

# Superior antitumoral activity of dimerized targeted single-chain TRAIL fusion proteins under retention of tumor selectivity

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Although targeting of the death receptors (DRs) DR4 and DR5 still appears a suitable antitumoral strategy, the limited clinical responses to recombinant soluble TNF-related apoptosis inducing ligand (TRAIL) necessitate novel reagents with improved apoptotic activity/tumor selectivity. Apoptosis induction by a single-chain TRAIL (scTRAIL) molecule could be enhanced >10-fold by generation of epidermal growth factor receptor (EGFR)-specific scFv-scTRAIL fusion proteins. By forcing dimerization of scFv-scTRAIL based on scFv linker modification, we obtained a targeted scTRAIL composed predominantly of dimers (Db-scTRAIL), exceeding the activity of nontargeted scTRAIL ~100-fold on Huh-7 hepatocellular and Colo205 colon carcinoma cells. Increased activity of Db-scTRAIL was also demonstrated on target-negative cells, suggesting that, in addition to targeting, oligomerization equivalent to an at least dimeric assembly of standard TRAIL *per se* enhances apoptosis signaling. In the presence of apoptosis sensitizers, such as the proteasomal inhibitor bortezomib, Db-scTRAIL was effective at picomolar concentrations *in vitro* (EC<sub>50</sub> ~2 × 10<sup>-12</sup> M). Importantly, *in vivo*, Db-scTRAIL was well tolerated and displayed superior antitumoral activity in mouse xenograft (Colo205) tumor models. Our results show that both targeting and controlled dimerization of scTRAIL fusion proteins provides a strategy to enforce apoptosis induction, together with retained tumor selectivity and good *in vivo* tolerance.

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The TNF-related apoptosis-inducing ligand (TRAIL; Apo2L/Dulanermin) as well as TRAIL-agonistic antibodies are able to kill a broad range of tumor cell lines *in vitro*, are well tolerated *in vivo* and thus applicable for therapy of cancer.<sup>1,2</sup> Nonetheless, clinical trials revealed that the use of soluble, recombinant TRAIL, given alone or in combination with other drugs, may be often insufficient to gain significant therapeutic effects. For example, in a phase II trial focusing on treatment of patients with relapsed follicular non-Hodgkin's lymphoma (NHL), the response rate of the anti-CD20 antibody rituximab was not improved when TRAIL was given in combination.<sup>3</sup> Accordingly, the improvement of TRAIL-based therapies should address the apparent shortcomings of present TRAIL reagents, namely short *in vivo* half-life and low specific bioactivity. In addition, sensitization of tumor cells toward the apoptosis-inducing activity of TRAIL appears particularly relevant.

Resistance to TRAIL-induced apoptosis can be caused by the expression levels and composition of proapoptotic and decoy TRAIL receptors as well as by intracellular mechanisms. The cell surface expression of death receptor (DR)4 or DR5 is a prerequisite for TRAIL-mediated apoptosis, but is insufficient in case of blocked intracellular pathways. Levels of inhibitor of apoptosis proteins, such as X-linked inhibitor of apoptosis protein (XIAP), have been shown to be critical determinants of TRAIL sensitivity in melanoma.<sup>4</sup> In hepatocellular carcinoma (HCC), several factors including the decreased expression of CD95 (Apo1/Fas),<sup>5</sup> activation of transcription factor nuclear factor-κB (NF-κB)<sup>6</sup> or overexpression of pro-inflammatory cytokines and their receptors<sup>7</sup> accompany such dysregulation of apoptotic signaling. However, TRAIL has been shown to induce cell death selectively in HCC when tumor cells are sensitized by chemotherapeutics like the multi-kinase inhibitor sorafenib<sup>8</sup> and the proteasome

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**Abbreviations:** Ac-DMQD-AMC, Ac-Asp-Met-Gln-Asp-7-Amino-4-methylcoumarin; ALT, alanine aminotransferase; CHX, cycloheximide; DR, death receptor; ECL, enhanced chemoluminescence; EGFR, epidermal growth factor receptor; Fab, antigen-binding fragment; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HRP, horseradish peroxidase; IMAC, immobilized metal affinity chromatography; i.p., intraperitoneal(ly); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NHL, non-Hodgkin's lymphoma; NSCLC, non-small cell lung cancer; PHH, primary human hepatocyte; RTK, receptor tyrosine kinase; s.c., subcutaneous(ly); scFv, single-chain Fv fragment; scTRAIL, single-chain TRAIL; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR, TRAIL receptor; V<sub>H</sub>, variable domain of immunoglobulin heavy chain; V<sub>L</sub>, variable domain of immunoglobulin light chain; XIAP, X-linked inhibitor of apoptosis protein; zVEDfmk, z-Asp-Glu-Val-Asp-fluoromethylketone; z-Val-Ala-Asp-fluoromethylketone

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inhibitor bortezomib.<sup>9,10</sup> The clinical relevance of TRAIL receptor (TRAILR)-directed therapy of HCC is emphasized by current studies<sup>11</sup> (see also <http://clinicaltrials.gov/ct2/show/study/NCT01258608>).

Different approaches have been taken to enhance specific apoptotic activity and maintain high tumor selectivity of recombinant TRAIL molecules. Thus, TRAIL mutants, acting predominantly on either DR4 or DR5, facilitate cell death induction, for example on DR5-positive tumor cells, without the need for membrane immobilization or crosslinking of soluble TRAIL.<sup>12,13</sup> Alternatively, TRAIL can be fused to antibodies directed against tumor markers. It has been shown for CD33-directed scFv-TRAIL that such fusion proteins are superior over antibody-targeted chemotherapeutics because of stronger cytotoxic activity and the minimization of side effects on normal nontransformed cells.<sup>14</sup> Correct trimerization and zinc coordination of recombinant TRAIL therapeutics seem to be crucial for biological activity and for nonreactivity toward nontransformed cells, in particular hepatocytes.<sup>15</sup> Our previous results showed that three extracellular domains of human TRAIL can be fused by peptide linkers to yield highly active single-chain TRAIL (scTRAIL) that can be further endowed with target-dependent activity by fusion to an antibody derivative.<sup>16</sup> Such antigen-binding scFv-scTRAIL molecules functionally mimic the natural membrane-expressed TRAIL and are more effective therapeutics than scTRAIL alone.<sup>16</sup> We here sought to develop a concept for controlled oligomerization of scFv-scTRAIL fusion proteins leading to improved expression and stability as well as increased functional activity under retention of tumor selectivity.

## Results

**Construction and preparation of scTRAIL fusion proteins.** For generation of functionally improved scFv-scTRAIL fusion proteins, scTRAIL was first genetically optimized for mammalian expression. Additionally, the linker motifs in the molecule were reduced from (GGGS)<sub>4</sub> to (GGGS)<sub>2</sub>, potentially making the protein more rigid and less prone to aggregation without affecting apoptosis induction (data not shown).

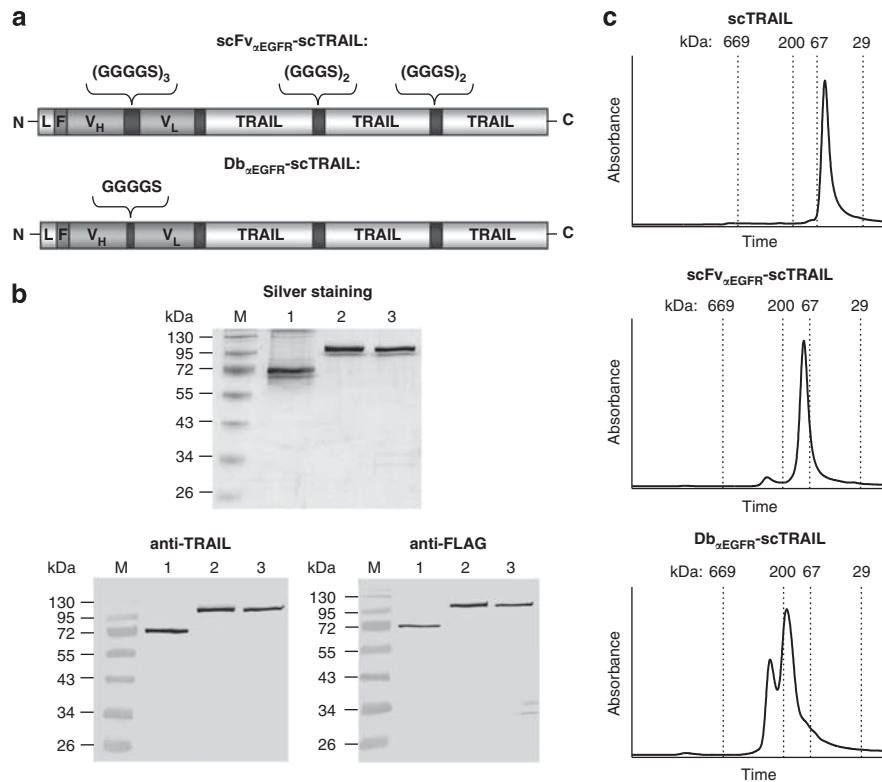
In this study, we used epidermal growth factor receptor (EGFR), an established tumor marker overexpressed in several carcinomas, including colon, lung and liver cancer,<sup>17</sup> as a model for targeting. The chimeric monoclonal antibody cetuximab (C225) is directed against the extracellular domain of EGFR and blocks receptor activation by interfering with ligand binding.<sup>18</sup> This antibody was approved for treatment of advanced metastatic colorectal cancer, squamous cell carcinoma of the head and neck, and is evaluated in non-small cell lung cancer (NSCLC).<sup>19,20</sup> A humanized single-chain Fv fragment (scFv) 'huC225' (RE Kontermann, unpublished data) derived from cetuximab was fused to the N-terminus of scTRAIL, yielding scFv<sub>z</sub>EGFR-scTRAIL (Figure 1a). The aim of this work was the improvement of the proapoptotic activity of scFv<sub>z</sub>EGFR-scTRAIL fusion proteins under retention of tumor selectivity, that is, nonreactivity toward normal, nonmalignant tissue. TRAIL bioactivity depends on the oligomerization

state, in particular relevant for TRAILR2 (DR5), which is poorly activated by soluble, trimeric forms of TRAIL.<sup>21,22</sup> As DR5 appears to be the major mediator of apoptosis signaling in the vast majority of carcinoma cells, we reasoned that a dimeric variant of scFv<sub>z</sub>EGFR-scTRAIL should combine tumor targeting with an even more enhanced DR5 activation. Dimerization was accomplished by reducing the linker between V<sub>H</sub> (variable domain of immunoglobulin heavy chain) and V<sub>L</sub> (variable domain of immunoglobulin light chain) from (GGGS)<sub>3</sub> to GGGGS as described for the so-called diabodies,<sup>23</sup> yielding Db<sub>z</sub>EGFR-scTRAIL.

