the other hand, TRAIL-R2 may be a key regulator of cholestatic liver injury by induction of apoptosis in cholangiocytes.³⁵ Consequently, the scFv–scTRAIL might be an appropriate therapeutic, which meets the criteria of high antitumor and low off-target activity. *In vivo* analyses in mice revealed that, on i.v. injection, the scFv–scTRAIL and the scTRAIL displayed biphasic pharmacokinetics. In the first phase, the calculated $t_{1/2\alpha}$ of ~ 35 min was similar for both proteins. In the second phase, the scFv–scTRAIL displayed a slightly increased half-life with $t_{1/2\beta}$ of ~ 160 min compared with 125 min for the scTRAIL. This strongly contrasts to some of the published data for homotrimeric TRAIL, indicating a half-life of 3–5 min ($t_{1/2\alpha}$) and 20 min ($t_{1/2\beta}$) in rodents.^{40,41} Our results rather match with pharmacokinetic data of LZ-TRAIL, a trimer-stabilized TRAIL variant ($t_{1/2\alpha}$: 1.3 h; $t_{1/2\beta}$: 4.8 h⁸). Most important, both single-chain TRAIL variants significantly reduced growth of established Colo205 tumors already at rather moderate doses, either on local or systemic treatment. In accordance with the *in vitro* bioactivity, the scFv–scTRAIL displayed superior tumoricidal effects *in vivo* compared with the scTRAIL. We propose that increased antitumoral activity is largely related to ErbB2-mediated tumor enrichment and the targeting-dependent increase in bioactivity rather than the slightly increased bioavailability. The observed re-growth of tumors upon cessation of treatment has also been reported with homotrimeric TRAIL and might overcome when the combination treatments with appropriate chemotherapeutics are conducted.^{9,10,40} Furthermore, we can exclude an effect of the ErbB2-specific antibody fragment scFvFRP5 on tumor

growth, as addition of TRAIL-neutralizing antibodies completely restored cell viability on incubation with scFv–scTRAIL. This is in accordance with previous reports showing that the mAb FRP5 did not affect tumor growth *in vivo*, despite even partial agonistic activity *in vitro*, evident from an increase in ErbB2 phosphotyrosine level and accelerated receptor turnover by antibody-mediated receptor crosslinking.⁴² In contrast, a monovalent scFvFRP5–ETA fusion protein did not induce ErbB2-tyrosine phosphorylation, indicating that the activity of a monovalent scFv fusion protein might be limited by the intrinsic turnover rate of its target receptor.⁴³ We have own preliminary data confirming an increase in ErbB2-phosphorylation on treatment of Colo205 cells with a conventional, homotrimeric scFvFRP5–TRAIL fusion protein, whereas treatment with the monovalent scFv–scTRAIL had no receptor-activating effect (data not shown). Accordingly, the single-chain design concept might be of advantage compared with a conventional fusion protein if target-antigen multimerization has to be avoided. Moreover, Stagg *et al.*⁴⁴ could show that combination of ErbB2 blockade and TRAIL-R2 activation can elicit potent synergistic antitumor activity. Thus, modifying the targeting module using an ErbB2-specific scFv antibody fragment also capable of inhibiting ErbB2 signaling might result in further increased ErbB2-specific antitumor activity of an scFv–TRAIL fusion protein. The here described antitumoral activity of the scFv–scTRAIL fusion protein, which resulted in clear reduction in tumor size under treatment, appears superior to that of published preclinical data with homotrimeric TRAIL, regarding both the applied dose and the treatment periods.^{7,8,40} However, detailed side-by-side comparison of different TRAIL reagents are required to confirm this observation.

In conclusion, our data provide evidence that bioactive single-chain TRAIL fusion proteins can be generated. Furthermore, fusion to a target-specific scFv antibody results in target-dependent increase in TRAIL-mediated cytotoxicity *in vitro*, likely due to efficient TRAIL-R2 activation, and improves antitumoral activity *in vivo*. Accordingly, this design concept represents a promising strategy to enhance TRAIL's antitumoral action and to minimize potential unwanted off-target actions on normal tissues.

Materials and Methods

Plasmids and cell lines. The pRESpuro–scTRAIL expression construct for human scTRAIL (aa 95–281) was generated by insertion of linker sequences, encoding four repetitions of the GGGS motif, between the three TRAIL modules as described previously for the construction of scTNF.²⁰

The single-chain antigen binding protein scFvFRP5 for the ErbB2-specific pRESpuro–scFv–scTRAIL expression construct was described previously.²¹ HEK293, Jurkat, Colo205, SKW6.4 and L929 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) or DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The HT1080 cells were a kind gift of W Rettig (Boehringer Ingelheim Pharma, Vienna, Austria). Cells were cultured in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 5% or 10% (SKW6.4) fetal calf serum (FCS, PAA Laboratories, Pasching, Austria).

Production and purification of recombinant proteins. The TRAIL fusion proteins were produced in HEK293 cells after stable transfection of the corresponding expression plasmids using Lipofectamine 2000 (Invitrogen) and selection of stable clones by limiting dilution. For protein production, stable clones were expanded and grown in RPMI 1640, 5% FCS, to 90% confluency and

subsequently cultured in serum-free OptiMem (Invitrogen) supplemented with 50 μ M ZnCl₂, replacing media two times every 3 days. Supernatants were pooled and recombinant proteins were purified by affinity chromatography using anti-FLAG mAb M2 agarose (Sigma-Aldrich, Steinheim, Germany). The bound proteins were eluted with 100 μ g/ml FLAG peptide (Sigma-Aldrich) and dialyzed against PBS. After concentrating purified proteins by dialysis on PEG35000, they were analyzed and quantified by SDS-PAGE, silver gel (Sigma-Aldrich) and gel filtration and stored at 4°C.