scFv<sub>z</sub>EGFR-scTRAIL and Db<sub>z</sub>EGFR-scTRAIL were produced and purified with yields of >3 mg highly pure protein per liter cell culture supernatant. SDS-PAGE and immunoblotting revealed single bands with molecular masses of ~70 kDa for scTRAIL and ~100 kDa for scFv<sub>z</sub>EGFR-scTRAIL and Db<sub>z</sub>EGFR-scTRAIL, matching the calculated values of 68 and 94 kDa (93 kDa for Db<sub>z</sub>EGFR-scTRAIL), respectively (Figure 1b). The size exclusion chromatography of scTRAIL and scFv<sub>z</sub>EGFR-scTRAIL (Figure 1c) indicated a monomeric composition for the majority of the protein (>94%). Interestingly, Db<sub>z</sub>EGFR-scTRAIL was present in a heterogeneous state with a major peak corresponding to the expected dimer and a minor peak representing putative trimers or tetramers. Upon separation of both isoforms, equal amounts of the higher-order isoforms and the dimer displayed *in vitro* identical bioactivity (data not shown) indicating that higher than dimeric forms of scTRAIL oligomers apparently do not further enhance apoptosis signaling.

**EGFR-specific binding of scTRAIL fusion proteins.** The specific binding of scTRAIL fusion proteins to EGF receptors was analyzed by flow cytometry of Huh-7 cells, displaying moderate EGFR expression compared with HepG2 cells with barely detectable EGFR (Supplementary Figure S1). ScFv<sub>z</sub>EGFR-scTRAIL and Db<sub>z</sub>EGFR-scTRAIL binding was detectable on Huh-7 and HepG2 cells, but competition of fusion protein binding by preincubation of cells with the anti-EGFR scFv huC225 (2 μM) was only possible on Huh-7 cells (Figure 2a). Moreover, Huh-7 cell binding of Alexa 488 (Life Technologies, Darmstadt, Germany) labeled EGFR-specific cetuximab was blocked by preincubation with 0.5 μM Db<sub>z</sub>EGFR-scTRAIL (Figure 2b). The intermediate fluorescence signal observed upon competition of fusion protein binding on Huh-7 cells likely reflects the binding of the scTRAIL moiety to TRAIL receptors and is consistent with the low but specific signal obtained from scFv<sub>z</sub>EGFR-scTRAIL and Db<sub>z</sub>EGFR-scTRAIL binding to TRAILR<sup>+</sup> EGFR<sup>-</sup> HepG2 cells. Binding competition of both fusion proteins with EGFR-specific antibody/scFv on EGFR<sup>+</sup> cells is evidence for the structural integrity and functionality of the scFv and diabody targeting modules of the fusion proteins.

Quantitative binding studies of scTRAIL fusion proteins to EGFR<sup>+</sup>, DR4<sup>+</sup>DR5<sup>+</sup> NCI-H460 cells revealed significantly different ( $P=0.003$ ) EC<sub>50</sub> values for scFv<sub>z</sub>EGFR-scTRAIL ( $3.6 \pm 0.3 \times 10^{-10}$  M) and Db<sub>z</sub>EGFR-scTRAIL ( $1.6 \pm 0.3 \times 10^{-10}$  M), implicating an avidity effect of the specific molecular composition of the divalent Db<sub>z</sub>EGFR-scTRAIL and therefore potentially superior targeting compared with scFv<sub>z</sub>EGFR-scTRAIL (Figure 2c). To investigate whether



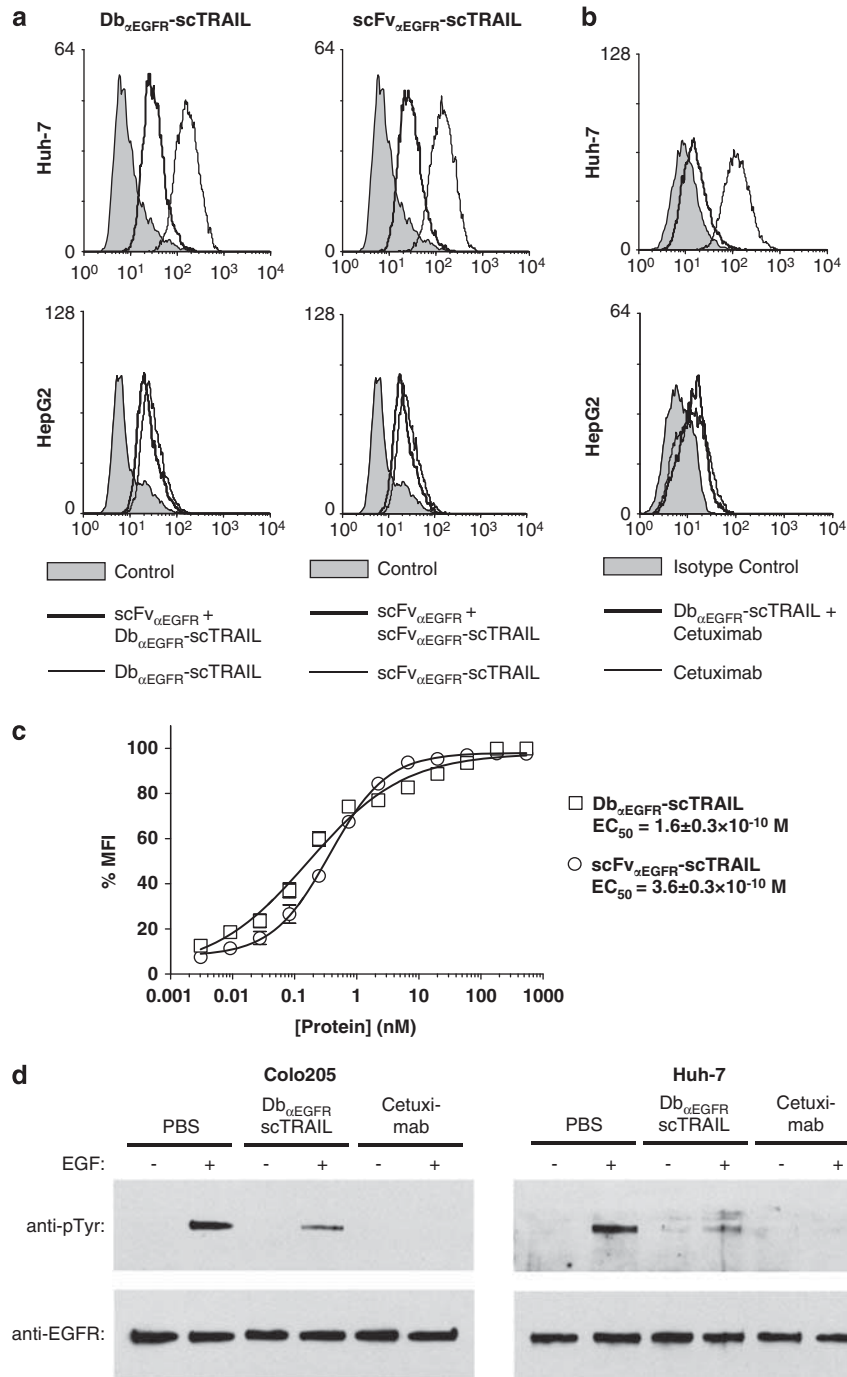
**Figure 1** Biochemical analysis of scTRAIL and scTRAIL fusion proteins. (a) Design of novel scTRAIL fusion proteins. Three human TRAIL domains (aa residues 95–281) were fused with (GGGS)<sub>2</sub> peptide linkers yielding single-chain TRAIL. EGFR-specific scFv antibody fragments comprising V<sub>H</sub> and V<sub>L</sub> were fused N-terminally to scTRAIL. (GGGGS)<sub>3</sub> or GGGGS peptide linkers between V<sub>H</sub> and V<sub>L</sub> were chosen to obtain a scFv or a diabody configuration, respectively. L, V<sub>H</sub> leader; F, FLAG tag. (b) Purified scTRAIL (1), scFv<sub>αEGFR</sub>-scTRAIL (2) and Db<sub>αEGFR</sub>-scTRAIL (3) were analyzed by reducing SDS-PAGE and silver staining (upper, 1 μg protein/lane) or immunoblotting (lower, 250 ng protein/lane) against TRAIL or FLAG. (c) scTRAIL and derived fusion proteins were separated by size exclusion chromatography. Thyroglobulin (669 kDa), β-amylase (200 kDa), bovine serum albumin (67 kDa) and carbonic anhydrase (29 kDa) were used as standard proteins

or not the cetuximab-derived, humanized scFv has retained in its divalent diabody format the functional activity of cetuximab, we checked for blocking of EGF-induced EGFR autophosphorylation by Db<sub>αEGFR</sub>-scTRAIL, whereby cetuximab served as a positive control. Functional blocking of EGF-stimulated receptor activation by the divalent Db<sub>αEGFR</sub>-scTRAIL could be demonstrated for both Colo205 (Figure 2d, left panel) and Huh-7 (Figure 2d, right panel) cells.

**Target-independent induction of cell death by scTRAIL fusion proteins.** To investigate the basic bioactivity of scTRAIL fusion proteins without the influence of targeting domains, we analyzed cell death induction on the target-negative cell lines HepG2 (hepatoma) and Jurkat (T-cell leukemia) and compared it with scTRAIL devoid of a targeting domain (Table 1 and Figure 3a). On HepG2 cells, Db<sub>αEGFR</sub>-scTRAIL exerted an ~10-fold increased bioactivity compared with scFv<sub>αEGFR</sub>-scTRAIL and a commercially available, highly active ‘KillerTRAIL’ (Figure 3a, left panel). Because of the EGFR deficiency of HepG2 cells, the proapoptotic activities of Db<sub>αEGFR</sub>-scTRAIL and scFv<sub>αEGFR</sub>-scTRAIL were not influenced by the presence of an at least sevenfold excess (70 nM) of the EGFR-blocking cetuximab (not shown). Accordingly, the bioactivity of scFv<sub>αEGFR</sub>-scTRAIL did not differ from that one of scTRAIL. On

EGFR<sup>-</sup>, DR4<sup>-</sup>DR5<sup>weak</sup> Jurkat cells, which are known to be sensitive for apoptosis induced by TRAIL oligomers, again we found a higher proapoptotic activity of Db<sub>αEGFR</sub>-scTRAIL compared with KillerTRAIL and, as expected, no reactivity toward non-oligomerized scFv<sub>αEGFR</sub>-scTRAIL or scTRAIL (Figure 3a, right panel), underlining the target-independent higher apoptotic activity of the dimeric scTRAIL fusion protein.

**EGFR-directed enhancement of cell death by scTRAIL fusion proteins.** The EGFR<sup>+</sup> human colon carcinoma Colo205 and liver carcinoma Huh-7 were chosen to demonstrate the enhancement in bioactivity achievable by the receptor targeting capacity of scFv<sub>αEGFR</sub>-scTRAIL and Db<sub>αEGFR</sub>-scTRAIL. Both cell lines require sensitization by either cycloheximide (CHX) or bortezomib to reveal efficient TRAIL-mediated apoptosis induction *in vitro* (Figure 3b). Compared with scTRAIL, the enhancement in bioactivity of the monovalent scFv<sub>αEGFR</sub>-scTRAIL was ~16-fold on Huh-7 cells and ~6-fold on Colo205 cells, whereby Colo205 expressed less EGFR compared with Huh-7 (Figure 3b and Supplementary Figure S1). Interference with EGFR binding by co-incubation with an excess of cetuximab (70 nM) revealed a right shift of the dose-response curve of scFv<sub>αEGFR</sub>-scTRAIL toward the EC<sub>50</sub> of nontargeted



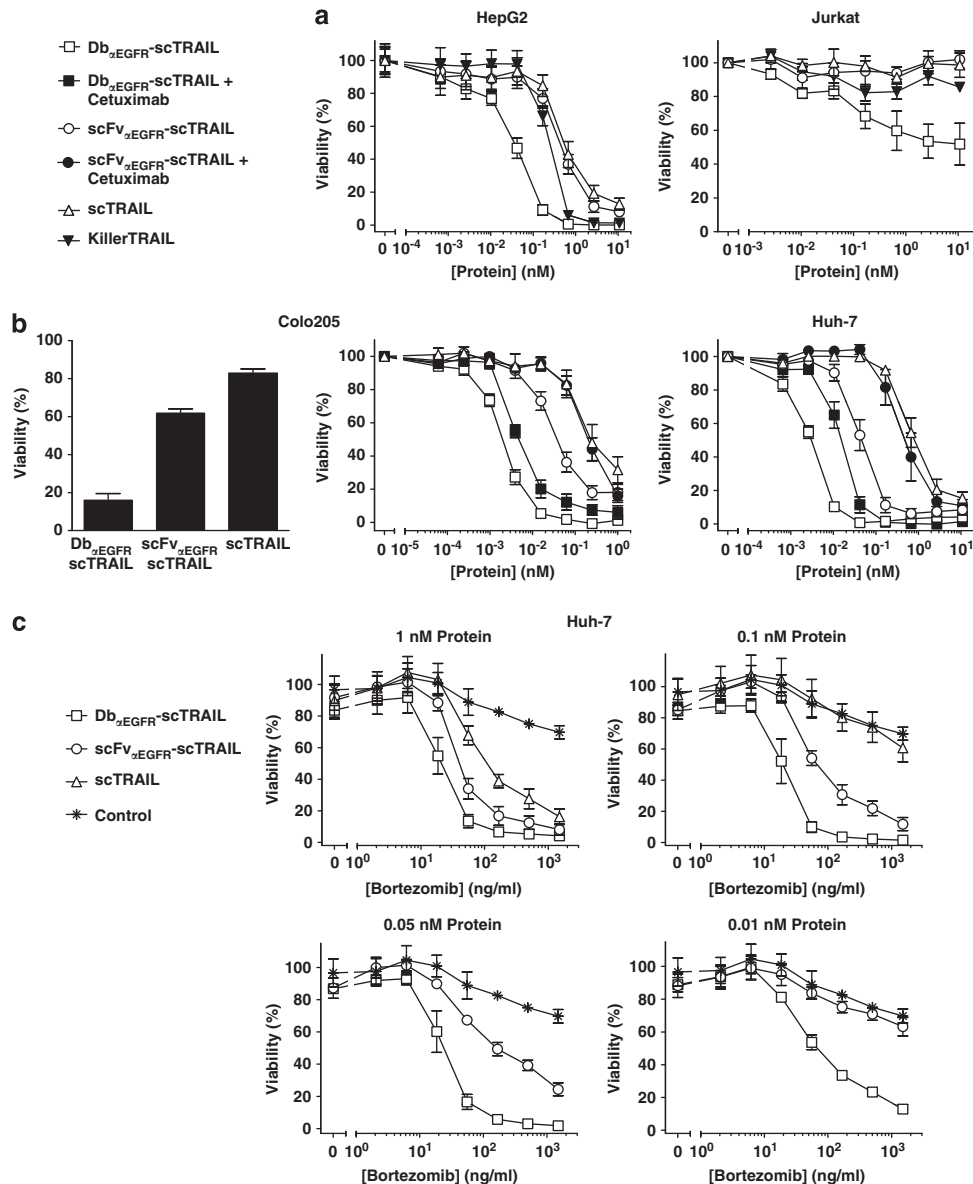
**Figure 2** Receptor interaction of EGFR-specific scTRAIL fusion proteins. **(a)** Binding of Db<sub>z</sub>EGFR-scTRAIL and scFv<sub>z</sub>EGFR-scTRAIL to target-negative HepG2 and target-positive Huh-7 cells (thin-lined histograms). Furthermore, cells were preincubated with an excess of anti-EGFR scFv before adding scTRAIL fusion proteins (bold histograms). Filled histograms, controls without scTRAIL fusion proteins. **(b)** Blocking of the binding of Alexa Fluor 488-labeled cetuximab to HepG2 and Huh-7 by an excess of Db<sub>z</sub>EGFR-scTRAIL (bold histograms). Thin-lined histograms, cetuximab alone; filled histograms, isotype controls. **(c)** Dose-response relationship of scTRAIL fusion protein binding to EGFR<sup>+</sup> NCI-H460 cells by indirect immunofluorescence flow cytometry to reveal concentration of half-maximum binding (EC<sub>50</sub>) (mean ± S.E.M., *n* = 4). **(d)** Colo205 and Huh-7 cells were serum-starved overnight and then incubated with 2 nM of Db<sub>z</sub>EGFR-scTRAIL, cetuximab and PBS for control, respectively. After 10 min, 50 ng/ml EGF was added and cells were incubated for additional 20 min followed by cell lysis. EGF receptors were immunoprecipitated using a specific mouse monoclonal antibody and subjected to SDS-PAGE followed by immunoblotting with phosphotyrosine antibody (top). Total amounts of EGFR were determined by reprobing the membrane with EGFR-specific rabbit polyclonal antibody (bottom)

scTRAIL (Table 1), pointing to an improvement of scTRAIL bioactivity by EGFR targeting. Db<sub>z</sub>EGFR-scTRAIL, representing a dimeric assembly of scFv-scTRAIL, showed an

~10-fold enhanced bioactivity compared with monovalent scFv<sub>z</sub>EGFR-scTRAIL and an ~100-fold enhancement compared with nontargeted scTRAIL. To further assess

**Table 1** EC<sub>50</sub> values (mean ± S.E.M.) of various TRAIL constructs on tumor cells in the presence of bortezomib/cycloheximide

	HepG2	Colo205	Huh-7
Db <sub>z</sub> EGFR-scTRAIL	5.4 ± 3.1 × 10 <sup>-11</sup> M	2.1 ± 0.3 × 10 <sup>-12</sup> M	2.7 ± 0.5 × 10 <sup>-12</sup> M
Db <sub>z</sub> EGFR-scTRAIL+Cetuximab		4.3 ± 0.4 × 10 <sup>-12</sup> M	1.6 ± 0.5 × 10 <sup>-11</sup> M
scFv <sub>z</sub> EGFR-scTRAIL	5.8 ± 2.7 × 10 <sup>-10</sup> M	2.8 ± 0.3 × 10 <sup>-11</sup> M	4.3 ± 1.2 × 10 <sup>-11</sup> M
scFv <sub>z</sub> EGFR-scTRAIL+Cetuximab		1.9 ± 0.4 × 10 <sup>-10</sup> M	5.0 ± 2.3 × 10 <sup>-10</sup> M
scTRAIL	5.3 ± 2.3 × 10 <sup>-10</sup> M	1.8 ± 0.5 × 10 <sup>-10</sup> M	7.0 ± 2.3 × 10 <sup>-10</sup> M
KillerTRAIL	2.4 ± 0.5 × 10 <sup>-10</sup> M		