Gel filtration. Protein samples were applied to a BioSuite250 HR SEC (300 \times 7.8) column (Waters, Millipore Corp., Milford, MA, USA) equilibrated in PBS and eluted at a flow rate of 0.5 ml/min.

Flow cytometry. Cells (5×10^5) were incubated for 2 h at 4°C with the indicated concentrations of the TRAIL fusion proteins. After washing the cells three times with PBS, 2% FCS, 0.02% sodium azide, bound fusion proteins were detected by anti-human TRAIL mAb (MAB687) (1 μ g/ml, R&D Systems, Wiesbaden, Germany) and fluorescein isothiocyanate-labeled rabbit anti-mouse IgG Ab (1 μ g/ml, Sigma-Aldrich). ErbB2 expression was detected by anti-c-neu (AB-5) (1 μ g/ml, Merck KGaA, Darmstadt, Germany) and fluorescein isothiocyanate-labeled rabbit anti-mouse IgG Ab (1 μ g/ml, Sigma-Aldrich). Alternatively, cell-bound scFv-scTRAIL was detected by TRAILR-Fc fusion proteins (5 μ g/ml TRAILR1-Fc and 3 μ g/ml TRAILR2-Fc) and fluorescein isothiocyanate-labeled anti-human IgG, Fc-specific antibodies (Sigma-Aldrich). For competition studies, FRP5 hybridoma supernatant or mouse IgG1 (Sigma-Aldrich) was added 30 min before addition of the scFv-scTRAIL.

Cell death assays. HT1080 cells (2×10^4 /well), Colo205 cells (5×10^4 /well), SKW6.4 cells (2×10^4 /well) or Jurkat cells (1×10^5 /well) were cultured in 100 μ l culture medium in 96-well plates. The next day, cells were treated in duplicates or triplicates with the indicated concentrations of the TRAIL fusion proteins or 'Killer-TRAIL' (Axxora Deutschland GmbH, Lörrach, Germany). After overnight incubation, cell viability was determined either by crystal violet staining (HT1080) or the MTT method (Jurkat, Colo205, SKW6.4),⁴⁵ except that for MTT staining a lysis buffer consisting of 15% SDS in DMF/dH₂O (1:1), pH4.5 (with 80% acetic acid) was used. To show target antigen- and TRAIL-R2-dependent induction of cell death, cells were preincubated for 30 min with FRP5 hybridoma supernatant⁴⁶ (~ 10 μ g/ml) or a competing ErbB2 peptide (10 μ g/ml) encompassing the FRP5-binding epitope²¹ or a neutralizing TRAIL-R2-specific Fab preparation (50 μ g/ml), respectively. For inhibition of TRAIL bioactivity, anti-TRAIL antibody 2E5 (1 μ g/ml, Axxora Deutschland GmbH) was used. For co-culture assays, 1×10^4 Colo205 and 1×10^4 L929 cells/well were seeded in 96-well plates. The next day, cells were challenged in duplicates with scTRAIL and scFv-scTRAIL, respectively. After additional 16 h, cell viability was determined using the MTT method. Cell death assays with HT1080 and Colo205 cells were performed in the presence of 2.5 μ g/ml cycloheximide (Sigma-Aldrich) to sensitize cells for the induction of cell death.

Pharmacokinetics. Animal care and all experiments performed were in accordance with federal guidelines and have been approved by the University and State authorities. Balb/c mice (female, 7 weeks, weight between 18 and 20 g, 3 mice/group) received an i.v. injection of 400 pmol of the recombinant proteins in a total volume of 100 μ l. In time intervals of 2, 6, 20, 60, 120, 240, 360 min and 24 h, blood samples (~ 100 μ l) were taken from the tail and incubated on ice. Clotted blood was centrifuged at 10 000 g for 10 min, 4°C, and serum samples were stored at -80°C. Serum concentrations of recombinant proteins were finally determined by ELISA (BD Biosciences, Heidelberg, Germany). For calculation, relative values of serum concentrations were analyzed with the first value (2 min) being set to 100%. GraphPad Prism Software was used for curve fitting by nonlinear regression. The pharmacokinetic parameters AUC, $t_{1/2\alpha}$ and $t_{1/2\beta}$ were calculated by Excel using the first 3 time points to calculate $t_{1/2\alpha}$ and the last three time points to calculate $t_{1/2\beta}$. Statistics were determined by the unpaired *t*-test.

Xenograft mouse models

Local treatment. Six-week-old female NMRI nu/nu mice (Janvier Laboratories, Le Genest-Saint-Isle, France) were injected s.c. with 3×10^6 Colo205 cells in 100 μ l PBS. Treatment started when tumors reached ~ 25 mm². Mice received 4 daily s.c. injections of 1 nmol of the various TRAIL fusion proteins in concentrated cell-culture supernatant. Supernatants were concentrated by dialysis on PEG35000.

The control group received 100 μ l concentrated cell-culture supernatant from empty vector-transfected cells at the same time intervals.

Systemic treatment. Colo205 cells (3×10^6) in 100 μ l PBS were s.c. inoculated at the left and right dorsal sides of 6-week-old female NMRI nu/nu mice. Treatment started 5 days after tumor cell inoculation when tumors reached ~ 25 mm². Mice received 8 daily i.v. injections of 1 nmol of the affinity purified TRAIL fusion proteins. The control group received 100 μ l PBS at the same time intervals. Tumor growth was monitored as described.⁴⁷

Conflict of interest

The authors declare no conflict of interest.

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