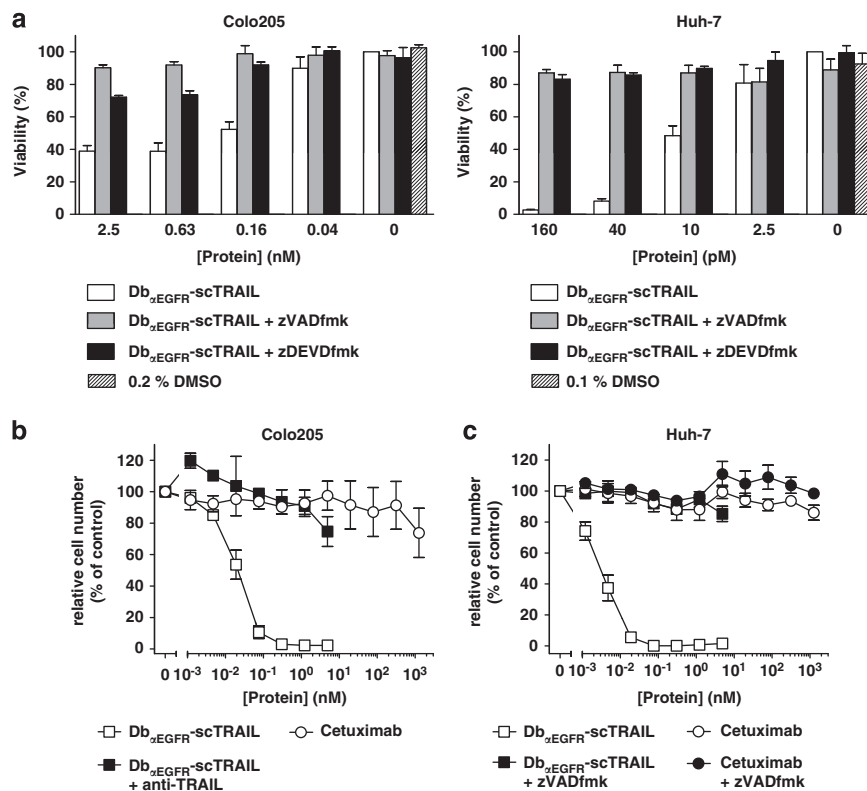


**Figure 3** Target-independent and EGFR-targeted induction of cell death by scTRAIL fusions. (a) HepG2 cells (left) were sensitized with 350 ng/ml bortezomib and treated with serial dilutions of Db<sub>z</sub>EGFR-scTRAIL (squares), KillerTRAIL (filled triangles), scFv<sub>z</sub>EGFR-scTRAIL (circles) or scTRAIL (open triangles). After 16 h, number of viable cells was determined using crystal violet staining (mean ± S.E.M., *n* = 4). Nonsensitized Jurkat cells (right) were assayed analogously, followed by determination of cell viability using the MTT method (mean ± S.E.M., *n* = 3). (b) Colo205 colon carcinoma (left and middle, mean ± S.E.M., *n* = 3) or Huh-7 hepatocarcinoma cells (right, mean ± S.E.M., *n* = 4) were sensitized with bortezomib (left, 400 ng/ml; right, 250 ng/ml) or CHX (middle, 2.5 μg/ml) and treated with 0.63 nM (left) or serial dilutions of Db<sub>z</sub>EGFR-scTRAIL (open squares), scFv<sub>z</sub>EGFR-scTRAIL (open circles) or scTRAIL (triangles). After 16 h, cell viability was determined using crystal violet staining (Huh-7) or the MTT method (Colo205). For quantification of the targeting effect, cells were preincubated with an excess of cetuximab (70 nM) before adding Db<sub>z</sub>EGFR-scTRAIL (filled squares) and scFv<sub>z</sub>EGFR-scTRAIL (filled circles). (c) Huh-7 cells were assayed essentially as in (b), but preincubated with constant concentrations of Db<sub>z</sub>EGFR-scTRAIL (squares), scFv<sub>z</sub>EGFR-scTRAIL (circles) or scTRAIL (triangles), followed by addition of serial dilutions of bortezomib (mean ± S.E.M., *n* = 3). Control (asterisk), bortezomib alone

apoptosis sensitization by bortezomib, Huh-7 cells were pretreated with a fixed dose of the scTRAIL fusion proteins followed by titration of bortezomib (Figure 3c). At a protein concentration of  $\geq 1$  nM, the tested reagents Db<sub>zEGFR</sub>-scTRAIL, scFv<sub>zEGFR</sub>-scTRAIL and scTRAIL did not differ in their ability to induce apoptosis in the presence of bortezomib. In contrast, at protein concentrations of  $\leq 0.1$  nM, the superior activity of targeted scTRAIL fusion proteins became visible, with Db<sub>zEGFR</sub>-scTRAIL as the most potent apoptosis inducer.

A nearly complete block of cell death by either pan-caspase (z-Val-Ala-Asp-fluoromethylketone (zVADfmk)) or caspase-3 selective (z-Asp-Glu-Val-Asp-fluoromethylketone (zDEVDfmk)) inhibitors (Figure 4a) and failure of necrostatin-1 to prevent or reduce cell death (data not shown) indicated that Huh-7 and Colo205 undergo predominantly apoptotic cell death upon treatment with scTRAIL fusion proteins. Cetuximab blocked EGF-induced autophosphorylation of EGF receptors (Figure 2d). It is well known that cetuximab only blocks ligand-dependent but not ligand-independent receptor tyrosine kinase (RTK) activity as a complete, divalent antibody but not as a monovalent antigen-binding fragment (Fab).<sup>24</sup> Moreover, on appropriate target cells, cetuximab can exert

growth-inhibitory and/or apoptosis-inducing activity,<sup>19</sup> suggesting a possible contribution of the divalent EGFR targeting domain of Db<sub>zEGFR</sub>-scTRAIL to the enhanced apoptosis induction. However, for the tumor cells studied here, we obtained no evidence for a TRAIL-independent cell death-inducing function of the targeting domain of Db<sub>zEGFR</sub>-scTRAIL. First, as for the monovalent targeted scFv<sub>zEGFR</sub>-scTRAIL, *in vitro* cotreatment of Db<sub>zEGFR</sub>-scTRAIL with 70 nM cetuximab resulted in a decrease in bioactivity to the level of nontargeted scTRAIL rather an increase in apoptosis (Figure 3b). Furthermore, cetuximab by itself, though blocking EGF-induced autophosphorylation of EGFR in Colo205 and in Huh-7 cells (Figure 2d), did not substantially affect growth of these two cancer cell lines in a 4-day culture (Figures 4b and c). Similarly, when Db<sub>zEGFR</sub>-scTRAIL-induced apoptosis was prevented, either by the presence of neutralizing anti-TRAIL antibodies in Db<sub>zEGFR</sub>-scTRAIL-treated Colo205 cell cultures (Figure 4b) or by treating Huh-7 cell cultures with pan-caspase inhibitors (Figure 4c), only a marginal growth inhibition was noted during the 4-day observation period. Together, for the cells and the *in vitro* conditions studied here, the data indicate that (1) Db<sub>zEGFR</sub>-scTRAIL-induced cell death requires TRAIL signaling and (2) blocking EGFR function by



**Figure 4** Caspase dependence of cell death and impact of the anti-EGFR diobody moiety of Db<sub>zEGFR</sub>-scTRAIL. (a) Colo205 cells (left) and Huh-7 cells (right) were sensitized with 25 and 250 ng/ml bortezomib, respectively, and treated with different concentrations of Db<sub>zEGFR</sub>-scTRAIL with or without the presence of pan-caspase inhibitor zVADfmk or caspase-3 inhibitor zDEVDfmk (both inhibitors 20  $\mu$ M for Colo205 and 10  $\mu$ M for Huh-7). After 16 h, cell viability was determined using MTT staining (Colo205) or crystal violet staining (Huh-7) and data were normalized using bortezomib-treated cells as control (mean  $\pm$  S.E.M.,  $n = 3$ ). (b)  $1 \times 10^4$  Colo205 cells per well were grown in 96-well plates using medium with 0.1% FCS. Upon stimulation with 50 ng/ml EGF and sensitization with 10 ng/ml bortezomib, cells were incubated with equimolar concentrations of Db<sub>zEGFR</sub>-scTRAIL (open squares), Db<sub>zEGFR</sub>-scTRAIL + anti-TRAIL mAb 2E5 (filled squares) or cetuximab (circles) for 4 days and cell number was assayed by the MTT method using bortezomib/EGF-treated cells as control for normalization (mean  $\pm$  S.E.M.,  $n = 2$ ). (c)  $1 \times 10^4$  Huh-7 cells per well were grown in 96-well plates and treated with 20 ng/ml bortezomib or with a combination of bortezomib and 10  $\mu$ M zVADfmk. Then, cells were incubated with equimolar concentrations of Db<sub>zEGFR</sub>-scTRAIL (open squares), Db<sub>zEGFR</sub>-scTRAIL + zVADfmk (filled squares), cetuximab (open circles) or cetuximab + zVADfmk (filled circles) for 3 days and cell viability was assayed by the MTT method using bortezomib-treated cells and bortezomib/zVADfmk-treated cells, respectively, as control for normalization (mean  $\pm$  S.E.M.,  $n = 3$ ).

the diabody module does not contribute to rapid apoptosis induction.

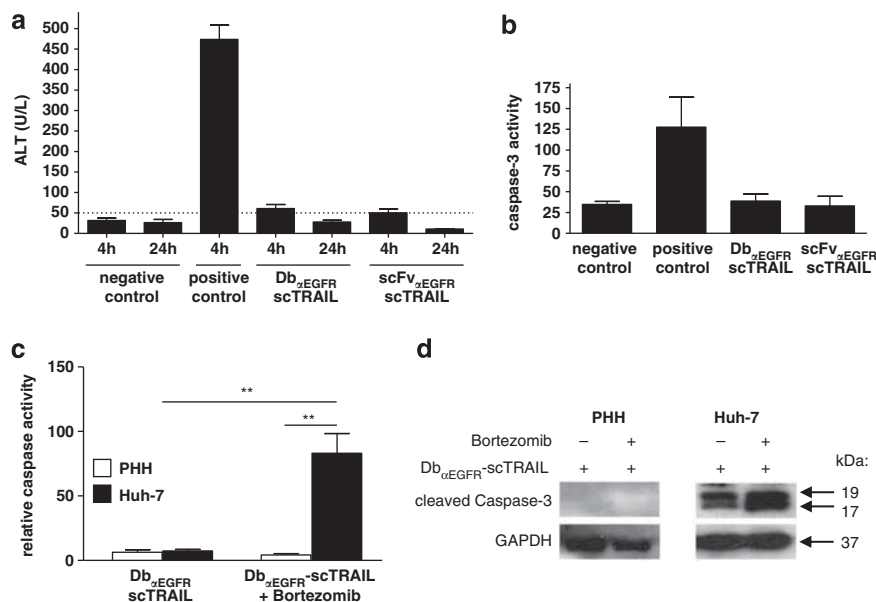
**In vivo tolerance of Db<sub>α</sub>EGFR-scTRAIL.** In order to investigate the *in vivo* properties of the novel fusion protein, we first checked for potential hepatotoxic effects of Db<sub>α</sub>EGFR-scTRAIL and monovalent scFv<sub>α</sub>EGFR-scTRAIL in comparison with aggregated Fas ligand used as a positive control. To this, serum alanine aminotransaminase (serum ALT) and liver caspase-3 activities were assayed in mice after application of a high dose of the protein (1 nmol). Both assays consistently revealed that a Db<sub>α</sub>EGFR-scTRAIL dose inducing maximum apoptosis *in vitro* did not cause acute liver toxicity *in vivo* (Figure 5). Thus, mean serum ALT activities at 4h after application of Db<sub>α</sub>EGFR-scTRAIL (61 U/l) or scFv<sub>α</sub>EGFR-scTRAIL (50 U/l) were only slightly and transiently raised to values considered as upper normal level (50 U/l) in humans and declined to baseline after 24h (Figure 5a). Analyses of caspase-3 activities from mouse liver tissue 24h (5h for positive control) after *in vivo* fusion protein application (Figure 5b) and histological analyses of liver tissue sections (Supplementary Figure S2) revealed no evidence for acute tissue toxicity of the applied reagents. Furthermore, a comparison of Huh-7 hepatoma cells and primary human hepatocytes (PHHs) for caspase-3 activation by Db<sub>α</sub>EGFR-scTRAIL in the presence of bortezomib showed a strong, bortezomib-dependent caspase-3 activation in the tumor cells, whereas normal liver cells were neither affected by the scTRAIL fusion protein alone nor in combination with bortezomib (Figure 5c). These results were confirmed by immunoblot analysis of cleaved caspase-3 in Huh-7 and PHHs, with no detectable caspase activation in combination-treated PHHs,

whereas robust caspase processing was detectable in sensitive Huh-7 carcinoma cells (Figure 5d).

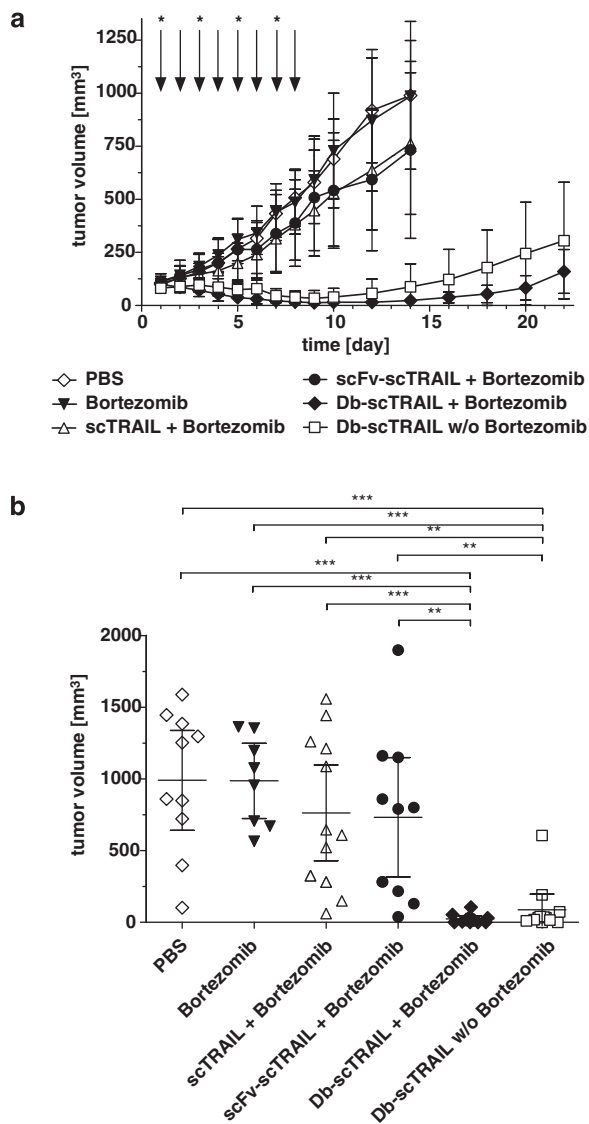
**Antitumoral activity of Db<sub>α</sub>EGFR-scTRAIL in a xenograft tumor model.** We investigated the *in vivo* antitumoral activity of Db<sub>α</sub>EGFR-scTRAIL in nude mice carrying Colo205 carcinomas (Figure 6). Given the *in vitro* data, showing superior bioactivity of Db<sub>α</sub>EGFR-scTRAIL compared with scFv<sub>α</sub>EGFR-scTRAIL or scTRAIL in particular at low protein concentrations, eight doses of 0.45 nmol protein were injected intraperitoneally (i.p.) in a daily regimen in combination with bortezomib cotreatment every second day. The systemic treatment started after establishment of solid, vascularized tumors, and tumor growth was monitored for 22 days. Bortezomib treatment by itself did not interfere with progressive tumor growth, whereas scTRAIL and scFv<sub>α</sub>EGFR-scTRAIL both delayed tumor growth but, at the low dosage applied, did not induce regression of tumors. In contrast, upon Db<sub>α</sub>EGFR-scTRAIL treatment, a strong reduction of tumor size and prolonged survival in all animals, with macroscopically undetectable tumors in 11/12 (+ bortezomib) and 9/12 (w/o bortezomib) cases, was recorded (Figure 6). Interestingly, under the treatment conditions applied, there was only a slight, but statistically not significant, benefit of cotreatment with bortezomib, although at termination of treatment the combination group presented with slower regrowth of tumors (Figure 6a).

## Discussion

In this study we present a new format of TRAIL fusion proteins based on directed dimerization with strongly



**Figure 5** Systemic and *in vitro* tolerance of scTRAIL fusion proteins to primary tissues. (a) Alanine aminotransaminase (ALT) activity in mouse serum after *in vivo* i.p. application of 1 nmol Db<sub>α</sub>EGFR-scTRAIL or scFv<sub>α</sub>EGFR-scTRAIL (mean ± S.E.M., n = 3). Positive control, 0.1 nmol Fas ligand fusion protein; negative control, PBS; Dotted line, upper normal level (50 U/l). (b) Caspase-3 activities in mouse liver biopsies at the end point of the previous experiment. (c) Relative caspase activity (fold increase compared with untreated) in primary human hepatocytes (PHHs, mean ± S.E.M., n = 5) or Huh-7 hepatocarcinoma cells (mean ± S.E.M., n = 7) after incubation with 1.1 nM Db<sub>α</sub>EGFR-scTRAIL in the presence or without 500 ng/ml bortezomib. Asterisks indicate statistical significance. (d) Cleavage of caspase-3 in PHH (left) and Huh-7 cells (right) after incubation with 500 ng/ml bortezomib, 1.1 nM Db<sub>α</sub>EGFR-scTRAIL or both was analyzed by immunoblotting



**Figure 6** Antitumor activity of TRAIL fusion proteins in a Colo205 xenograft tumor model. **(a)** Tumor volume as a function of time after i.p. application of PBS (open diamonds), bortezomib (filled triangles), scTRAIL + bortezomib (open triangles), scFv<sub>α</sub>EGFR-scTRAIL + bortezomib (circles), Db<sub>α</sub>EGFR-scTRAIL + bortezomib (filled diamonds) or Db<sub>α</sub>EGFR-scTRAIL only (squares). Arrows, protein application; asterisks, bortezomib application; symbols, mean of tumor volumes ± 95% confidence interval (CI), *n* = 12 tumors/treatment group. **(b)** Individual tumor volumes at day 14. Bars, mean of tumor volumes ± 95% CI

increased tumor-targeted bioactivity and devoid of systemic toxicity of these TRAIL fusion proteins. We and others previously showed that proapoptotic ligands of the TNF family can be engineered to exert stronger antitumor activity by creation of single-chain molecules<sup>25</sup> and by targeting death ligands to cell surface-expressed tumor antigens by fusion to a tumor-specific scFv.<sup>16,26</sup> Tumor-targeted scFv-TRAIL fusion proteins can mimic the naturally membrane-bound form of the ligand enabling DR4- and particularly DR5-mediated apoptosis induction, which is poorly exerted by soluble forms of TRAIL.<sup>21,27,28</sup> In support of this, we have recently shown that a fusion protein comprising scFv and single-chain TRAIL

is a preferred format compared with nontargeted TRAIL in terms of bioactivity *in vitro* and *in vivo*.<sup>16</sup>

Aside from targeting through scFv, bioactivity of death ligands can be considerably increased through oligomerization.<sup>29</sup> However, this may lead to generalized systemic toxicity, particularly evident for acute liver toxicity induced by Fas-specific reagents.<sup>30</sup> Similarly, for TRAIL, off-target toxicity of uncontrolled oligomeric forms of TRAIL has been reported.<sup>31</sup> We reasoned that a combination of antigen targeting and controlled TRAIL oligomerization should, on respective target cells, result in an effective activation of TRAIL DRs, and in particular DR5, which is the major proapoptotic TRAILR in carcinoma cells.<sup>32</sup> With the forced dimerization of scFv<sub>α</sub>EGFR-scTRAIL fusion proteins by shortening the linkers between V<sub>H</sub> and V<sub>L</sub> to five amino-acid residues, we generated a Db<sub>α</sub>EGFR-scTRAIL fusion protein exhibiting bivalent antigen binding and dimeric scTRAIL configuration, which meets the above criteria. In addition to the dimers, a minor species of higher molecular mass, likely trimeric or tetrameric fusion proteins, was detected by gel filtration of affinity-purified Db<sub>α</sub>EGFR-scTRAIL. We assume that the scTRAIL moiety of the fusion protein has an additional influence on oligomerization, because higher-order oligomeric species were not found when the αEGFR diabody was expressed as a single protein (data not shown). However, we propose that such oligomeric assemblies do not provide a further advantage with regard to bioactivity, compared with the dimeric Db<sub>α</sub>EGFR-scTRAIL form. First, cell death assays using the separated species gave no hint for a different bioactivity of both isoforms. Second, an engineered protein format with an at least trimeric assembly of scFv<sub>α</sub>EGFR-scTRAIL showed again no better activity *in vitro* as compared with Db<sub>α</sub>EGFR-scTRAIL (A Benk and M Siegemund, unpublished data).

We found that Db<sub>α</sub>EGFR-scTRAIL has a higher affinity to target-positive tumor cells than scFv<sub>α</sub>EGFR-scTRAIL, evident from an approximately twofold lower EC<sub>50</sub> at 4 °C. Both the diabody configuration and the dimeric status of scTRAIL could contribute to this apparent affinity increase. However, we attribute this largely to a better avidity of the bivalent diabody targeting module, as dynamic clustering of TRAILRs and therefore stable receptor ligand interactions are prevented under the applied assay conditions. Independent evidence for increased avidity of Db<sub>α</sub>EGFR-scTRAIL is obtained from the reduced efficacy of cetuximab in competing the binding of Db<sub>α</sub>EGFR-scTRAIL to EGFR<sup>+</sup> cells as compared with scFv<sub>α</sub>EGFR-scTRAIL. Accordingly, we postulate that a more stable receptor interaction of Db<sub>α</sub>EGFR-scTRAIL supports clustering and subsequent DISC formation of death receptors in specific membrane compartments, for example lipid rafts, which have been proposed to play an important role in DR4 and DR5 signal transduction.<sup>33,34</sup> Our data support this reasoning by showing enhanced bioactivity of the Db<sub>α</sub>EGFR-scTRAIL molecules, to which both targeting and the dimeric/oligomeric assembly of the TRAIL molecule itself contribute, resulting in an overall greater 100-fold increased bioactivity on target-positive tumor cells. In contrast, on target-negative tumor cell lines like HepG2 or Jurkat, the dimeric scTRAIL configuration exclusively seems to contribute to an activity increase compared with scTRAIL.



The Db<sub>z</sub>EGFR-scTRAIL comprises two scFvs derived from the mAb cetuximab, an EGF-blocking, antagonistic antibody reported to be capable of growth inhibition and/or apoptosis induction in some tumor cells.<sup>19,20</sup> Although we confirmed blocking of EGF-induced EGFR autophosphorylation in the tumor cells studied here, it was interesting to note that this RTK inhibition did not apparently contribute to the enhancement of TRAIL-induced apoptosis, at least as detectable within short-term *in vitro* assays. Lack of an active contribution of EGFR blocking to apoptosis sensitivity was particularly evident from a right shift in the dose-response curves to a level of nontargeted TRAIL molecules when cetuximab was added to Db<sub>z</sub>EGFR-scTRAIL-treated cells. Moreover, the block of Db<sub>z</sub>EGFR-scTRAIL-mediated cell death by TRAIL-neutralizing antibodies confirms that the apoptotic signal emanated from TRAILRs. Accordingly, the strongly enhanced apoptotic activity of Db<sub>z</sub>EGFR-scTRAIL is an intrinsic molecular feature of this bifunctional protein, in which EGFR targeting and presentation of TRAIL to its cognate DRs cannot be mimicked by cooperative action of two independent molecules, one interfering with EGFR activity and one inducing apoptosis via TRAIL DRs. However, these *in vitro* studies do not rule out a contribution of EGFR inhibition by the scFv module in the observed *in vivo* antitumoral activity of Db<sub>z</sub>EGFR-scTRAIL, where several additional parameters aside from basal apoptosis sensitivity affect tumor outgrowth and responsiveness to drug treatment.

Strongly enhanced bioactivity and improved pharmacokinetics of targeted scTRAIL fusion proteins (see Schneider *et al.*<sup>16</sup> and this study) appear mandatory to reach significant clinical responses under TRAIL therapy. However, considering previous reports on the potential loss of tumor selectivity of aggregated or artificially crosslinked TRAIL forms,<sup>15,35,36</sup> the oligomeric nature of Db<sub>z</sub>EGFR-scTRAIL demands a thorough analysis of potential off-target action and systemic toxicity. Toward this end, we obtained evidence that Db<sub>z</sub>EGFR-scTRAIL was surprisingly well tolerated in mice at doses up to 3 mg/kg with no evidence for acute hepatotoxicity, judged from ALT levels and histopathological analyses. *In vitro* Db<sub>z</sub>EGFR-scTRAIL treatment of primary human hepatocytes with or without apoptosis sensitizer bortezomib did not induce caspase activation, thus fully confirming the mouse *in vivo* data. Additionally, healthy human liver tissue from *in vitro* cultures proved to be resistant toward Db<sub>z</sub>EGFR-scTRAIL in the presence of the apoptosis sensitizer bortezomib also (H Bantel *et al.*, unpublished data).

On top of a good *in vivo* tolerance, a superior *in vivo* antitumoral activity of Db<sub>z</sub>EGFR-scTRAIL against xenotransplanted Colo205 cells was found. Interestingly, in this *in vivo* tumor model, Db<sub>z</sub>EGFR-scTRAIL was very effective even in the absence of the apoptosis sensitizer bortezomib, although after termination of short-term therapy, tumor regrowth was detectable earlier in this group as compared with the combination therapy group. A potentially greater benefit of combination therapy might become evident under a different therapeutic regimen and/or only in long-term studies. In contrast, in preliminary *in vivo* studies with the hepatocellular carcinoma cells Huh-7, the antitumoral activity of different scTRAIL derivatives, including Db<sub>z</sub>EGFR-scTRAIL, was only revealed in the presence of bortezomib (A Vogel, unpublished

data), which corresponded to the strict dependence of *in vitro* TRAIL sensitivity on bortezomib of this particular cell line. This is in accordance with published data of bortezomib as an effective sensitizer of apoptosis.<sup>9,37</sup>

In conclusion, we have shown that by genetic engineering, controlled dimerization and tumor targeting can be combined to create bifunctional fusion proteins with enforced apoptosis induction under maintenance of systemic tolerance, which exceeds the functional activities of conventional and other antigen-targeted TRAIL molecules. Specifically, combining targeting domains with intrinsic antagonistic activity toward a targeted growth factor receptor with death ligands engineered for improved apoptotic activity could potentially synergize to further enhance the antitumor activity. Although for the tumor model studied here, this bifunctionality of Db<sub>z</sub>EGFR-scTRAIL appeared dispensable for *in vitro* apoptosis induction, it might be of particular relevance *in vivo* for tumors that are still growth factor dependent. Because of the modular nature of the fusion protein, the concept shown here for EGFR targeting can be exploited for a broad range of tumors for which selective cell surface markers are known.

## Materials and Methods

**Plasmids and cell lines.** The pRESpuro-scTRAIL expression construct for human scTRAIL was obtained by *EcoRI/NotI* cloning of a synthesized sequence encoding three TRAIL modules (aa residues 95–281) connected by (GGGS)<sub>2</sub> linkers into a construct described previously.<sup>16</sup>

For generation of the EGFR-specific pCR3-scFv-scTRAIL expression construct, a synthesized coding sequence of a humanized scFv 'huC225' (RE Kontermann, unpublished data) was amplified using the oligonucleotides 5'-CGAGGTGCAGCTGGTCGAG-3' and 5'-TGC GGCCGCTCTCTTGATTC-3'. Next, this template was annealed with the oligonucleotide 5'-ATATATCTCGAGGCCGCGACTACAAAGACGATGACGATAAAGGAGCCGAGGTGCAGCTGGTCGAG-3' to insert an *XhoI* site and a FLAG tag coding sequence. After strand elongation, the oligonucleotides 5'-ATATATCTCGAGGCCGCGAC-3' and 5'-ATATGAATCTGCGCCGCTCTCTTGATTC-3' were used for PCR. The product was cloned by *XhoI/EcoRI* into pCR3 (Invitrogen, Karlsruhe, Germany), carrying a V<sub>H</sub> leader and a dummy scFv-scTRAIL sequence. The scTRAIL coding sequence of this construct<sup>16</sup> was then substituted with scTRAIL ((GGGS)<sub>2</sub> linkers) via *EcoRI/XbaI*.

The EGFR-specific pCR3-Db-scTRAIL expression construct was derived from pCR3-scFv<sub>z</sub>EGFR-scTRAIL by shortening the scFv linker from (GGGS)<sub>3</sub> to GGGGS. Therefore, two PCR products were generated using the oligonucleotides (1) 5'-CCCACAGCCTCGAGGCCAG-3' and (2) 5'-GAGCCGCCACCGCACTAG-3' as well as (3) 5'-CTAGTGGCGGTGGCGGCTCTGATATTCAGCTGACCAGTCC-3' and (4) 5'-TGAATTCGCGCCGCTCTC-3'. After annealing of the products at the underlined regions and strand elongation, the whole sequence was amplified by the oligonucleotides (1) and (4) followed by *XhoI/NotI* cloning into pCR3-scFv<sub>z</sub>EGFR-scTRAIL.

HEK293, HepG2, NCI-H460, Colo205 and Jurkat cells were obtained from the ATCC (Manassas, VA, USA). Huh-7 cells were obtained from the JCRB (Osaka, Japan). Cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 5% fetal calf serum (FCS; HyClone, South Logan, UT, USA), excepting HepG2, Colo205 and Huh-7, where 10% FCS was added to the medium. PHHs were isolated according to a well-defined standard procedure.<sup>38</sup>

**Production and purification of recombinant proteins.** TRAIL fusion proteins were produced in HEK293 cells as published,<sup>16</sup> and purified from supernatants first by immobilized metal affinity chromatography (IMAC) based on affinity of TRAIL to Ni-NTA-Agarose (Qiagen, Hilden, Germany). After elution with 100 mM imidazol in IMAC buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 300 mM NaCl) and dialysis against PBS, the proteins were further purified by affinity chromatography using anti-FLAG mAb M2 agarose (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol. ScTRAIL was purified by M2 agarose only. After elution of bound proteins with 100 µg/ml FLAG peptide (Peptides & elephants, Potsdam, Germany) and dialysis against PBS, proteins were concentrated with

Vivaspin devices (PES membrane with 50 or 10 kDa molecular weight cutoff; Sartorius Stedim, Aubagne, France). The protein concentration was measured with a spectrophotometer (NanoDrop Products, Wilmington, DE, USA) and aliquots were stored at  $-80^{\circ}\text{C}$ .

**Immunoprecipitation and protein analysis.** For immunoprecipitations, cells were lysed on ice in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM sodium fluoride, 20 mM  $\beta$ -glycerophosphate, 1 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% SDS, 0.25% sodium deoxycholate) with Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) and lysates were clarified by centrifugation ( $16\,000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ). Then, 1.5 mg lysate protein was incubated with 1.5  $\mu\text{g}$  mouse anti-EGFR Ab-13 mAb (Neomarkers, Fremont, CA, USA) under gentle shaking at  $4^{\circ}\text{C}$  overnight. Immune complexes were captured with protein G sepharose (KPL, Gaithersburg, MD, USA) and washed three times with RIPA buffer. Proteins were analyzed by SDS-PAGE and western blotting using mouse anti-phosphotyrosine P-Tyr-100 mAb (Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-EGFR 1005 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Enhanced chemoluminescence (ECL; Pierce Biotechnology, Rockford, IL, USA) was used for visualization.

Caspases were detected by immunoblotting using a rabbit polyclonal antibody against cleaved caspase-3 (Cell Signaling Technology). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control was detected with a rabbit polyclonal antibody (Cell Signaling Technology). HRP-conjugated secondary antibodies (Zymed Laboratories, San Francisco, CA, USA) and ECL were used for visualization.

Affinity-purified scTRAIL fusion proteins were analyzed by SDS-PAGE and silver staining (Sigma-Aldrich). For western blotting, anti-TRAIL MAB687 (R&D Systems, Wiesbaden, Germany) and anti-FLAG M2 mAbs (Sigma-Aldrich) were used, followed by anti-mouse alkaline phosphatase-coupled secondary antibody (Sigma-Aldrich) for detection. For size exclusion chromatography, proteins were applied to a BioSuite250 HR SEC  $300 \times 7.8$  HPLC column (Waters, Milford, MA, USA) equilibrated in PBS and eluted at a flow rate of 0.5 ml/min.

**Flow cytometry.**  $5 \times 10^5$  cells were suspended in PBA buffer (PBS, 0.025% BSA, 0.02%  $\text{NaN}_3$ ) and incubated for 1 h at  $4^{\circ}\text{C}$  with the scTRAIL fusion proteins (2  $\mu\text{g}/\text{ml}$ ). After washing the cells three times with PBA buffer, bound proteins were detected by anti-human TRAIL mAb MAB687 (2.5  $\mu\text{g}/\text{ml}$ ) and fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG Ab (1:200, Sigma-Aldrich), followed by three washing steps with PBA each. For blocking of scTRAIL fusion protein binding to EGFRs, a divalent variant of scFv huC225 (scFv huC225Cys, 50  $\mu\text{g}/\text{ml}$ , kindly provided by Celonic GmbH, Jülich, Germany) was added 30 min before incubation with scFv<sub>zEGFR</sub>-scTRAIL or Db<sub>zEGFR</sub>-scTRAIL, respectively. TRAIL receptors were detected by anti-TRAIL R1 mAb MAB347 and anti-TRAIL R2 mAb MAB6311 (4  $\mu\text{g}/\text{ml}$  each, R&D Systems) in conjunction with anti-mouse IgG-FITC. EGF receptors were detected by a phycoerythrin-labeled anti-human EGFR mAb sc-101 (4  $\mu\text{g}/\text{ml}$ , Santa Cruz Biotechnology). For binding competition, purified Db<sub>zEGFR</sub>-scTRAIL (50  $\mu\text{g}/\text{ml}$ ) was added 30 min before incubation with Alexa Fluor 488-coupled (Life Technologies) mAb cetuximab (1  $\mu\text{g}/\text{ml}$ ).

Binding of scTRAIL fusion proteins to EGFR-expressing NCI-H460 cells was analyzed by flow cytometry at  $4^{\circ}\text{C}$  as described.<sup>39,40</sup> Cells were incubated with anti-TRAIL mAb MAB687 (2.5  $\mu\text{g}/\text{ml}$ ) and FITC-labeled rabbit anti-mouse IgG Ab (1:200) in the presence of cell-bound protein or without (background control). Data were fitted with Prism (GraphPad Software, La Jolla, CA, USA) from four independent binding curves, and mean  $\text{EC}_{50}$  values  $\pm$  S.E.M. were calculated. For statistical analysis, Student's *t*-test was applied.

**Cell death assays.** Huh-7 ( $2.5 \times 10^4$  per well), HepG2 ( $3 \times 10^4$  per well), Colo205 ( $5 \times 10^4$  per well) or Jurkat cells ( $1 \times 10^5$  per well) were grown in 100  $\mu\text{l}$  culture medium in 96-well plates for 24 h, followed by treatment with serial dilutions of scTRAIL fusion proteins or 'KillerTRAIL' (Enzo Life Sciences, Lörrach, Germany) in triplicates. For positive control, cells were killed with 0.25% Triton X-100. Cell death assays were performed in the absence or presence of bortezomib (20–250 ng/ml for Huh-7, 350 ng/ml for HepG2, 10–400 ng/ml for Colo205; Selleck Chemicals, Houston, TX, USA) or CHX (2.5  $\mu\text{g}/\text{ml}$  for Colo205; Sigma-Aldrich). Bortezomib/CHX was added 30 min before incubation with the proapoptotic ligands to sensitize carcinoma cells for cell death induction. Appropriate concentrations of caspase inhibitors zVADfmk and zDEVDfmk (10 mM stock solutions in DMSO; Enzo Life Sciences) were added to the

bortezomib solution. Where applicable, the EGFR signaling pathway was induced by 50 ng/ml EGF (Sigma-Aldrich). Alternatively, cells were preincubated for 30 min with the proapoptotic ligands followed by addition of serial dilutions of bortezomib. After 16 h of incubation, cell viability was determined either by crystal violet staining (Huh-7, HepG2) or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Jurkat, Colo205).<sup>41</sup> For the MTT assay, cells were lysed in DMF/H<sub>2</sub>O (1:1), 15% SDS, pH 4.5. To demonstrate target antigen-dependent induction of cell death, cells were preincubated for 30 min with competing cetuximab mAb (10  $\mu\text{g}/\text{ml}$ , Merck KGaA, Darmstadt, Germany) or alternatively scFv huC225Cys (10  $\mu\text{g}/\text{ml}$ ). For inhibition of TRAIL bioactivity, neutralizing anti-TRAIL antibody 2E5 (1  $\mu\text{g}/\text{ml}$ , Enzo Life Sciences) was used.

**ALT and caspase activities.** Groups of three CD1 mice (Janvier, Le Genest-St-Isle, France) were treated i.p. with 1 nmol Db<sub>zEGFR</sub>-scTRAIL and scFv<sub>zEGFR</sub>-scTRAIL, 0.1 nmol FasL fusion protein (positive control) and PBS (negative control). Blood samples were taken from the tail after 4 and 24 h and incubated on ice. Clotted blood was centrifuged ( $10\,000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ) and serum samples were stored at  $-80^{\circ}\text{C}$ . Activity of ALT was determined by an enzymatic assay (BIOO Scientific, Austin, TX, USA). To determine caspase-3 activity in the liver tissue, mice were killed after 24 h (positive control after 5 h) and liver biopsies were taken. Homogenates were prepared in lysis buffer (200 mM NaCl, 20 mM Tris, 1% NP-40, pH 7.4). Then, 10  $\mu\text{g}$  of protein was analyzed by conversion of the fluorogenic substrate Ac-Asp-Met-Gln-Asp-7-Amino-4-methylcoumarin (Ac-DMQD-AMC; Enzo Life Sciences). Caspase activity in PHH and Huh-7 cells was determined as published.<sup>42</sup>

**Xenograft mouse tumor model.** Female NMRI nu/nu mice (Janvier), 8 weeks old, were injected subcutaneously (s.c.) with  $3 \times 10^6$  Colo205 cells in 100  $\mu\text{l}$  PBS at left and right dorsal sides. Treatment started 6 days after tumor cell inoculation when tumors reached  $\sim 100 \text{ mm}^3$ . Mice received 8 daily i.p. injections of 0.45 nmol of the affinity-purified TRAIL fusion proteins. On days 1, 3, 5 and 7 of treatment, mice received additionally 5  $\mu\text{g}$  bortezomib in 100  $\mu\text{l}$  PBS i.p. 3 h before protein injection. The control groups received 100  $\mu\text{l}$  PBS or 5  $\mu\text{g}$  bortezomib at the same time intervals. Tumor growth was monitored as described.<sup>16,43</sup> Tukey's test was applied for statistics.

### Conflict of Interest

University of Stuttgart has filed a patent application on the TRAIL fusion proteins described in this report with K Pfizenmaier, RE Kontermann and M Siegemund named as inventors. The other authors declare no conflict of interest.

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1. Amm HM, Oliver PG, Lee CH, Li Y, Buchsbaum DJ. Combined modality therapy with TRAIL or agonistic death receptor antibodies. *Cancer Biol Ther* 2011; **11**: 431–449.
2. Holland PM. Targeting Apo2L/TRAIL receptors by soluble Apo2L/TRAIL. *Cancer Lett* 2011; e-pub ahead of print 8 January 2011; doi:10.1016/j.canlet.2010.11.001.
3. Belada D, Mayer J, Czuczman MS, Flinn IW, Durbin-Johnson B, Bray GL. Phase II study of dulanerin plus rituximab in patients with relapsed follicular non-Hodgkin's lymphoma (NHL). *ASCO Meeting Abstracts* 2010; **28**: 8104.
4. Hörnle M, Peters N, Thayaparasingham B, Vörsmann H, Kashkar H, Kulms D. Caspase-3 cleaves XIAP in a positive feedback loop to sensitize melanoma cells to TRAIL-induced apoptosis. *Oncogene* 2011; **30**: 575–587.
5. Volkmann M, Schiff JH, Hajjar Y, Otto G, Stilgenbauer F, Fiehn W et al. Loss of CD95 expression is linked to most but not all p53 mutants in European hepatocellular carcinoma. *J Mol Med* 2001; **79**: 594–600.
6. Chan C, Yau T, Jin D, Wong C, Fan S, Ng IO. Evaluation of nuclear factor-kappaB, urokinase-type plasminogen activator, and HbX and their clinicopathological significance in hepatocellular carcinoma. *Clin Cancer Res* 2004; **10**: 4140–4149.
7. Haybaeck J, Zeller N, Wolf MJ, Weber A, Wagner U, Kurrer MO et al. A lymphotoxin-driven pathway to hepatocellular carcinoma. *Cancer Cell* 2009; **16**: 295–308.
8. Koehler B, Urbanik T, Vick B, Boger R, Heeger S, Galle P et al. TRAIL-induced apoptosis of hepatocellular carcinoma cells is augmented by targeted therapies. *World J Gastroenterol* 2009; **15**: 5924–5935.

9. Ganten TM, Koschny R, Haas TL, Sykora J, Li-Weber M, Herzer K *et al*. Proteasome inhibition sensitizes hepatocellular carcinoma cells, but not human hepatocytes, to TRAIL. *Hepatology* 2005; **42**: 588–597.
10. Chen K, Yeh P, Hsu C, Hsu C, Lu Y, Hsieh H *et al*. Bortezomib overcomes tumor necrosis factor-related apoptosis-inducing ligand resistance in hepatocellular carcinoma cells in part through the inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *J Biol Chem* 2009; **284**: 11121–11133.
11. Sun W, Nelson D, Alberts SR, Poordad F, Leong S, Teitelbaum UR *et al*. Phase Ib study of mapatumumab in combination with sorafenib in patients with advanced hepatocellular carcinoma (HCC) and chronic viral hepatitis. *J Clin Oncol* 2011; **29** (Suppl 4) abstract 261.
12. MacFarlane M, Kohlhaas SL, Sutcliffe MJ, Dyer MJS, Cohen GM. TRAIL receptor-selective mutants signal to apoptosis via TRAIL-R1 in primary lymphoid malignancies. *Cancer Res* 2005; **65**: 11265–11270.
13. van der Sloot AM, Tur V, Szegedzi E, Mullally MM, Cool RH, Samali A *et al*. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. *Proc Natl Acad Sci USA* 2006; **103**: 8634–8639.
14. ten Cate B, Bremer E, de Bruyn M, Bijma T, Samplonius D, Schwemlein M *et al*. A novel AML-selective TRAIL fusion protein that is superior to Gemtuzumab Ozogamicin in terms of in vitro selectivity, activity and stability. *Leukemia* 2009; **23**: 1389–1397.
15. Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B *et al*. Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* 2001; **7**: 383–385.
16. Schneider B, Munkel S, Krippner-Heidenreich A, Grunwald I, Wels WS, Wajant H *et al*. Potent antitumor activity of TRAIL through generation of tumor-targeted single-chain fusion proteins. *Cell Death Dis* 2010; **1**: e68.
17. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* 2000; **19**: 3159–3167.
18. Naramura M, Gillies SD, Mendelsohn J, Reisfeld RA, Mueller BM. Therapeutic potential of chimeric and murine anti-(epidermal growth factor receptor) antibodies in a metastasis model for human melanoma. *Cancer Immunol Immunother* 1993; **37**: 343–349.
19. Ng M, Cunningham D. Cetuximab (Erbix) – an emerging targeted therapy for epidermal growth factor receptor-expressing tumours. *Int J Clin Pract* 2004; **58**: 970–976.
20. Doody JF, Wang Y, Patel SN, Joynes C, Lee SP, Gerlach J *et al*. Inhibitory activity of cetuximab on epidermal growth factor receptor mutations in non small cell lung cancers. *Mol Cancer Ther* 2007; **6**: 2642–2651.
21. Wajant H, Moosmayer D, Wüest T, Bartke T, Gerlach E, Schönherr U *et al*. Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. *Oncogene* 2001; **20**: 4101–4106.
22. Berg D, Stühmer T, Siegmund D, Müller N, Giner T, Dittrich-Breiholz O *et al*. Oligomerized tumor necrosis factor-related apoptosis inducing ligand strongly induces cell death in myeloma cells, but also activates proinflammatory signaling pathways. *FEBS J* 2009; **276**: 6912–6927.
23. Holliger P, Prospero T, Winter G. Diabodies<sup>™</sup>: small bivalent and bispecific antibody fragments. *Proc Natl Acad Sci USA* 1993; **90**: 6444–6448.
24. Fan Z, Lu Y, Wu X, Mendelsohn J. Antibody-induced epidermal growth factor receptor dimerization mediates inhibition of autocrine proliferation of A431 squamous carcinoma cells. *J Biol Chem* 1994; **269**: 27595–27602.
25. Krippner-Heidenreich A, Grunwald I, Zimmermann G, Kühnle M, Gerspach J, Sterns T *et al*. Single-chain TNF, a TNF derivative with enhanced stability and antitumor activity. *J Immunol* 2008; **180**: 8176–8183.
26. Gerspach J, Wajant H, Pfizenmaier K. Death ligands designed to kill: development and application of targeted cancer therapeutics based on proapoptotic TNF family ligands. *Results Probl Cell Differ* 2009; **49**: 241–273.
27. Bremer E, Kuijlen J, Samplonius D, Walczak H, de Leij L, Helfrich W. Target cell-restricted and -enhanced apoptosis induction by a scFv: sTRAIL fusion protein with specificity for the pancreatic carcinoma-associated antigen EGp2. *Int J Cancer* 2004; **109**: 281–290.
28. Bremer E, Samplonius DF, Peipp M, van Genne L, Kroesen B, Fey GH *et al*. Target cell-restricted apoptosis induction of acute leukemic T cells by a recombinant tumor necrosis factor-related apoptosis-inducing ligand fusion protein with specificity for human CD7. *Cancer Res* 2005; **65**: 3380–3388.
29. Wajant H, Gerspach J, Pfizenmaier K. Engineering death receptor ligands for cancer therapy. *Cancer Lett* 2011; e-pub ahead of print 13 January 2011; doi:10.1016/j.canlet.2010.12.019.
30. Schneider P, Holler N, Bodmer JL, Hahne M, Frei K, Fontana A *et al*. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J Exp Med* 1998; **187**: 1205–1213.
31. Koschny R, Walczak H, Ganten TM. The promise of TRAIL – potential and risks of a novel anticancer therapy. *J Mol Med* 2007; **85**: 923–935.
32. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002; **2**: 420–430.
33. Song JH, Tse MCL, Bellail A, Phuphanich S, Khuri F, Kneteman NM *et al*. Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and nonapoptotic signals in non small cell lung carcinoma cells. *Cancer Res* 2007; **67**: 6946–6955.
34. Psahoulia FH, Drosopoulos KG, Doubravska L, Andera L, Pintzas A. Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. *Mol Cancer Ther* 2007; **6**: 2591–2599.
35. Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR *et al*. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med* 2000; **6**: 564–567.
36. Ganten TM, Koschny R, Sykora J, Schulze-Bergkamen H, Büchler P, Haas TL *et al*. Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. *Clin Cancer Res* 2006; **12**: 2640–2646.
37. Koschny R, Holland H, Sykora J, Haas TL, Sprick MR, Ganten TM *et al*. Bortezomib sensitizes primary human astrocytoma cells of WHO grades I to IV for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Clin Cancer Res* 2007; **13**: 3403–3412.
38. Nussler AK, Nussler NC, Merk V, Brulport M, Schormann W, Hengstler JG. The Holy grail of hepatocyte culturing and therapeutic use. In: Santini M (ed). *Regenerative Medicine Today*. Springer International: Berlin, Germany, 2009, pp: 283–320.
39. Hopp J, Hornig N, Zettlitz KA, Schwarz A, Fuss N, Müller D *et al*. The effects of affinity and valency of an albumin-binding domain (ABD) on the half-life of a single-chain diabody-ABD fusion protein. *Protein Eng Des Sel* 2010; **23**: 827–834.
40. Benedict CA, MacKrell AJ, Anderson WF. Determination of the binding affinity of an anti-CD34 single-chain antibody using a novel, flow cytometry based assay. *J Immunol Methods* 1997; **201**: 223–231.
41. Wüest T, Gerlach E, Banerjee D, Gerspach J, Moosmayer D, Pfizenmaier K. TNF-Selectokine: a novel prodrug generated for tumor targeting and site-specific activation of tumor necrosis factor. *Oncogene* 2002; **21**: 4257–4265.
42. Seidel N, Volkmann X, Länger F, Flemming P, Manns MP, Schulze-Osthoff K *et al*. The extent of liver steatosis in chronic hepatitis C virus infection is mirrored by caspase activity in serum. *Hepatology* 2005; **42**: 113–120.
43. Kim TH, Youn YS, Jiang HH, Lee S, Chen X, Lee KC. PEGylated TNF-related apoptosis-inducing ligand (TRAIL) analogues: pharmacokinetics and antitumor effects. *Bioconjug Chem* 2011; **22**: 1631–1637.



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